New insights into the role of breast cancer resistance protein in endocrine and metabolic processes

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New insights into the role of breast cancer resistance protein in endocrine and metabolic processes

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General introduction
ATP binding cassette transporters

The ATP binding cassette (ABC) transporter family forms one of the largest membrane-bound protein families encoded by the human genome and a total of 49 genes of human ABC protein have been identified and sequenced. Based on the arrangement of molecular structural components, i.e., nucleotide binding domains and topologies of transmembrane domains, human ABC transporters are classified into seven different subfamilies (A to G). Before the first transporter genes were cloned, it was long known that resistant sublines were formed after incubating cancer cell lines with chemotherapeutic agents. In 1976, Juliano and Ling were the first to report that this resistance was associated with a particular 170 kD glycoprotein, which was later named P-glycoprotein (P-gp). Its gene was cloned for the first time in 1986 and called multidrug resistance gene 1 (mdr1). Nowadays, the eukaryotic ABC transporters are well characterized and are known to transport a variety of molecules, against steep concentration gradients at the expense of ATP, across the plasma membrane as well as across intracellular membranes of the endoplasmic reticulum, peroxisomes, lysosomes, and mitochondria. Nearly all ABC proteins are efflux transporters, except for the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7), which acts as a chloride ion channel, and the sulfonylurea receptors SUR1/ABCC8 and SUR2/ABCC9 that regulate K+ channels. Efflux transporters have an important role in tissue defense through the removal of harmful molecules. Collectively, ABC proteins are capable of transporting a wide variety of substrates including hydrophilic, lipophilic, cationic, anionic, and neutrally charged molecules as well as their conjugated metabolites, as reviewed by Leslie et al. Transporters that have been extensively studied in the context of tissue defense and drug resistance include P-gp, multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) (table 1). These transporters are found in many organs and tissues in the body, including epithelia of e.g. the intestine, liver and kidney, as well as tissues with a tight barrier function like the blood-brain barrier (BBB), blood-placenta barrier, blood-testis barrier (BTB) and the choroid plexus as part of the blood-cerebrospinal fluid (CSF) barrier, however, expression levels differ per organ. For example, P-gp and BCRP are most abundant in the BBB while in the liver MRP2 is most abundantly expressed and in the kidney proximal tubule cells, MRP4 and P-gp are highly expressed. The efflux transporters are expressed either at the basolateral (MRP1 and -3) or apical (P-gp, BCRP, and MRP2) cell membrane, except for MRP4, the localization of which is dependent on the cell type either in the apical (renal proximal tubule cells and BBB) and basolateral membranes (hepatocytes and choroid plexus epithelium).
Table 1. List of important human xenobiotic efflux transporters, their genes, localization, and function

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Gene</th>
<th>Size (aa*)</th>
<th>Tissue</th>
<th>Localization</th>
<th>Substrate specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1/P-gp</td>
<td>ABCB1</td>
<td>1280</td>
<td>Lung, intestine, colon, liver, kidney, BBB, choroid plexus, testis and placenta</td>
<td>Apical membrane</td>
<td>Broad substrate specificity; preference for hydrophobic, amphipathic and cationic molecules</td>
</tr>
<tr>
<td>MRP1</td>
<td>ABCC1</td>
<td>1530</td>
<td>Lung, heart and skeletal muscle, intestine, colon, liver, kidney, BBB, choroid plexus, adrenal gland, tests, ovary and placenta</td>
<td>Basolateral membrane (but BBB; apical)</td>
<td>Hydrophobic drugs and glutathione, glucuronide, and sulfate conjugates</td>
</tr>
<tr>
<td>MRP2</td>
<td>ABCC2</td>
<td>1545</td>
<td>Lung, intestine, liver, kidney, BBB, choroid plexus and placenta</td>
<td>Apical membrane</td>
<td>Unconjugated organic anions and glutathione, glucuronide, sulfate, and heavy metal conjugates</td>
</tr>
<tr>
<td>MRP3</td>
<td>ABCC3</td>
<td>1527</td>
<td>Lung, intestine, colon, liver, kidney, adrenal gland</td>
<td>Basolateral membrane</td>
<td>Drug conjugates to glutathione and other organic anions</td>
</tr>
<tr>
<td>MRP4</td>
<td>ABCC4</td>
<td>1325</td>
<td>Lung, intestine, colon, liver, kidney, BBB, choroid plexus, testis and ovary</td>
<td>Dual</td>
<td>Broad substrate specificity including bases, (cyclic) nucleotides, and nucleosides</td>
</tr>
<tr>
<td>MXR/ABCP/BCRP</td>
<td>ABCG2</td>
<td>655</td>
<td>Lung, intestine, colon, liver, kidney, BBB, choroid plexus, adrenal gland, testis, ovary and placenta</td>
<td>Apical membrane</td>
<td>Broad substrate specificity; preference for acids and drug conjugates</td>
</tr>
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</table>

Data obtained from published reviews.11,15,17

* aa = amino acids
In renal proximal tubule cells, hepatocytes and enterocytes, basolaterally-located ABC transporters pump molecules from the cells towards the blood stream and in the apical membrane, they drive urinary and biliary excretion and prevent intestinal uptake of their substrates. Transporters located on the apical or luminal membrane of endothelial cells which form a tight barrier, e.g. BBB, blood-CSF barrier and blood-placenta barrier, prevent molecules from entering specific tissues by pumping them into the blood stream. Membranes from other endothelial cells contain pores via which small molecules can freely diffuse into and out of cells. Still, transporters are present in the membranes of these cells which can pump molecules against a steep concentration gradient. By this, transporters often provide protection against harmful molecules, nevertheless, their role can be disadvantageous when eliciting drug resistance. Transporter-mediated treatment failure is a major clinical complication in cancer therapy. Their role in drug resistance is reinforced by the fact that expression levels of transporters are tightly regulated and reduced intracellular drug accumulation is often attributed to an overexpression of efflux proteins. This thesis focusses on BCRP, a member of the G subfamily of ABC transporters.

**Breast cancer resistance protein (BCRP)**

The human ABCG subfamily (as well as the murine and rat Abcg subfamily) contains five members, i.e. ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8. Most of the ABC transporters are full transporters, containing two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) on one polypeptide. Family members of the human ABCG subfamily, however, are so-called half transporters that have one NBD at the N-terminus and one TMD, which possesses six transmembrane helices, at the C-terminus (figure 1): a reverse orientation compared to all other ABC genes. These half transporters require dimerization to form a functional transporter.\(^\text{17}\)

In 1998, Doyle et al.\(^\text{18}\) were the first to report a gene, responsible for the novel resistance phenotype found in mitoxantrone-treated human MCF-7 breast cancer cells. They named the gene BCRP since it was cloned from a subline of breast cancer cells. Soon thereafter, Allikmets et al.\(^\text{19}\) described a nearly identical transporter, termed ABCP as this ABC transporter was found to be highly expressed in placenta. Then, another group cloned a gene responsible for the high degree of resistance to mitoxantrone observed in the colon carcinoma cell line S1-M1-80 and named it mitoxantrone resistance gene, or MXR.\(^\text{20}\) The genes described proved to be nearly identical when their sequences became available and the BCRP/ABCP/MXR gene was placed in the G subfamily of ABC transporters and was assigned the name ABCG2.\(^\text{9}\)
In two ways BCRP is a misfit between its fellow subfamily members. Firstly, the other four members, the intracellularly located ABCG1 and ABCG4, and the plasma membrane located ABCG5 and ABCG8, are involved in transmembrane transport of phospholipids and sterols (like cholesterol)\textsuperscript{21-24}, while BCRP is a nonsterol transporter.\textsuperscript{25} Secondly, BCRP functions solely as a homodimer, whereas ABCG5 and ABCG8 are known to form a functional heterodimer, which promotes the efflux of cholesterol and sitosterol.\textsuperscript{21,26,27} Also, ABCG1 has been described to heterodimerize with ABCG4 to form a functional transporter.\textsuperscript{25,28}

Dimerization of two adjacent BCRP molecules is realized via disulfide bonds, which are formed in the lumen of the endoplasmic reticulum. The extracellular cysteine- (Cys-) 603 is important for dimer formation as an interpeptide disulfide bond links both Cys-603 residues of two monomeric BCRP molecules (figure 1). Because of the high homology of amino acid sequences in the extracellular loop between the human \textit{ABCG2} gene and its orthologues in monkey, pig, cow, dog, rat, mouse, and fowl, it is assumed that the cysteine residue corresponding to Cys-603 is critically involved in Bcrp homodimer formation in those species as well.\textsuperscript{27} Removal of Cys-603 abolishes dimerization without affecting expression and plasma membrane-targeting of the transporter. Two other cysteine residues in the extracellular loop, \textit{i.e.} Cys-592 and Cys-608, appear to be essential for protein stability as well as for plasma membrane-targeting of BCRP.\textsuperscript{29,30} These cysteine residues are considered to form an intramolecular disulfide bond, as illustrated in figure 1.
General introduction

Other posttranslational modifications of BCRP include N-glycosylation and phosphorylation. BCRP is often present in polarized cells, which are characterized by their asymmetric cell surface organization (the apical and the basolateral surface). The delivery mechanism for membrane-bound proteins in these cells is more complex than those of non-polarized cells. It has been demonstrated that N-glycosylation of BCRP plays an important role in protein folding, maintenance of protein stability and the translocation of a BCRP dimer to the apical plasma membrane.32,33 Besides, N-glycosylation of BCRP appears to enhance ubiquitin-mediated proteasomal degradation.34 Sequence analyses revealed three potential N-linked glycosylation sites in human BCRP35 and two in ratBcrp36. Moreover, BCRP is phosphorylated at threonine 362, which promotes BCRP dimerization and ultimately its plasma membrane localization.37

BCRP in blood-organ barriers

The majority of BCRP is localized to the plasma membrane where it can export molecules out of the cell. Many epithelia in the body express BCRP, e.g. ovary, prostate, intestine, liver, kidney, and lactating mammary gland, as well as tissues with a tight barrier function like the blood-organ barriers.15,16,38,39 These barriers consist of capillary endothelial cells whose tight junctions between adjacent cells are highly developed, which prevent small molecules to easily reach the organ (figure 2). By this tight barrier as well as by efflux transporters, which are present in the endothelial cells and remove those molecules that were able to pass the barrier, the particular organ is protected from harmful molecules. The BBB is the blood-organ barrier that separates the circulating blood from the brain interstitial fluid. Schinkel et al.40 were the first to show the importance of efflux transporters in the BBB when they found an unusual high mortality in Mdr1a-/- knockout mice after treatment with the centrally neurotoxic antiparasitic agent, ivermectin. Next to P-gp, BCRP is abundantly expressed in the BBB (figure 2A).41 Many drugs are BCRP substrates and BBB transporter function is advantageous when the particular drug is aimed to target peripherally located receptors because BCRP limits brain drug exposure and potentially coincided side effects. However, anti-epileptics, antidepressants, and anti-psychotics target receptors in the brain and, therefore, BCRP-mediated limitation of brain exposure can obviously be disadvantageous. BCRP is also present in the choroid plexus, the so-called blood-CSF barrier, where it facilitates transport of substrates from the blood towards the CSF (figure 2B). The CSF provides a highly controlled and stable environment for the central nervous system and allows for homeostatic regulation by distribution of neuroendocrine factors, electrolytes and nutrients, and removal of metabolic
waste. CSF is formed as plasma is filtered from the blood through the epithelial cells of the choroid plexus, which is located in the major cisternae of the third and fourth ventricles of the brain. Obviously, it is difficult to measure drug concentrations at the target site of drugs that act on the central nervous system. Therefore, often drug concentration in the CSF was used as a surrogate.42

Another blood-organ barrier is the blood-placenta barrier. The placenta is an organ that brings maternal and fetal blood circulations into proximity, allowing mutual interchange of nutrients and waste products. In the placenta, BCRP is highly expressed at the membrane of syncytiotrophoblasts and helps to prevent potentially harmful molecules from entering the fetal compartment. BCRP expression was also found on the luminal (fetal-facing) surface of fetal blood vessels of the villous core (figure 2C) and is thought to regulate the transfer of various endogenous compounds to the fetus.43 Though, syncytial BCRP expression is much higher as compared to BCRP expression in fetal blood vessels. Studies using a dually perfused human placenta model and Bcrp-deficient animal models showed that fetal exposure to topotecan, genistein, nitrofurantoin, and glyburide was limited by BCRP.44-47 Its expression
levels have been shown to decrease throughout pregnancy, leaving the fetus susceptible to drugs that are administered to pregnant women (i.e. synthetic glucocorticoids, selective serotonin reuptake inhibitors, glyburide, antiretrovirals) at later stages of the pregnancy. In this respect, the regulation of BCRP is intensively studied. Steroid hormones are thought to play an important role in this, as explained further in this chapter.

In the testis, the blood-testis barrier (BTB) physically divides the seminiferous epithelium into the basal and the apical compartment, thereby creating a testicular microenvironment that is pivotal for mammalian steroidogenesis and spermatogenesis. Unlike other blood-organ barriers, the BTB consists of several cell types that together protect the developing germ cells. Tight junctions, adherens junctions and gap junctions between the Sertoli cells, which nourish and support the developing germ cells throughout the stages of spermatogenesis, prevent molecules to easily reach the adluminal compartment of the seminiferous epithelium. The active part of the BTB consists of efflux transporters. P-gp, BCRP, MRP1 and MRP4 are most abundantly expressed and are located in the Sertoli cells, steroid producing Leydig cells or capillary endothelium. BCRP is only expressed in the luminal membranes of testicular capillary endothelium (figure 2D). The positioning of the efflux transporters in the BTB suggests that their substrates are transported out of the seminiferous tubules to prevent entry and accumulation of harmful molecules in the testes and to protect the developing germ cells.

**Role of BCRP in physiology and pathology**

Nowadays, BCRP is recognized as a xenobiotic transporter that plays a major role in multidrug resistance and a myriad of BCRP substrates have been described, among them xenobiotics like chemotherapeutic agents, tyrosine kinase inhibitors, antivirals, HMG-CoA reductase inhibitors, and flavonoids. Besides the protective role of BCRP against xenobiotic compounds, accumulating evidence shows that BCRP also transports endogenous compounds. Uric acid is such an endogenous substrate for BCRP. It is a weak organic acid and the end product of purine nucleotides degradation in humans. Elevated plasma uric acid levels, i.e. hyperuricemia, are related to a variety of pathologies, including gout, cardiovascular disease and chronic kidney disease. Gout is the most common form of inflammatory arthritis caused by sodium uric acid crystal precipitation. Formation of these crystals is also the cause of nephrolithiasis. Hyperuricemia is caused by either an increase in uric acid production or by a decrease in its excretion. Efflux transporters, like BCRP, located on the apical membrane of the kidney proximal tubule cells play an important role in the elimination of uric acid.
vital role of BCRP in uric acid homeostasis can clearly be observed in patients suffering from hyperuricemia due to single nucleotide polymorphisms (SNPs) that render the transporter inactive, such as the common SNP C421A encoding the Q141K mutation. BCRP is also involved in the elimination of several porphyrins, like protoporphyrin IX, hematoporphyrin and heme. Heme is the functional group of hemoproteins and crucial for many cellular processes. However, excess of free heme can be detrimental to tissues by mediating oxidative and inflammatory injury. Heme scavengers act as the first line of defense against the injurious actions of heme, however, they may be saturated when exposed to large amounts of heme. Downstream, several putative defense systems against intracellular heme are known, such as heme oxygenase-1 (HO-1), but also the efflux transporter BCRP. HO-1 degrades heme into biliverdin, iron, and carbon monoxide. A possible interplay between the two proteins has not yet been described.

Furthermore, BCRP was also found to be a highly conserved feature in a wide variety of stem cells. At first it was noticed that a distinct subpopulation of cells, side-population cells, accumulated less dye when using the fluorescent DNA dye Hoechst33342 to stain mouse bone marrow cells. Later, Zhou et al. identified Bcrp as being responsible for preservation of the side-population and its potential role in the regulation of hematopoietic development has been suggested. However, the transporter does not appear to be necessary for normal hematopoiesis as Bcrp-deficient mice are viable with normal numbers of stem cells, despite the complete absence of side-population cells. BCRP has been shown to provide protection to the hematopoietic stem cells against cytotoxic substrates. Nowadays, it is known that side-population cells not only reside within the bone marrow but just as well in non-hematopoietic organs. These non-hematopoietic side-population cells can differentiate into many cell types and thereby contribute to tissue repair. In side-population cells, BCRP/Bcrp expression falls dramatically with the differentiation of pluripotent stem cells. Krishnamurthy et al. studied the role of BCRP in these cells and they suggested that the transporter provides protection against intracellular accumulation of heme-related molecules (e.g. porphyrins) to enable cell survival under conditions of hypoxia.

Furthermore, it has been demonstrated that BCRP is expressed in alveolar epithelial cells of the mammary gland during pregnancy and lactation, where it actively secretes a variety of drugs, carcinogens, and toxins into milk. In apparent contradiction with its detoxifying role elsewhere in the body, this BCRP-mediated contamination of milk exposes suckling infants to potentially harmful molecules. Yet, the evolutionarily conserved expression and induction of BCRP in the lactating mammary gland suggests an important physiological function. Therefore, van Herwaarden et al. proposed that BCRP might function to provide the milk
with essential nutrients such as riboflavin (vitamin B2) and possibly also biotin (vitamin B7), offsetting the coincidental risk of contaminating milk with harmful molecules. Interestingly, the endogenous BCRP substrates folic acid and vitamin B12 were not actively secreted into milk by murine Bcrp.

**BCRP and steroid hormones**

The localization of BCRP in the plasma membrane is shown to be sex specific. The sex steroids estradiol, progesterone and testosterone have been shown to impact BCRP expression. In estrogen-responsive cells, estradiol down-regulates mRNA expression of BCRP and BCRP function is impeded as estradiol initiates its internalization and stimulates proteosomal degradation. Estradiol signals through the estrogen receptors ERα and ERβ.51,75-78 Progesterone upregulates BCRP expression via a progesterone response element in the promoter, as shown in a cancer cell line derived from human placental choriocarcinoma.79 The inductive effects of testosterone on BCRP mRNA expression were shown by Tanaka et al.78 in kidneys and liver of castrated rats and mice in combination with sex steroid treatment. Besides regulatory properties, steroids are also linked with transport activity. BCRP transports conjugated steroids, such as dehydroepiandrosterone sulfate (DHEAS), estrone sulfate (E1S) and, to a lesser extent, estradiol glucuronide (E217βG).80-83 Moreover, a role for BCRP in transport of androgens in prostate stem cells84 and transport of estradiol in membrane vesicles from *Lactococcus lactis* containing functional human BCRP85 was suggested. Attempts to confirm a similar role for BCRP expressed in mammalian cells have failed until now, suggesting that the membrane micro-environment is of importance in transporting the highly lipophilic hormones. A steroid binding site in BCRP has been proposed by two independent groups using an *in silico* docking approach.86,87 These findings suggest a role for BCRP in hormone metabolism and regulation, however, the molecular bases of protein-steroid interactions are yet unclear.50

**In vitro and in vivo models to study BCRP and related ABC transporters**

Methods to study ABC transporter function vary from organ perfusion systems, tissue cultures, isolated subcellular fractions (*e.g.* microsomes) to cloned and expressed single proteins. With these methods transport processes can be studied at various levels of complexity. Most of the *in vitro* methods make use of transporter-overexpression cell models, generated either by stable transfection or by transient transfection or transduction. A common and fast assay
that allows for the rapid screening and identification of potential substrates and inhibitors is the ATPase assay. In this assay, membranes of transporter-overexpressing cells are incubated with potential substrates. Since transporter-mediated efflux requires ATP hydrolysis, hence, stimulation of ATPase activity suggests that the compound is a possible substrate or inhibitor. Another high-speed method is the membrane vesicle transport assay (also called uptake assay). Membrane vesicles are prepared from transporter-overexpressing cells. Both right-side-out and inside-out oriented vesicles are generated, however, only transport of molecules into the cavity of inside-out oriented vesicles can be measured (figure 3).

Figure 3. A schematic illustration of a rapid screening method of human BCRP-molecule interactions using inside-out membrane vesicles isolated from BCRP-overexpressing HEK293 cells. Using the energy derived from ATP hydrolysis, substrate molecules (green) are transported into the vesicle by BCRP that can be inhibited by specific inhibitors (orange). (Figure was created using images from Servier Medical Art; http://www.servier.com/Powerpoint-image-bank)
In this thesis, we used membrane vesicles prepared from transporter-overexpressing human embryonic kidney (HEK293) cells, established using baculovirus transductions that were produced using the Bac-to-Bac system (Invitrogen). Using this assay, substrates and transport inhibitors can be identified, as well as their kinetic characteristics ($K_m$ or $K_i$). Furthermore, the model is useful in predicting drug-drug interactions. However, the assay is only suitable for assessing the transport of hydrophilic (polar) compounds as lipophilic (non-polar) compounds often diffuse out of the vesicles after the incubation period, hence, transporter-mediated uptake is difficult to determine.

Besides, these compounds easily stick to the filters and therefore high background signals disturb transport measurements. For determining transport of non-polar compounds, often cell assays are used. In this thesis, we used Madin-Darby canine kidney (MDCKII) cells stably overexpressing BCRP and P-gp. Transport of molecules can be studied using MDCK cell monolayers in normal well plates or transwells. Both assays highly depend on passive diffusion of the test compound over the cell membrane, as efflux can be determined only after the compound has entered the cell. In the accumulation assay, cell monolayers are loaded with the compound of interest together with a transport inhibitor. Since active transport out of cells is much faster than passive diffusion back into cells, and transporters mediate the efflux of molecules against concentration gradients, a difference in accumulation in transporter-overexpressing cells compared to control cells demonstrates the involvement of the transporter in the efflux of the test compound. In transwell transport assays, polarized cells, grown on a semi-permeable membrane, form a barrier between the apical compartment and the basolateral compartment of the well. By adding the compound to either the apical or the basolateral compartment, apical-to-basolateral transport or basolateral-to-apical transport can be determined to delineate the bidirectional transport of the compounds.

If the substrate is available in radioactively labeled form, the accumulation assay is often the method of choice because it is faster and less laborious than the transwell transport assay. However, when analyzing substrates by analytical methods like high-pressure liquid chromatography (HPLC), which is often combined with tandem mass spectrometry (LC-MS/MS), it is much easier to extract the target molecules out of media (transwell assay) than out of cellular matrices (accumulation assay). These in vitro methods are able to identify novel substrates and transporter inhibitors. Besides, transport kinetics and drug-drug interactions can be studied. Nevertheless, the relevance of a transporter in the disposition of a certain compound is usually studied in vivo.
Chapter 1

The development of transporter knockout animals has provided us with more insight into the function and characteristics of ABC transporters, however, these models are not devoid of limitations. Compensatory gene expression and protein synthesis of other transporters may counteract the lack of the specific gene.93 Yet, short-term exposure to transporter inhibitors is often not that straightforward. Inhibitors not always affect the transporters completely or they are not specific for one transporter. Furthermore, some inhibitors only affect the transporter at cytotoxic concentrations and are, therefore, not suitable for in vivo utilization. For BCRP/Bcrp, fumitremorgin C (FTC) is an effective and specific inhibitor but neurotoxic effects disqualify its use in vivo. Its derivative Ko143 appeared to be more potent than FTC in vitro and has been used in in vivo experiments.94,95 Also GF120918 (elacridar), which potently inhibits both P-gp and BCRP, is used in animals and is shown to be extremely well tolerated in mice as well as in human.95-97 Using these in vivo models, the effect of BCRP on the disposition of certain molecules can be studied by comparing accumulation of substrates in plasma and organs of wild type and Bcrp-deficient or Bcrp-inhibited animals. When analyzing the role of BCRP in a specific organ, in situ or ex vivo perfusion models are often used, i.e. kidney (urinary elimination), intestine (absorption), BBB (brain penetration), liver (biliary excretion) and placenta (transplacental passage) perfusion models. Furthermore, combining confocal microscopy with quantitative digital image analysis, isolated brain capillaries from rats and mice can be studied and visualized ex vivo. With this state-of-the-art technique, it is not only possible to study the transport route of a certain molecule but allows to identify Bcrp expression modulators as well. Ultimately, combining the results of different molecular, cellular and in vivo studies are required to identify the exact role of individual transporters in the distribution of certain compounds.

Aim and outline of this thesis

Research on the role of BCRP in most organs has until now been focused predominantly on the restriction of drug penetration and its protective function against the toxic effects of exogenous compounds. Yet, accumulating evidence shows that BCRP transports endogenous molecules just as well. Most of these substrates are transported out of organs into the blood, however, in some tissues the direction of transport is the opposite, like in the mammary gland, liver, kidneys and choroid plexus. Together with its broad range of substrates, this emphasizes the efficiency of BCRP as a transporter and suggests that, while there are redundant mechanisms of xenobiotic protection, the protein is also very important in physiology. The aim of this thesis was to investigate the role of the efflux transporter BCRP in endocrine and metabolic processes.
Chapters 2, 3 and 4 focus on BCRP/Bcrp in endocrine organs. In chapter 2, the presence and localization of Bcrp was investigated in endocrine organs of wild type mice. Moreover, the interaction of various steroid hormones with BCRP activity was studied. Chapters 3 and 4 describe the interaction between several endocrine disrupting chemicals that, in males, predominantly target the testis, and several efflux transporters present in the BTB using in vitro methods (chapter 3). Furthermore, the role of BCRP in the disposition of one of these endocrine disruptors, bisphenol A, was examined in vivo in mice. The results of this study are described in chapter 4.

The role of BCRP in important metabolic processes is described in chapters 5, 6 and 7. The protection against heme, which induces oxidative stress, by two significant protective proteins, HO-1 and BCRP is described in chapter 5. Furthermore, the role of BCRP and MRP4 in the regulation of kynurenic acid levels in the brain (chapter 6) and in the kidneys of hyperuricemic mice (chapter 7) was investigated. The thesis concludes with chapter 8, where the implications of this research and future perspectives are discussed.
Chapter 1

References


Chapter 1


General introduction


Chapter 1


Localization of breast cancer resistance protein in endocrine organs and inhibition of its transport activity by steroid hormones

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Abstract

Breast cancer resistance protein (BCRP) is known for its protective function against the toxic effects of exogenous compounds. In addition to this, a role in the transport of endogenous compounds has also been described. Since BCRP in the plasma membrane was shown to be regulated by sex steroids, we investigated the presence and possible role of BCRP in steroid hormone producing organs. Therefore, the presence and localization of Bcrp was investigated in endocrine organs of wild type mice. Furthermore, the interaction of various steroid hormones with human BCRP activity was studied. Quantitative PCR revealed Bcrp mRNA in the pituitary and adrenal glands, pancreas, ovary, testis and adipose tissue. Immunohistochemistry revealed the presence of Bcrp in the cortex of the adrenal gland and in plasma membranes of adipocytes. In the pituitary gland, pancreas, ovary and testis, Bcrp was mainly located in the capillaries. The interaction between BCRP and twelve steroid hormones was studied using membrane vesicles of HEK293-BCRP cells. Estradiol, testosterone, progesterone and androstenedione inhibited BCRP-mediated uptake of $^3$H-estrone sulfate ($E_1S$) most potently, with calculated inhibitory constant ($K_i$) values of $5.0 \pm 0.2$ µM, $36 \pm 14$ µM, $14.7 \pm 1.3$ µM and $217 \pm 13$ µM, respectively. BCRP function was attenuated non-competitively, which implies an allosteric inhibition of BCRP-mediated $E_1S$ transport by these steroids. In conclusion, localization of Bcrp in endocrine organs together with the efficient allosteric inhibition of the efflux pump by steroid hormones are suggestive for a role for BCRP in steroid hormone regulation.
Introduction

Breast Cancer Resistance Protein (BCRP; ABCG2) is a member of the ATP Binding Cassette (ABC) superfamily of transmembrane proteins involved in the transport of a variety of molecules against steep concentration gradients at the expense of ATP. BCRP is a half transporter that requires dimerization to become a fully active efflux pump and was originally discovered in a breast cancer cell line resistant to chemotherapeutics. Nowadays, BCRP is recognized as a xenobiotic transporter that plays a major role in multidrug resistance. The efflux pump is present in various tissues with a barrier function, including the placenta, prostate, small intestine, brain, colon, liver, mammary gland and kidney. Besides the protective role of BCRP against accumulating xenobiotic compounds, a role in the transport of endogenous compounds has also been described, such as the transport of folic acid, heme, urate and uremic toxins. Moreover, BCRP transports conjugated steroids, such as dehydroepiandrosterone sulfate (DHEAS), estrone sulfate (E1S) and estradiol glucuronide (E217βG). The localization of BCRP in the plasma membrane is shown to be sex specific, which is suggested to be due to the suppressive effect of estradiol and the inductive effect of testosterone. Also, progesterone has been shown to regulate BCRP transcription in cancer cell lines, including BeWo cells, a cell line derived from human placental choriocarcinoma. Moreover, a role for BCRP in transport of androgens was suggested in prostate stem cells and transport of estradiol was demonstrated in membrane vesicles from Lactococcus lactis containing functional human BCRP, indicating that the efflux pump might also play a role in steroid action. In this respect, the presence of BCRP was described for some hormone producing organs, but for some organs, only BCRP mRNA content or presence of the protein by Western blot was shown. In the present study, we investigated murine organs, including the pituitary and adrenal glands, pancreas, kidney, ovary, testis and adipose tissue for the presence and localization of Bcrp. Furthermore, we evaluated the effects of several sex steroids on BCRP-mediated substrate transport using membrane vesicles of baculovirus transduced HEK293 cells. Our results show presence of the efflux pump in steroid producing organs. In addition, the sex steroids estradiol, testosterone, progesterone and androstenedione inhibited BCRP function in a concentration-dependent manner.
Chapter 2

Materials and methods

RNA isolation and quantitative PCR
All procedures involving animals were approved by the Animal Experimental Committee of the Radboudumc. Bcrp mRNA was assessed in the adrenal gland, pituitary gland, epididymal fat pad, abdominal fat pad, pancreas, testis, ovary and the kidney of Friend leukemia virus B (FVB) mice (Charles River Laboratories, Germany). Isolated organs were immediately snap frozen in liquid nitrogen until further analysis. Fat pads, adrenal glands and pituitaries were homogenized using micropestles. Other organs were homogenized in frozen state using a Mikro-dismembrator U (Sartorius B. Braun Biotech Int., Melsungen, Germany). To avoid RNA degradation, the metal cylinders were washed with 0.5 M NaOH prior to use. Subsequently, total RNA was isolated using a NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer’s instructions. Immediately, a reverse transcriptase reaction was performed with 250 ng RNA using random primers (Invitrogen, Breda, the Netherlands) and an Omniscript® RT kit (Qiagen, Hilden, Germany), following manufacturer’s recommendations. Synthesized cDNA was used for quantitative PCR, performed in a StepOnePlus™ Real-Time PCR system by means of the TaqMan® protocol (Applied Biosystems, Warrington, UK). Bcrp mRNA concentration was normalized to the mRNA concentration of the housekeeping gene β-actin. The primer-probe sets were obtained from Applied Biosystems (β-actin; 4352933E, Bcrp; Mm00496364_m1).

Immunohistochemistry
The localization of Bcrp was assessed by immunohistochemistry. Organs from Bcrp−/− mice were used as negative controls. The knockout mice were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands) and were bred and housed at the Central Animal Laboratory of the Radboudumc. The organs were fixed by cardiac perfusion. Briefly, the mice were anesthetized with a single, lethal i.p. injection of 100 mg/kg pentobarbital (Nembutal, 60 mg/ml). The hearts of anesthetized mice were exposed and the right atrium was clipped with surgical scissors. A 23-gauge needle was inserted into the left ventricle and phosphate buffered saline (PBS) containing heparin (300 ml/h) was administered. Subsequently, the mice were perfused with a 4% w/v formaldehyde solution, freshly prepared from paraformaldehyde. The organs were removed and fixed in a 4% w/v formaldehyde solution for 24 h and embedded in paraffin, except for fat pads, which were fixed in Bouin’s fixative immediately after the perfusion fixation. Sections of 5 µm were mounted on 3-aminopropyltriethoxysilane (APES) coated slides and dried for 2 h at 57 °C.
Localization of Bcrp in endocrine organs and inhibition of its transport activity by steroid hormones

After deparaffinization with xylene and rehydration, the slides were heated in sodium citrate buffer (pH 6) at 100 °C for 15 min. Endogenous peroxidase was blocked with 1.5% v/v \( \text{H}_2\text{O}_2 \) for 30 min. After blocking with nonimmune rabbit serum, the slides were incubated with primary antibody against mouse Bcrp (BXP-9, 1:20; Kamiya Biomedical Company, Seattle, WA, USA) overnight at 4 °C. The biotinylated secondary antibody (rabbit-anti-rat, 1:500; Acris Antibodies GmbH, Herford, Germany) was incubated for 30 min followed by a 30 min incubation with standard avidin-biotin complex (ABC; Brunschwig Chemie, Amsterdam, the Netherlands). Next, DAB chromogene was used for visualization. Slides were counterstained with Mayer’s hematoxylin, dehydrated, and mounted with DPX. Sections were evaluated by means of a light microscope (Leica DM 6000 B) and digitized using a Leica DFC480 digital camera (Leica Microsystems, Wetzlar, Germany).

Transduction of Human Embryonic Kidney (HEK293) cells and isolation of membrane vesicles

Increased synthesis of human BCRP and MRP3 in HEK293 cells was established using baculoviruses, which were produced using the Bac-to-Bac system (Invitrogen, Breda, the Netherlands) with BacMamVSV-EX-hBCRP (pENTR221-hBCRP; HsCD00044371; Harvard Institute of Proteomics, Harvard Medical School, Boston, MA, USA) and BacMamVSV-EX-hMRP3 (sequence of MRP3 was equal to GenBank accession number NM_003786), as described previously by Wittgen et al.\(^28\). As a control, the enhanced yellow fluorescent protein (eYFP) was introduced into the baculoviruses as well. Crude membranes of HEK293-BCRP,-MRP3,-eYFP cells were isolated, resuspended in TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) and membrane vesicles were prepared according to a previously described method.\(^29\) Total protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Veenendaal, the Netherlands). Crude membrane vesicles were dispensed in aliquots, snap frozen in liquid nitrogen, and stored at -80 °C until further use.

Western blot analysis

Membrane vesicles were prepared for gel electrophoresis by incubation with Laemmli sample buffer (consisting of 0.5 M tris-HCl pH 6.8, 8% w/v sodium dodecyl sulfate (SDS), 40% w/v glycerol, 0.08% w/v bromophenol blue and 0.4 M \( \beta \)-mercapto-ethanol) for 10 min at 65 °C. Proteins (15 µg total protein per sample) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and blotted onto a nitrocellulose membrane using a dry blot system (iBlot; Invitrogen, Breda, the Netherlands). The membrane was incubated overnight at 4 °C with mouse-anti-hBCRP antibody (BXP-21, 1:200; Kamiya Biomedical
Company, Seattle, WA, USA) or mouse-anti-hMRP3 antibody (M_III-21, 1:200; Abcam, Cambridge, UK). Primary antibodies were detected using fluorescently labelled goat-anti-mouse secondary antibody (IRdye800; 1:10,000 Rockland Immunochemicals, Boyertown, PA, USA). Signals were visualized using the Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

**BCRP-mediated estrone sulfate (E$_1$S) uptake and inhibition by steroids**

Uptake of [$^3$H]-E$_1$S into HEK293-BCRP and -eYFP membrane vesicles was performed using an assay which was well established in our lab.$^{16,28,29}$ A reaction mix consisting of TS buffer supplemented with 4 mM ATP/AMP, 10 mM MgCl$_2$ and various concentrations of [$^3$H]-E$_1$S at pH 7.4 was added to 7.5 µg of membrane vesicles (based on total protein content). After an incubation of 60 sec at 37 ºC to enable ATP-dependent uptake, the reaction was stopped by placing the samples on ice and by addition of ice-cold TS buffer. Reaction mix was removed and the vesicles were washed by means of a rapid filtration technique using glass fiber filter plates (Millipore, Etten-Leur, the Netherlands). Scintillation fluid was added to the filters and the amount of radioactivity was determined using a scintillation counter (Tri-Carb® 2900TR; Perkin Elmer, Waltham, MA, USA). Reference samples were measured to calculate the amount of transported E$_1$S. ATP-dependent transport was calculated by subtracting values measured in the presence of AMP from those measured in the presence of ATP. Net BCRP-mediated E$_1$S transport was calculated by subtracting ATP-dependent E$_1$S uptake in HEK293-eYFP vesicles from that of HEK293-BCRP vesicles. Linearity of E$_1$S uptake in time was determined using 250 nM E$_1$S.

The effects of several androgens, estrogens and progestagens (all obtained from Sigma-Aldrich, St. Louis, MO, USA or Steraloids Inc., Newport, RI, USA) on vesicular E$_1$S uptake were assessed by performing the above-mentioned transport assay in the presence of 50 µM of the steroid. All steroid hormones were dissolved in ethanol. Maximum ethanol concentrations of 1.3% v/v were used in uptake experiments and solvent controls were included to exclude non-specific effects. The inhibition by the steroid hormones estradiol, testosterone, progesterone and androstenedione on vesicular E$_1$S uptake were studied in more detail. Therefore, the membrane vesicles were incubated in the presence of increasing concentrations of the steroid at three different concentrations of [$^3$H]-E$_1$S (50, 100, and 250 nM). In studies with androstenedione, the glass fiber filter plate was pre-incubated with 50 mg/ml BSA for one hour at 37 ºC and the reaction mix was supplemented with 0.2 mg/ml BSA, to reduce the background which is caused by adhesion to the filter plate. The sidedness of the membrane vesicles was not determined as ATP-dependent uptake can occur only in inside-out vesicles. The relative inhibition by steroids was expressed as percentage of maximum uptake.
Localization of Bcrp in endocrine organs and inhibition of its transport activity by steroid hormones

Inhibition of MRP3-mediated estradiol glucuronide (E$_2$17βG) uptake by steroids

The effects of estradiol, testosterone, progesterone and androstenedione on MRP3 activity was determined by the same method as described above, using E$_2$17βG as a substrate. HEK293-eYFP and -MRP3 membrane vesicles were incubated with 80 nM [³H]-E$_2$17βG with or without the steroids, for 3 min at 37 °C. The washing steps were performed using PVDF filter plates (Millipore, Etten-Leur, the Netherlands). These parameters were adapted from Wittgen et al. 28. Unlabeled E$_2$17βG (100 µM) was used as a positive control for inhibition of MRP3 activity.

Data analysis

Relative Bcrp mRNA concentration was normalized for the cycle threshold (Ct) value of the endogenous reference gene β-actin (delta Ct; dCt) and depicted as the reciprocal of dCt (mean ± SEM). Differences in mRNA concentration between male and female organs were assessed by means of a Student’s t-test, considering P < 0.05 significant. Fold differences in Bcrp mRNA levels between the organs were calculated using 2$^{dCt\text{ organ1}-dCt\text{ organ2}}$. Uptake experiments were performed in triplicate in three independent batch isolations. Michaelis-Menten analysis was used to study transport kinetics. Inhibition curves are depicted as mean ± SEM of three independent experiments. Curve fitting was performed by non-linear regression analysis. The mode of inhibition was determined using Dixon’s method combined with linear regression analysis to estimate the inhibitory constant (K). A representative of one experiment is depicted. Differences in MRP3-mediated E$_2$17βG uptake were assessed by means of a one-way ANOVA test followed by Dunnett’s post hoc test. All analyses were performed with GraphPad Prism software (version 5.02, GraphPad Software Inc., San Diego, CA, USA).

Results

Bcrp mRNA analysis in murine organs

Bcrp mRNA levels in murine endocrine organs were determined by means of quantitative PCR (figure 1). For comparison, mRNA levels in kidneys were evaluated, which are known to be very high. 8 The relative Bcrp mRNA concentration was normalized to the mRNA concentration of the endogenous reference gene β-actin. The variation in cycle threshold (Ct) values of β-actin indicated that the qPCR reactions were reproducible (SEM ≤ 0.92). Bcrp mRNA was present in all organs tested. The relative mRNA concentration in the adrenal gland, pituitary gland, epididymal and abdominal fat, the pancreas and in the ovary was comparable and
found to be more than 30 times lower as compared to mRNA levels in the kidney. *Bcrp* mRNA levels in the testis was about four times higher than in the other organs, but 9-fold lower as compared to the kidney. Interestingly, mRNA levels of *Bcrp* in male mice were slightly higher than in female mice (*P* < 0.01 for the kidney and *P* < 0.05 for other organs), while there was no difference in *β-actin* mRNA concentration between samples from males and females.

**Figure 1.** Relative *Bcrp* mRNA concentration in wild type mouse organs, normalized for the cycle threshold (Ct) value of the endogenous reference gene *β-actin* (delta Ct; dCt). For all experiments, tissues from six mice were individually analyzed, except for the adrenal gland and the pituitary gland. For those, three samples of two pooled organs were analyzed. Bars represent the reciprocal of mean dCt ± SEM of independent experiments (N=3-6). *P* < 0.05; **P** < 0.01 compared to *Bcrp* mRNA concentration in males by Student’s *t*-test. There was no difference in *β-actin* mRNA concentration between males and females. The SEM of *β-actin* Ct values was ≤ 0.92.

**Localization of Bcrp in murine organs**

Immunohistochemical analysis revealed localization of Bcrp protein in numerous murine organs. Organ sections of *Bcrp<sup>−/−</sup>* mice were used to determine nonspecific staining. Both male and female organ sections were evaluated, but no differences in localization of the protein between male and female were found (data not shown). Therefore, only organs from female mice are shown here, except for the testis. Figure 2 depicts Bcrp localization in organs with a barrier function, viz. kidney, brain and liver. In the kidney, Bcrp staining was most prominently observed in the proximal tubular lumen, the brush border. Also in Bowman’s capsule brush border, which is present in murine nephrons, Bcrp was localized. The distal tubular lumen did not contain Bcrp. No Bcrp staining was found in the negative control sections (*Bcrp<sup>−/−</sup>*).
Localization of Bcrp in endocrine organs and inhibition of its transport activity by steroid hormones

Figure 2. Representative immunohistochemical images of Bcrp distribution in wild type (left panels) and Bcrp−/− (negative control; right panels) mouse kidney, brain tissue and liver. Because of an absence of gender difference in localization, only organs from female mice are shown. Perfusion fixed, paraffin embedded organ-sections were incubated with a primary antibody against mouse Bcrp (BXP-9). DAB chromogen staining (brown) visualizes Bcrp localization. The sections were counterstained with Mayer’s hematoxylin. Bars 100 µm. Inserts represent details of Bcrp positivity and corresponding negative controls. Bcrp was present in the brush border of proximal tubule and in the brush border of Bowman’s capsules (arrow) of the kidney and in the capillaries of the brain and liver.
Furthermore, Bcrp was localized in the capillaries of the brain and liver. Besides presence of Bcrp in organs with a barrier function, the protein was found in endocrine organs (figure 3 and 4). In the sinusoidal capillaries in adrenal gland cortex, Bcrp was clearly localized. In addition to this, some cells showed intracellular Bcrp staining in the zona glomerulosa and zona reticularis, but neither in the zona fasciculata nor in the medulla of the adrenal gland intracellular Bcrp staining was found. In the capillaries of the anterior pituitary gland, Bcrp was localized at the apical surface of the endothelial cells. The hormone producing cells of the pituitary gland showed no Bcrp-specific staining. The adipocytes in both epididymal and abdominal fat pads showed Bcrp positivity, which was absent in Bcrp−/− sections. No differences in localization of the pump were found between epididymal and abdominal fat pads, therefore, only micrographs of epididymal fat pads were shown in figure 3. Bcrp was not located in the endocrine and exocrine cells of the pancreas (figure 4), but endothelial cells lining the lumen of the capillaries in the pancreas showed a very subtle Bcrp staining, when comparing wild type organs with the negative control organs. Blood vessels of the testis, located between the seminiferous tubules, were Bcrp positive. On the other hand, the interstitial cells of Leydig and the Sertoli cells, which were located within the seminiferous tubules, did not show Bcrp staining. Furthermore, the transport protein was localized in endothelial cells lining the lumen of ovarian blood vessels. Interestingly, not all vessels were Bcrp positive, but Bcrp was present in all small capillaries.

Presence and activity of BCRP in isolated membrane vesicles

Increased synthesis of human BCRP in HEK293 cells was established by transduction with baculovirus containing the recombinant bacmide DNA. Western blot analysis performed on isolated membrane vesicles demonstrated that the transduction was successful, as indicated by the band at ~75 kD (figure 5a). The negative control, consisting of membrane vesicles isolated from HEK293-eYFP cells, showed no presence of BCRP. Transport activity was determined by incubating the membrane vesicles with [3H]-E1S at 37 °C. Time-dependent uptake showed linearity up to 120 s (data not shown). Michaelis-Menten analysis (figure 5b) revealed a Km of 4.5 ± 0.4 µM and a Vmax of 332 ± 12 pmol/mg·min−1, after incubating the vesicles for 60 s at 37 °C. In control (eYFP) vesicles, a maximum uptake rate of 6 pmol/mg·min−1 was found.
Localization of Bcrp in endocrine organs and inhibition of its transport activity by steroid hormones

Figure 3. Representative immunohistochemical images of Bcrp distribution in wild type (left panels) and Bcrp^{-/-} (negative control; right panels) mouse adrenal gland, pituitary gland and epididymal fat. Because of an absence of gender difference in localization, only organs from female mice are shown. Perfusion fixed, paraffin embedded organ-sections were incubated with a primary antibody against mouse Bcrp (BXP-9). DAB chromogene staining (brown) visualizes Bcrp localization. The sections were counterstained with Mayer’s hematoxylin. Bars 100 µm for adrenal gland and 50 µm for pituitary gland and epididymal fat. (a) zona glomerulosa, (b) zona fasciculata, (c) zona reticularis of the adrenal gland are indicated. Inserts represent details of Bcrp positivity and corresponding negative controls. Bcrp was located in the sinusoidal capillaries in adrenal gland cortex, in the capillaries of the pituitary gland and in adipocytes.
Figure 4. Representative immunohistochemical images of Bcrp distribution in wild type (left panels) and Bcrp−/− (negative control; right panels) mouse pancreas (female), testis and ovary. Perfusion fixed, paraffin embedded organ-sections were incubated with a primary antibody against mouse Bcrp (BXP-9). DAB chromogene staining (brown) visualizes Bcrp localization. The sections were counterstained with Mayer’s hematoxylin. Bars 100 µm. Inserts represent details of Bcrp positivity and corresponding negative controls. Blood vessels in the testis and ovary showed distinct Bcrp positivity, whereas in the pancreas, they showed very subtle Bcrp staining.
Localization of Bcrp in endocrine organs and inhibition of its transport activity by steroid hormones

Figure 5. (a) Western blot analysis of HEK293-eYFP- and -BCRP membrane vesicles. BCRP was detected using mouse-anti-BCRP antibody (BXP-21). A Michaelis-Menten plot (b) revealed net BCRP-mediated uptake of E$_1$S. Vesicles were incubated with increasing concentrations of [H]-E$_1$S for 60 s at 37 °C in the presence of AMP or ATP. Net ATP-dependent uptake was calculated by subtraction of AMP values. Control (eYFP) vesicles showed maximum uptake of 6 pmol/mg*min$^{-1}$. Graph represents means ± SEM of triplicate measurements in a representative experiment.

**Inhibition of transport activity of BCRP and MRP3 by steroids**

The effects of several androgens, estrogens and progestagens on BCRP-mediated E$_1$S uptake was assessed by incubating the membrane vesicles with [H]-E$_1$S in the presence of 50 µM of the steroid for 60 s at 37 °C. A substrate concentration of 250 nM E$_1$S was used (figure 5B, which was well below the $k_m$ of E$_1$S uptake to ascertain initial uptake kinetics. Estradiol, testosterone, progesterone and androstenedione inhibited the efflux pump most potently (Table 1) and were, therefore, selected to be studied in more detail. Concentration-dependent inhibition of BCRP-mediated uptake of E$_1$S was found for all four steroids (figure 6a-d). Progesterone was able to inhibit BCRP activity for >90% within the concentration-range tested. Estradiol, testosterone and androstenedione inhibited the efflux pump for 80%, 86% and 70%, respectively, at the highest possible concentrations tested. We were not able to assess whether the steroids were able to fully inhibit the efflux pump using our vesicular transport system because this was limited by the maximum solubility of the steroids in ethanol.
Chapter 2

Table 1. Effect of steroids (50 µM) on BCRP-mediated vesicular E₁S uptake

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Vesicular E₁S uptake (%)</th>
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<tr>
<td>Cholesterol</td>
<td>71.9 ± 5.2</td>
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<tr>
<td>Pregnenolone</td>
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<tr>
<td>17α-hydroxypregnenolone</td>
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<tr>
<td>Dehydroepiandrosterone</td>
<td>58.7 ± 7.8</td>
</tr>
<tr>
<td>Androstenediol</td>
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<tr>
<td>Progesterone</td>
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<tr>
<td>17α-hydroxyprogesterone</td>
<td>108.0 ± 18.7</td>
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<tr>
<td>Androstenedione</td>
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</tr>
<tr>
<td>Testosterone</td>
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</tr>
<tr>
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<tr>
<td>Estrone</td>
<td>ND</td>
</tr>
<tr>
<td>Estradiol</td>
<td>40.4 ± 8.4 a</td>
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<tr>
<td>Estriol</td>
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Values are shown as mean ± SEM of one experiment performed in triplicate. Vesicular E₁S uptake is expressed as a percentage of maximum uptake after incubation with 250 nM [³H]-E₁S with or without 50 µM of the steroid for 60 s at 37 °C. ND, Not determined.

The most potent inhibitors of BCRP-mediated E₁S uptake were selected to be studied in more detail.

Figure 6. Concentration-dependent inhibition of net BCRP-mediated E₁S uptake by estradiol (a), testosterone (b), progesterone (c), and androstenedione (d). Vesicles were incubated with 250 nM [³H]-E₁S and increasing concentrations of the steroid, for 60 s at 37 °C in the presence of AMP or ATP. AMP values were subtracted from ATP values. Net BCRP-mediated E₁S uptake was calculated by subtraction of corresponding eYFP values and expressed as a percentage of maximum uptake. Curve fitting was performed by non-linear regression analysis. Graphs represent means ± SEM of three independent experiments.
Because of their lipophilicity, steroids move readily through the plasma membrane and potentially might interfere with other membrane transporters. To exclude the possibility that the inhibitory effects on BCRP activity resulted from such a nonspecific phenomenon, the steroids were tested for their inhibitory effects on the activity of another ABC transporter, viz. MRP3. Unlike substrates for other ABC transporters, such as MRP1, MRP2 and P-glycoprotein, MRP3 substrates do not overlap with the ones known to be transported by BCRP. Because of this, we did not expect steroids to interact with MRP3. Increased synthesis of human MRP3 in HEK293 membranes was confirmed using Western blot analysis (figure 7a). Concentrations of the steroids which were able to inhibit BCRP activity for more than 50% were used to determine their effects on MRP3 activity, using [3H]-E$_{17}^{\beta}$G as a substrate. Under these conditions, none of the steroids inhibited MRP3-mediated [3H]-E$_{17}^{\beta}$G uptake (figure 7b). Instead, testosterone and progesterone even stimulated MRP3 activity. Moreover, the positive control, unlabeled E$_{17}^{\beta}$G (100 µM), did inhibit [3H]-E$_{17}^{\beta}$G uptake significantly, to 19.4 ± 0.6 % of maximum uptake ($P < 0.001$).

![Figure 7](image.png)

**Figure 7.** (a) Western blot analysis of HEK293-eYFP- and -MRP3 membrane vesicles. MRP3 was detected using mouse-anti-MRP3 antibody (M3II-21). (b) Effects of estradiol, testosterone, progesterone and androstenedione on MRP3 activity. Membrane vesicles were incubated with 80 nM [3H]-E$_{17}^{\beta}$G (substrate) and indicated concentrations of the steroids, for three min at 37 °C. The effect of the steroids on net MRP3-mediated E$_{17}^{\beta}$G uptake is expressed as a percentage of uptake in negative control samples. Bars represent means ± SEM of three independent experiments. Differences in MRP3-mediated E$_{17}^{\beta}$G uptake were assessed by means of a one-way ANOVA test followed by Dunnett’s post hoc test.
Dixon plot analysis

To determine the mode of interaction, we measured concentration-dependent effects of the steroids on BCRP activity at three different E1S concentrations. Figure 8 depicts Dixon plots of the data, which were analyzed by linear regression. The three lines representing steroid inhibition at different E1S concentrations of the four steroids intersected all at the x-axis, indicating a non-competitive inhibitory effect. The apparent inhibitory constant (Ki) values were 5.0 ± 0.2 µM for estradiol, 36 ± 14 µM for testosterone, 14.7 ± 1.3 µM for progesterone, and 217 ± 13 µM for androstenedione.

Figure 8. Dixon plots of inhibitory effects of estradiol (a), testosterone (b), progesterone (c), and androstenedione (d) on BCRP-mediated E1S uptake. Membrane vesicles were incubated with indicated concentrations of [3H]-E1S and increasing concentrations of the steroid. The reciprocal of transport velocity (1/V) was calculated and the mode of inhibition was determined using Dixon’s method combined with linear regression analysis to estimate the inhibitory constant (Ki) values; 5.0 ± 0.2 µM (a), 36 ± 14 µM (b), 14.7 ± 1.3 µM (c), 217 ± 13 µM (d). A representative of one experiment, out of three independent measurements, is depicted.
Discussion

This study reports a clear overview of Bcrp presence in several murine organs as well as its cellular localization in these tissues. In addition to adrenal gland, pancreas and testis, we show for the first time localization of Bcrp in ovary, pituitary gland and adipose tissues. Moreover, we report an efficient inhibitory effect of steroid hormones on BCRP function. Estradiol, testosterone, progesterone and androstenedione attenuated BCRP-mediated E$_1$S transport in a non-competitive manner, which implies an allosteric inhibition of BCRP function by these steroids.

Presence of BCRP in various tissues with a barrier function, including placenta, prostate, small intestine, brain, colon, liver, mammary gland and kidney has been shown previously. Investi- gations on the presence of the efflux pump in endocrine organs received much less attention and localization of the protein has hardly been described. We assessed Bcrp on both mRNA level as well as protein presence in endocrine organs. In addition, endocrine organs from Bcrp$^{-/-}$ mice were used for comparison, allowing discrimination between background and Bcrp-specific staining. In accordance with the findings of Langmann et al., who quantified Bcrp mRNA in various human tissues, we found Bcrp mRNA in mouse kidney, testis and adrenal gland. While our results show highest mRNA levels in the kidney, Langmann et al. described highest mRNA levels in the testis. This could be attributed to interspecies differences. We described previously that Bcrp mRNA levels were much higher in mouse kidneys compared to rat and human kidneys. Additionally, our results revealed that for all organs tested, mRNA concentrations of Bcrp in male mice were significantly higher than in females, which is in agreement with previously published findings. Despite the clear gender differences in Bcrp mRNA levels, there were no differences in localization of the protein between male and female organs (data not shown). Tanaka et al. described male-predominant Bcrp gene expression in rat kidney and mouse liver. Moreover, Merino et al. found differences in the disposition of the BCRP substrate, nitrofurantoin, between male and female mice, with higher plasma levels in females. They also reported higher hepatic protein levels of human BCRP in men compared to women, determined by Western blot analysis using crude membrane fractions from human liver. The precise role of the efflux pump in males vs. females was beyond the scope of the present study, and further research should elucidate the consequences of this gender difference.

In accordance with previous findings, immunohistochemical analysis revealed that Bcrp was prominently located in the brush borders of proximal tubule epithelium of the murine kidney. Furthermore, Bcrp was found to be present in adrenal gland, pituitary gland, testis
and ovary, where it was located predominantly in endothelial cells lining blood vessel walls. In agreement, Maliepaard et al.\textsuperscript{9} reported Bcrp localization in venous and capillary endothelial cells of almost all tissues.

In addition to the arterioles in the adrenal gland cortex, Bcrp was found to be present in the cells lining the zona glomerulosa and zona reticularis, which secrete mineralocorticosteroids and androgens, respectively. The latter is in agreement with the findings of Fetsch et al.\textsuperscript{7}. For the pituitary gland, we are the first to reveal Bcrp localization in the capillaries. Bcrp presence in blood vessels was also found in the pancreas, though, it was low as compared to the other organs studied. Presence of Bcrp in the pancreas was described previously, but mainly in pancreatic progenitor cells.\textsuperscript{31,32} In contrast to Fetsch et al., who reported Bcrp localization in the islet and acinar cells, we did not find Bcrp in the endocrine and exocrine cells of the pancreas.

In ovary, Bcrp was clearly present in capillaries and some larger blood vessels and in testis the capillaries showed positive staining as well. The fact that not all vessels in ovary tissue were Bcrp positive, might be characteristic for the dynamic environment of the estrous cycle, where there is a constant regeneration and degradation of capillaries. The degrading capillaries lose their endothelial function and thereby endothelial protein is decreased. Tanaka et al.\textsuperscript{23} reported Bcrp in ovary and testis of mice. In accordance with our results, they demonstrated that Bcrp protein levels were higher in testis than in ovary. Enokizono et al.\textsuperscript{33} did not find evidence for Bcrp activity when evaluating genistein accumulation in the ovaries of wild-type and Bcrp\textsuperscript{-/-} mice, but they did find Bcrp activity in murine testis. It was reported that the interstitial cells of Leydig\textsuperscript{7} and spermatogonial cells\textsuperscript{34} of the testis localized Bcrp. Conversely, our findings could not endorse these reports. Though, presence of Bcrp in the lumina of testicular blood vessels was evident.\textsuperscript{6}

In both epididymal and abdominal fat pads, we found Bcrp to be present in mature adipocytes, which has as yet not been described. Adipocytes are metabolically active cells that play a key role in the control of the body’s energy balance by the secretion of resistin, adiponectin, and leptin. Moreover, adipocytes are involved in steroid hormone regulation since they highly express aromatase (\textit{CYP19A1}), which is responsible for a key step in the biosynthesis of estrogens.\textsuperscript{35} Therefore, it will be interesting to unravel further the role of Bcrp in adipocytes. Another important endocrine organ, which we did not evaluated, is the mammary gland. Steroids highly influence the growth and function of the mammary gland. It is, therefore, interesting to study the presence and function Bcrp this organ. In 2005, Jonker et al.\textsuperscript{5} published the localization of BCRP/Bcrp in breast tissue of mice, cows and humans. They clearly showed that the transporter was localized in mouse mammary gland epithelium.
during late pregnancy and in lactating mice but presence of the protein was negligible in the mammary gland of nonpregnant and nonlactating mice. It would be interesting to study the mechanism by which Bcrp synthesis and function is influenced during pregnancy and lactation.

In all the organs we analysed, Bcrp was principally found in the endothelial layer of sinusoids, capillaries and veins. The fact that Bcrp was located in the canalicular membrane of the liver indicates that the efflux pump may be involved in excretion processes in the liver, similar to other ABC transporters, such as P-gp and MRP2. In the brain and in the testis, the endothelial cells form tight junctions, creating a blood-tissue barrier, preventing substances to freely enter the tissue. Here, efflux transporters, such as Bcrp, are thought to protect the brain and developing sperm cells against toxic agents. In other organs, normal endothelium consists of loosely connected endothelial cells and is known to be quite permeable for several substances. Nevertheless, the fact that Bcrp is so clearly present in these endothelial cells and it is able to transport substances against a concentration gradient indicates a contribution of the efflux pump to transport compounds across the endothelium. Although, the endocrine organs, obviously, have a significant secretory function, we did not find Bcrp localized in endocrine and exocrine cells, except for adipocytes and the adrenal gland. Hence, the efflux pump may contribute to the transport of steroids, or their derivates, into the blood stream.

Using Dixon’s method, we proved indirectly that estradiol, testosterone, progesterone and androstenedione are efficient inhibitors, but likely not transported by BCRP via the same binding site as E$_2$.S. The question whether steroids are substrates for BCRP has been discussed before and contradictory findings have been reported. Most studies support the conclusion that steroids interact but are not substrates for BCRP, which is in agreement with the present findings. Nevertheless, we cannot completely rule out the possibility of steroids being transported by BCRP. The idea that there is no need for steroids to be transported actively over the plasma membrane because of their high lipophilicity is outdated, since it has been described that steroids are substrates for other ABC transporters. Besides, steroids are derived from cholesterol, which itself is transported by other members of the ABCG subfamily. We have undertaken studies to measure steroid uptake in HEK293-BCRP membrane vesicles, but unreliable results were obtained due to the large passive permeability of these lipophilic compounds.

To exclude the possibility that the inhibitory effects of the steroids on BCRP activity were nonspecific, their effects on the activity of MRP3 was tested. None of the steroids inhibited MRP3-mediated E$_2$-17βG uptake. Interestingly, however, testosterone and progesterone stimulated this uptake. Stimulation of MRP3 has been described before, although, the
mechanism explaining these complex stimulatory effects has not yet been elucidated.\textsuperscript{28,42,43} The fact that MRP3 function was not inhibited by the steroids indicates that the inhibition of BCRP activity by the steroids was a transporter-specific effect. Estradiol, testosterone, progesterone and androstenedione inhibited BCRP activity for more than 70%. However, the concentrations needed to cause this inhibitory effect were rather high compared to plasma levels. These concentrations are indicative of the affinity of the steroids for BCRP compared to the affinity of the substrate (E$_1$S) for BCRP, which is known to be very high. When the affinity of the steroids is much lower, then the concentrations needed to cause an effect are obviously much higher. Furthermore, the steroids themselves are very lipophilic and are therefore prone to stick to plastic tubes and/or wells, which might result in an over-estimation of the actual concentration. This problem was acknowledged earlier by Tanneberger et al.\textsuperscript{44} To predict the clinical relevance of the interaction between steroids and BCRP, knowledge on intracellular steroid concentrations is important. Plasma steroid levels are not relevant for predicting their effects on a membrane transporter that transports molecules from the inside to the outside of the cell. Intracellular levels are, so far, unknown and difficult to determine. One can imagine that intracellular concentrations in organs responsible for the production of hormones may be much higher than plasma levels and, thereby, could potentially influence BCRP function \textit{in vivo}. Not only BCRP function is altered by steroids, as shown in our results, also membranal localization of BCRP is highly influenced by sex steroids. Imai \textit{et al.}\textsuperscript{22} found that estrogens post-transcriptionally downregulated BCRP in estrogen-responsive cancer cells. They found estradiol-mediated reduction in BCRP protein in MCF-7 cells, however, not on mRNA level. This downregulation was counteracted by gene silencing of estrogen receptor-\textalpha (ER\textalpha), indicating that ER\textalpha is necessary for the suppression of BCRP protein. In agreement, Hartz \textit{et al.}\textsuperscript{21} described that estradiol signals through ER\textbeta and ER\textalpha to initiate Bcrp internalization and acts via ER\textbeta to stimulate proteosomal degradation of Bcrp in murine brain capillaries. In contrast, Ee \textit{et al.}\textsuperscript{20} found that estradiol enhanced Bcrp mRNA levels in cells stably expressing ER\textalpha, at similar estradiol concentrations. Ovariectomy and castration of mice and rats, solely or in combination with sex steroid treatment, revealed regulation of Bcrp mRNA by estradiol and testosterone.\textsuperscript{23} In human placental BeWo cells, progesterone and estradiol significantly increased and decreased BCRP mRNA levels, respectively.\textsuperscript{45} Estradiol by itself likely downregulated BCRP through an estrogen receptor (ER), while progesterone alone upregulated BCRP via a mechanism other than progesterone receptor (PR).

In conclusion, we found Bcrp to be present in numerous murine endocrine organs, including ovary, pituitary gland and adipose tissues. Furthermore, the efficient inhibition of BCRP-
mediated transport by estradiol, testosterone, progesterone and androstenedione implies a clear interaction between the steroids and the efflux pump. Together, our results support the speculation that BCRP has a role in steroid hormone regulation. Further research will address this hypothesis.

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References


Localization of Bcrp in endocrine organs and inhibition of its transport activity by steroid hormones


Chapter 2


Localization of Bcrp in endocrine organs and inhibition of its transport activity by steroid hormones
Endocrine disruptors differentially target ATP-binding cassette transporters in the blood-testis barrier and affect Leydig cell testosterone secretion in vitro

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Chapter 3

Abstract

Endocrine disrupting chemicals (EDCs) are considered to cause testicular toxicity primarily via interference with steroid hormone function. Alternatively, EDCs could possibly exert their effects by interaction with ATP-binding cassette (ABC) transporters that are expressed in the blood-testis barrier. Here, we investigated the effects of bisphenol A (BPA), tetrabromobisphenol A (TBBPA), bis(2-ethylhexyl) phthalate (DEHP), mono(2-ethylhexyl) phthalate (MEHP), perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) on breast cancer resistance protein (BCRP), multidrug resistance protein 1 and 4 (MRP1,4), and P-glycoprotein (P-gp) using membrane vesicles overexpressing these transporters. BPA solely inhibited BCRP activity whereas TBBPA, PFOA and PFOS inhibited all transporters tested. No effect was observed for the phthalates. Using transporter-overexpressing Madin-Darby canine kidney (MDCKII) cells, we show that BPA and PFOA, but not TBBPA, are transported by BCRP, whereas none of the compounds were transported by P-gp. To investigate the toxicological implications of these findings, testosterone secretion and expression of steroidogenic genes were determined in murine Leydig (MA-10) cells upon exposure to the selected EDCs. Only BPA and TBBPA concentration-dependently increased testosterone secretion by MA-10 cells to 6- and 46-fold of control levels, respectively. Inhibition of the Mrp’s by MK-571 completely blocked testosterone secretion elicited by TBBPA, which could not be explained by coinciding changes in expression of steroidogenic genes. Therefore, we hypothesize that transporter-mediated efflux of testosterone precursors out of MA-10 cells is inhibited by TBBPA resulting in higher availability for testosterone production. Our data show the toxicological and clinical relevance of ABC transporters in EDC risk assessment related to testicular toxicity.
**Introduction**

Male sub- and infertility is an increasing problem in Western society. Epidemiological trends show an increase in infertility and requests for assisted reproductive techniques. This increasing trend is observed not only as a consequence of lifestyle factors or side effect of therapeutic agents but is also suggested to be a consequence of environmental exposures. It is estimated that of all infertile couples, 20% of the cases can be attributed to male factors solely and that male factors are contributory in another 30-40%.

Yet, male infertility is a complex problem of which possible causes are still poorly understood.

In adult males, spermatogenesis is driven by the gonadotropins luteinizing hormone (LH) that stimulates steroidogenesis in Leydig cells (LCs) and follicle-stimulating hormone (FSH) that acts on Sertoli cells. Local testicular steroidogenesis, i.e. production of androgens and estrogens, is essential in regulating spermatogenesis and involves multiple cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes. Maturation and differentiation of LCs coincides with decreased expression of genes related to adhesion and increased expression of steroidogenic enzymes (i.e. 3β-HSD). Moreover, the LH receptor (LHr), steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), and 17α-hydroxylase/20-lyase (CYP17A1) expression are induced upon differentiation, resulting in adult LCs that primarily produce testosterone upon LH stimulation.

The testicular microenvironment is pivotal for mammalian steroidogenesis and spermatogenesis. In the testis, the blood-testis barrier (BTB) physically divides the seminiferous epithelium into a basal and an apical compartment thereby playing a crucial role in the differentiation of spermatogonia into spermatocytes. In the active part of the BTB, like in various other tissues with a barrier function (i.e. blood-brain barrier, placenta), ATP-binding cassette (ABC) transporters are present that protect the organ from internal exposure to harmful substances and influence the disposition and metabolism of xenobiotics. These transporters are located at the cellular membranes of Leydig cells, Sertoli cells, and capillary endothelial cells. Efflux transporters are involved in the transport of a variety of molecules against steep concentration gradients at the expense of ATP. The most abundant efflux transporters in the BTB include breast cancer resistance protein (BCRP/ABCG2), P-glycoprotein (P-gp/ABCB1), and multidrug resistance proteins 1 and 4 (MRP1,4/ABCC1,4). Differential expression of these transporters can be observed throughout various parts of the BTB. In adult human testis, Leydig cells express P-gp, MRP1, and MRP4 but not BCRP.

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positioning of the efflux transporters in the BTB suggests that their substrates are transported out of the seminiferous tubules to prevent entry and accumulation of xenobiotics in the testes and to protect the germ cells. Often, exposure to endocrine disrupting chemicals (EDCs), i.e. compounds that interfere with hormone biosynthesis, metabolism or action, is suggested to contribute to the increasing incidence of male sub- and infertility. Consequently, the possible risk of several high-volume chemicals that are used to enhance the usability and safety of consumer products, and which have shown endocrine activity, is under continuous debate. Examples of these chemicals include bisphenol A (BPA), tetrabromobisphenol A (TBBPA), bis(2-ethylhexyl) phthalate (DEHP), mono(2-ethylhexyl) phthalate (MEHP), perfluorooctanoic acid (PFOA), and perfluorooctanesulfonic acid (PFOS). People in developed countries are exposed daily to these chemicals and, as a result, continuous exposure occurs in blood, liver, kidneys, spleen, gall bladder, and also in testes.\textsuperscript{10-12} Several studies have shown that these EDCs can affect steroidogenesis \textit{in vitro} and \textit{in vivo}\textsuperscript{11,13-18} and, as such, may act as testicular toxicants. However, for risk assessment purposes it is extremely important to consider the toxicokinetics of these EDCs and to date it is unclear whether they can affect the activity of ABC transporters present in the BTB.

Here, we describe the interaction of six commonly suggested EDCs, i.e. BPA, TBBPA, DEHP, MEHP, PFOA, and PFOS, with the transport activity of the human efflux transporters BCRP, P-gp, MRP1 and MRP4 that can be found in the BTB, using a membrane vesicle interaction assay. Furthermore, Madin-Darby canine kidney (MDCKII) cells stably overexpressing BCRP or P-gp were used to study transport of BPA, TBBPA, and PFOA. Also, the effects of these EDCs and the role of the ABC transporters on testosterone secretion and steroidogenic gene expression were determined in P-gp-, Mrp1- and Mrp4-expressing MA-10 mouse Leydig cells.

Materials and methods

Chemicals

Selected EDCs (supplementary figure 1), bisphenol A (BPA; >99%; CAS# 80-05-7), tetrabromobisphenol A (TBBPA; >97%; CAS# 79-94-7), perfluorooctanoic acid (PFOA; ≥98%; CAS# 335-67-1), and perfluorooctane sulfate (PFOS; ≥98%; CAS# 1763-23-1) were purchased from Sigma-Aldrich Co. (Zwijndrecht, the Netherlands). Diethylhexyl phthalate (DEHP; >98.0%; CAS# 117-81-7) was acquired from TCI Europe N.V. (Zwijndrecht, Belgium) and mono-ethylhexyl phthalate (MEHP; >97.5%; CAS# 4376-20-9) from Wako Chemicals GmbH (Neuss, Germany). [6,7-\textsuperscript{3}H(N)]-estrone sulfate ([\textsuperscript{3}H]-E\textsubscript{1}S; 54.3 Ci/mmol) and [6,7-\textsuperscript{3}H(N)]-estradiol 17-β-d-glucuronide ([\textsuperscript{3}H]-E\textsubscript{2}\textsubscript{17}βG; 41.8 Ci/mmol) were obtained from Perkin
Elmer (Groningen, the Netherlands). [3H]-N-methylquinidine ([3H]-NMQ; 85 Ci/mmol) was purchased from Solvo Biotechnology (Szeged, Hungary), [3,5,7-3H(N)]-methotrexate ([3H]-MTX; 25.3 Ci/mmol) from Moravek (Brea, CA, USA) and [3H(G)]-BPA (25 Ci/mmol) and [14C]-PFOA (55 mCi/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). All radiochemicals were solved in ethanol.

**Transduction of Human Embryonic Kidney (HEK293) cells and preparation of membrane vesicles**

Overexpression of human BCRP, P-gp, MRP1, and MRP4 in HEK293 cells was established using baculoviruses, which were produced using the Bac-to-Bac system (Invitrogen, Breda, the Netherlands), as described previously. As a control, vesicles from HEK293 cells overexpressing the enhanced yellow fluorescent protein (control) were used. Crude membranes were isolated, resuspended in TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) and membrane vesicles were prepared according to the previously described method. Total protein content was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Veenendaal, the Netherlands). Crude membrane vesicles were dispensed in aliquots, snap frozen in liquid nitrogen, and stored at -80 °C until further use.

**Transport interaction assay**

The interaction of EDCs with transporter activity was assessed by adding a reaction mix consisting of TS buffer supplemented with 4 mM ATP/AMP, 10 mM MgCl$_2$, radiolabelled substrates and various concentrations of EDCs to 7.5 µg of membrane vesicles. The following substrates were used; 250 nM [3H]-E$_1$S for BCRP, 100 nM [3H]-NMQ for P-gp, 133 nM [3H]-E$_1$17βG for MRP1, and 200 nM [3H]-MTX for MRP4. After an incubation of 60 sec (BCRP and P-gp) or 5 min (MRP1 and MRP4) at 37 ºC to enable ATP-dependent uptake, the reaction was stopped by placing the samples on ice and by addition of ice-cold TS buffer. Reaction mix was removed and the vesicles were washed by means of a rapid filtration technique using glass fiber filter plates (BCRP and P-gp) or PVDF filter plates (MRP1 and MRP4) (Millipore, Etten-Leur, the Netherlands). Scintillation fluid was added to the filters and the amount of radioactivity was determined using a scintillation counter (Tri-Carb® 2900TR; Perkin Elmer, Waltham, MA, USA). Reference samples were measured to calculate the amount of transported substrate. ATP-dependent transport was calculated by subtracting values measured in the presence of AMP from those measured in the presence of ATP. Net transporter-mediated transport was calculated by subtracting ATP-dependent uptake in control vesicles from that of transporter-overexpressing vesicles.
Accumulation assay in MDCKII cells

To determine transport of BPA, TBBPA, and PFOA, Madin-Darby canine kidney (MDCKII) cells stably overexpressing BCRP or P-gp were used, which were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands) and Dr. M. Gottesman (Laboratory of Cell Biology, National Cancer Institute, USA), respectively. Cells were seeded in 24-well plates and grown until confluency. For the accumulation assay, cells were washed with Hank’s balanced salt solution (HBSS), supplemented with 10 mM HEPES (pH 7.4), twice before 30 min preincubation with optiMEM (reduced serum medium; Invitrogen; Breda, the Netherlands) at 37 °C. Thereafter, cells were incubated with optiMEM containing ³H-BPA (40 nM), unlabeled TBBPA (1 µM), or ¹⁴C-PFOA (1 µM) with or without the BCRP inhibitor Ko143 (1 µM; Sigma-Aldrich Co., Zwijndrecht, the Netherlands) or the BCRP and P-gp inhibitor elacridar (2 µM; GF120918; Sequoia Research Products Limited, Pangbourne, United Kingdom). After one h at 37 °C, cells were washed twice with ice-cold HBSS/HEPES + 0.5% BSA and twice with ice-cold HBSS. For scintillation counting, cells were lysed with 1 M NaOH. Subsequently, HCl (1 M) was added and lysates were transferred into scintillation tubes containing scintillation fluid. Reference samples were measured to calculate the amount of transported substrate. For TBBPA measurements, cells were suspended after 30 min trypsin incubation and lysed by 50% acetonitrile. TBBPA content was determined by LC-MS/MS analysis.

LC-MS/MS analysis of TBBPA

After the accumulation assay, rigorously pipetted samples were centrifuged for 10 min at 16,000 x g and subsequently injected into the LC-MS/MS system that consisted of an Accela HPLC system (Thermo scientific, Breda, the Netherlands) equipped with a C18 UPLC column (Acquity UPLC HSS T3, 1.8 µm, 2.1 x 100 mm; Waters Corporation, Milford, Massachusetts, USA). Separation was performed at a flow rate of 200 µl/min with eluent A (50% acetonitrile/50% water) and eluent B (100% acetonitrile) under the following gradient conditions: 0 min, 0% eluent B, 5 min, 80% eluent B, 7 min, 80% eluent B, 8 min, 0% eluent B, 13 min, 0% eluent B. The eluate was directly passed into a TSQ Vantage tandem mass spectrometer (Thermo scientific) equipped with an electrospray ionization source. Negative electrospray ionization was achieved using a nitrogen sheath gas with ionization voltage at 2500 V. The capillary temperature was set at 350 °C. Detection of TBBPA was based on isolation of the deprotonated molecular ion, [M-H]+. Subsequently, MS/MS fragmentations and selected reaction monitoring were performed. The following SRM transitions were used: m/z 542,7 (parent ion) to m/z 447,8 and 419,9 (both product ions). A calibration curve of TBBPA was used to quantify the amount of transport. Acquired data were processed with Thermo Xcaliber software (Thermo scientific).
MA-10 cell culture and exposure to EDCs and/or inhibitors

The MA-10 cell line, derived from mouse Leydig tumor cells, was kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, Iowa, USA). Cells were cultured in 1:1 Dulbecco’s Modified Eagle Medium/F-12 nutrient mixture (Ham) with phenol red (DMEM/F-12 1:1, #11320; Gibco, Life Technologies Europe BV, Bleiswijk, the Netherlands) supplemented with 15% HyClone (Thermo Fisher Scientific, Waltham, USA), 2% HEPES (1 M), and 1% penicillin/streptomycin (Gibco). Cells were maintained at 37°C in a humidified atmosphere (95%) at 5% CO₂. Flasks and plates were coated at room temperature with 0.1% gelatin (Attachment Factor Protein; Gibco), 45 min before use.

For gene expression assessment, MA-10 cells were plated in gelatin pre-coated 12-wells plates (Greiner, the Netherlands) at a density of 6 x 10⁵ cells per well. For testosterone secretion measurements, MA-10 cells were seeded at a density of 2 x 10⁵ cells per well onto pre-coated 24-wells plates. After 24 h, the medium was replaced with medium containing the EDCs and/or inhibitors (at a maximum solvent concentration of 0.1% v/v). Gene expression was determined after a 6-hour exposure and testosterone levels were measured after 24 hours.

Gene expression of ABC transporters and steroidogenic enzymes in MA-10 cells

After a 6-hour exposure of MA-10 cells, the effects of the EDCs on expression of transporter and steroidogenic genes with and without transporter inhibition were determined by quantitative polymerase chain reaction (qPCR). First, total RNA was isolated from MA-10 cells by phenol-chloroform extraction using RNA InstaPure according to instructions provided by the manufacturer (Eurogentec, Liège, Belgium). Purity and concentration of the isolated RNA samples was determined at absorbance wavelengths of 230, 260, and 280 nm using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). RNA samples were diluted to a concentration of 66.7 µg/ml by addition of RNAse free water and stored at -80 °C until further use. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.) and diluted 10 times. qPCR was performed with reaction mixtures containing 12.5 µl iQ SYBR green supermix (Bio-Rad Laboratories, Inc.), 1 pmol forward primer, 1 pmol reversed primer, 0.5 µl RNAse free water, and 10 µl diluted cDNA. Supplementary table 1 describes the sequences of the primer pairs used including the reference gene β-actin. All primers span an exon-exon junction to ensure mRNA amplification only and were run through National Center for Biotechnology Information (NCBI) Blast (nucleotide non-redundant database) to confirm specificity. Also, efficiency was determined for all primer pairs. The mixtures were placed in the MyiQ™iCycler (Bio-Rad Laboratories,
Inc.) and heated to 95 °C for 3 min, following 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45 s. To ensure exclusion of primer dimers and other non-specific products formed, a melt curve was run afterwards.

**Testosterone secretion**

After a 24-h exposure of MA-10 cells to the EDCs, medium was removed and stored at -80 °C until analysis. Secretion of testosterone by MA-10 cells was measured in a 50 µl aliquot using a commercially available radioimmunoassay (RIA) kit (Beckman Coulter GmbH, Krefeld, Germany) according to manufacturer’s instructions.

**Results**

**Effects of EDCs on BCRP, P-gp, MRP1, and MRP4 transport activity**

To investigate the effects of the selected EDCs (supplementary figure 1) on activity of the ABC transporters, we performed an uptake assay using membrane vesicles overexpressing the specific transporters incubated with known substrates\[^8,19,20\] at concentrations of the EDCs ranging from 1 to 100 µM. Figure 1 shows the calculated net transporter-mediated substrate uptake depicted as percentage of maximum transport activity. BPA did not influence the activity of P-gp, MRP1, and MRP4. However, exposure to 100 µM of BPA resulted in a 77% reduction of BCRP-mediated E\(_2\)S uptake (figure 1A). TBBPA inhibited the activity of all four transporters tested (figure 1B). At 10 µM, TBBPA inhibited BCRP and MRP4 activity with 39% and 25%, respectively. At 100 µM TBBPA the activity of all transporters was reduced with > 85%. For P-gp and MRP4, additional concentrations between 10 and 100 µM TBPPA were tested and a clear concentration-dependent decrease in transport activity was seen with corresponding IC\(_{50}\) values of 22.9 and 24.0 µM for P-gp and MRP4, respectively. Both phthalates DEHP and MEHP, did not interact with any of the transporters tested at concentrations up to 100 μM (figure 1C-D). PFOA and PFOS inhibited the activity of all transporters tested, mainly at 100 µM (figure 1E-F). The strongest effect was observed on P-gp activity with an inhibition at 100 µM of 71% and 84% by PFOA and PFOS, respectively.
EDCs differentially target ABC transporters in the BTB and affect Leydig cell testosterone secretion

Figure 1. Transport activity of BCRP, P-gp, MRP1, and MRP4 upon exposure to various concentrations of (A) BPA; (B) TBBPA; (C) DEHP; (D) MEHP; (E) PFOA; and (F) PFOS. Membrane vesicles overexpressing the transporters were incubated with $[^3H]$-labeled substrates and increasing concentrations of EDCs for 60 s (BCRP and P-gp) or 5 min (MRP1-4) at 37 °C in the presence of AMP or ATP. The following substrates were used; 250 nM $[^3H]$-E1S for BCRP, 100 nM $[^3H]$-NMQ for P-gp, 133 nM $[^3H]$-E17βG for MRP1, and 200 nM $[^3H]$-MTX for MRP4. AMP values were subtracted from ATP values. Net transporter-mediated substrate uptake was calculated by subtraction of corresponding control values and expressed as percentage of maximum uptake. Bars represent means ± SEM of 3-4 independent experiments. Inhibition of transporter activity by the EDCs was assessed by means of a one-way ANOVA test followed by Dunnett’s post hoc test; (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

EDC accumulation in transporter-overexpressing MDCKII cells

An inhibition of transport by the EDCs does not necessarily imply that the compounds are substrates for the transporters. To investigate whether the EDCs tested acted as competing substrates, thereby inhibiting transporter activity, we determined the accumulation of BPA, TBBPA, and PFOA in MDCKII cells stably overexpressing BCRP or P-gp. Overexpression of BCRP significantly reduced the accumulation of BPA and PFOA to 20% and 52% of control cells, respectively (figure 2A and E). This inhibition was reversed by the BCRP inhibitor Ko143. TBBPA accumulated to similar levels in BCRP overexpressing cells as compared to control cells (figure 2C) indicating that this compound may not be a substrate for the transporter. Furthermore, in P-gp-overexpressing cells, no difference was found between BPA, TBBPA, or PFOA accumulation as compared to control cells (figure 2B, D, and F) indicating that these compounds are most likely not substrates for P-gp.
Figure 2. Accumulation of BPA, TBBPA or PFOA in MDCKII cells with or without overexpression of BCRP (A, C, and E) or P-gp (B, D, and F). Cells were incubated with either 40 nM [\(^3\)H]-BPA, 1 µM unlabeled TBBPA or 1 µM [\(^{14}\)C]-PFOA in absence or presence of BCRP inhibitor Ko143 (1 µM; A,C,E black bars) or BCRP and P-gp inhibitor elacridar (2 µM; B,D,F black bars) for one h at 37 °C. BPA and PFOA accumulation were determined by scintillation counting and TBBPA accumulation was estimated by LC-MS/MS analysis. Bars represent means ± SEM of 3-4 independent experiments. Significance between control cells and inhibitor treated cells (***, \(P < 0.001\)) was assessed by means of a Student’s \(t\)-test.
Effects of EDCs on testosterone secretion by MA-10 cells with and without transporter inhibition

Testosterone production by Leydig cells is prerequisite for proper spermatogenesis. To study the effect of EDCs on testosterone secretion and the role of ABC transporters in this process, we used the murine Leydig (MA-10) cell line. First, a qPCR was performed to confirm the presence of the ABC transporters in MA-10 cells. In table 1, the relative mRNA levels (ΔΔCt values transcript/β-actin) of Mrp1, Mrp4, and P-gp in MA-10 cells after a 24-hour exposure to vehicle (0.1% v/v DMSO) or 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; 100 µM) are shown. In accordance with previously described tissue distribution, Bcrp expression was undetectable in the MA-10 cells (data not shown). To investigate the influence of the selected EDCs on testosterone secretion, MA-10 cells were exposed to the compounds for 24 hours, either alone or in combination with known ABC transporter inhibitors. Basal testosterone levels in MA-10 media were 6.6 ± 1.0 pg/ml. Complete inhibition of testosterone production was elicited by inhibiting Cyp17 activity using SU10603, indicating the presence of a de novo steroidogenic pathway in this cell line. Steroidogenesis could be induced by LH (not shown) and 8-Br-cAMP (figure 3A), which increased the testosterone concentration in the medium to 3.2 ± 0.4 ng/ml. Inhibiting Mrp or P-gp activity by MK-571 or PSC 833, respectively, did not affect basal nor 8-Br-cAMP-induced testosterone levels. Next, the effects of the selected EDCs on testosterone levels in the medium were determined. DEHP, MEHP, PFOA, and PFOS had no effect on testosterone levels at concentrations up to 30 µM (figure 3B). In contrast, BPA and TBBPA concentration-dependently increased testosterone levels in the media up to 6- and 46-fold of control levels, respectively. Co-exposing the cells to BPA (10 µM) and PSC 833, but not MK-571, did increase testosterone concentrations even further (12-fold of DMSO control level; figure 3C). When MA-10 cells were co-exposed to TBBPA in combination with MK-571, testosterone levels did not increase above vehicle control levels and were statistically significantly lower than upon TBBPA-treatment alone (figure 3D). After co-exposure of cells to TBBPA and PSC 833, testosterone levels did not statistically significantly change compared to cells treated with TBBPA alone (figure 3D).
Table 1. Relative mRNA levels (ΔΔCt values transcript/β-actin) of ABC transporters and steroidogenic genes in MA-10 cells after a 24-h exposure to vehicle (0.1% v/v DMSO) or 8-Br-cAMP (100 μM). Data are expressed as means ± SD of 3 independent experiments that were performed in triplicate. Significance was calculated by a Student’s t-test. # = significantly different from vehicle-treated cells; (# P < 0.05, ## P < 0.01 and ### P < 0.001).

<table>
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<tr>
<th>Exposure</th>
<th>Mrp1</th>
<th>Mrp4</th>
<th>P-gp</th>
<th>StAR</th>
<th>Cyp11A1</th>
<th>Cyp17</th>
<th>3β-HSD</th>
<th>17β-HSD</th>
<th>5αRed1</th>
<th>LHr</th>
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<tr>
<td>DMSO</td>
<td>0.76 ± 0.26</td>
<td>0.79 ± 0.31</td>
<td>0.71 ± 0.46</td>
<td>0.02 ± 0.00</td>
<td>0.47 ± 0.09</td>
<td>0.11 ± 0.06</td>
<td>0.55 ± 0.11</td>
<td>0.44 ± 0.35</td>
<td>0.91 ± 0.29</td>
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</tr>
<tr>
<td>8-Br-cAMP</td>
<td>0.65 ± 0.16</td>
<td>0.72 ± 0.22</td>
<td>0.78 ± 0.56</td>
<td>1.10 ± 0.60 #</td>
<td>2.03 ± 0.24 ###</td>
<td>2.35 ± 0.51 ###</td>
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<td>1.73 ± 1.10</td>
<td>1.87 ± 0.34 #</td>
<td>2.69 ± 2.28</td>
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Abbreviations: Mrp, multidrug resistance protein; P-gp, P-glycoprotein; StAR, steroidogenic acute regulatory protein; Cyp, cytochrome P450; HSD, hydroxysteroid dehydrogenase; 5αRed1, 5α-reductase type 1; LHr, luteinizing hormone receptor.
EDCs differentially target ABC transporters in the BTB and affect Leydig cell testosterone secretion

**Figure 3.** Testosterone secretion by MA-10 cells after a 24-h exposure to (A) Cyp17 inhibitor SU10603 (2 µM), Mrp inhibitor MK-571 (100 µM), P-gp inhibitor PSC 833 (5 µM), 8-Br-cAMP (100 µM) or a combination; (B) various concentrations of EDCs; (C) various concentrations of BPA alone or in combination with MK-571 or PSC 833 and (D) various concentrations of TBBPA alone or in combination with MK-571 or PSC 833. Testosterone was measured using a commercially available RIA. Data are represented as means ± SEM of 3 independent experiments that were performed in triplicate. Significance was assessed using a Student’s t-test (A), a one-way ANOVA test followed by Dunnett’s post hoc test for individual curves (B), and a two-way ANOVA test followed by Bonferroni’s post hoc test for multiple curves (C-D). # Significantly different from vehicle-treated cells; * Significantly different from uninhibited BPA or TBPPA-treated cells at the same concentration; (# or * P < 0.05).

**EDCs mediated ABC transporter and steroidogenic gene expression in MA-10 cells**

Next, we investigated whether changes in testosterone levels of MA-10 cells were a result of altered steroidogenic gene expression upon EDC exposure. No changes in gene expression of Mrp1, Mrp4, and P-gp were observed upon exposure to 8-Br-cAMP (table 1), nor upon exposure to the Mrp inhibitor MK-571, the P-gp inhibitor PSC 833 or any of the EDCs at
10 µM (supplementary figure 2). Exposure of MA-10 cells to 8-Br-cAMP caused an increase in expression of the steroidogenic genes StAR (67-fold), Cyp11A1 (4-fold), Cyp17 (24-fold), and 5α-reductase type 1 (5αRed1; 2-fold) compared to vehicle-treated cells (figure 4A). This indicates that increased testosterone levels in media of 8-Br-cAMP-treated cells (figure 3A) were a result of an increased testosterone production. Concurring with the effects on testosterone levels, DEHP, MEHP, PFOA and PFOS did not affect steroidogenic gene expression in MA-10 cells (supplementary figure 3). Interestingly, while exposure to BPA or TBBPA alone significantly increased testosterone levels in MA-10 media, the statistically significant effects on steroidogenic gene expression were only minor. BPA only slightly induced Cyp11A1 (1.5-fold) expression and TBBPA moderately induced StAR (1.2-fold), Cyp11A1 (1.3 fold), and Cyp17 (1.3 fold) expression (figure 4B and 5C, respectively). This suggests that the changes in testosterone secretion by MA-10 cells after BPA or TBBPA exposure are most likely not (solely) attributable to an increased production of testosterone as a result of upregulation of steroidogenic genes.

The next step was to determine whether gene expression in 8-Br-cAMP, BPA or TBBPA-exposed MA-10 cells was affected during (P-gp or Mrp) transporter inhibition. Exposure to PSC 833 or MK-571 did not affect basal expression of the steroidogenic genes (supplementary figure 3). However, when cells were exposed to MK-571 in combination with 8-Br-cAMP, a 2-fold increase in StAR gene expression was observed compared to 8-Br-cAMP alone, albeit non-statistically significantly different from cells exposed to 8-Br-cAMP alone (figure 4A). Furthermore, 8-Br-cAMP in combination with MK-571 showed a decrease in Cyp17 (2-fold) and 5αRed1 (2-fold) gene expression compared to 8-Br-cAMP treated cells (figure 4A). A significant increase in StAR gene expression (2-fold) was observed in MA-10 cells exposed to the combination of TBBPA with MK-571 compared to cells exposed to TBBPA alone (figure 4C). No effect of MK-571 on the expression of other steroidogenic genes studied in combination with 8-Br-cAMP, BPA or TBBPA was observed. PSC 833 did not significantly affect steroidogenic gene expression levels in 8-Br-cAMP, BPA or TBBPA-exposed MA-10 cells (figure 4A, B and C). Taken together, these data indicate that changes in testosterone levels in MA-10 cell media upon BPA or TBBPA exposure do not arise from concurrent changes in steroidogenic enzyme activity following altered gene expression.
Figure 4. Expression of genes involved in steroidogenesis in MA-10 cells after a 24-h exposure to (A) 8-Br-cAMP (100 μM); (B) BPA (10 μM) or (C) TBBPA (10 μM) alone or in combination with MK-571 (100 μM) or PSC 833 (5 μM). Data are represented as means ± SEM of 3 independent experiments that were performed in triplicate. Significance was assessed by means of a Student’s t-test. # Significantly different from vehicle-treated cells; * Significantly different from 8-Br-cAMP, BPA or TBBPA-treated cells; (# or * P < 0.05). Dotted lines indicate the reference level of the DMSO control.
Chapter 3

Discussion

The present study shows for the first time differential inhibitory effects of BPA, TBBPA, PFOA and PFOS on BCRP, P-gp, MRP1, and MRP4 transport activities. Moreover, BPA and PFOA appear to be substrates for BCRP. Our data further indicate that exposure to BPA and TBBPA can affect testosterone secretion from murine Leydig cells, which appears to be only partly due to increased steroidogenic enzyme expression. Possibly, increased availability of precursors for testosterone production via decreased ABC transporter-facilitated excretion of testosterone precursors, plays a role in this process.

The BTB provides structural and protective support to developing germ cells. The BTB is constituted by coexisting tight junctions, testis-specific atypical adherens junctions, desmosomes, and gap junctions. The active part of the BTB consists of drug efflux transporters that are differentially expressed throughout various parts of the BTB. Drug efflux transporters are a major determinant of xenobiotic kinetics and, consequently, of testicular exposure to potentially harmful compounds. From this perspective, the interaction of EDCs with ABC transporters is of great toxicological relevance. Our study shows that BPA, TBBPA, PFOA and PFOS differentially inhibit the activity of BCRP, P-gp, MRP1 or MRP4. Interestingly, BPA specifically inhibits BCRP but not P-gp, MRP1 or MRP4 activity. Furthermore, we demonstrated that BPA is transported by BCRP and not by P-gp. In accordance, Mazur et al. recently reported that BPA is a potential substrate for BCRP, but not for P-gp, based on an indirect ATP consumption assay. In contrast, the halogenated derivative of BPA, TBBPA, is not transported by BCRP or P-gp even though TBBPA is a potent inhibitor of all four transporters tested. Based on these data, TBBPA can be classified as a non-competitive transport inhibitor, yet detailed kinetic studies are needed to confirm this. PFOA also appeared to be a substrate for BCRP and, considering the structural similarity and inhibitory potency, it is likely that PFOS is also a BCRP substrate.

ABC transporters play an important role in the distribution of chemicals into several tissues, including the testis. Several single nucleotide polymorphisms (SNPs) have been reported that render transporters inactive causing substrates to accumulate in tissues. Moreover, drug-induced inhibition of ABC transporter activity can occur. For example, several commonly used drugs have been described to modulate BCRP function. BCRP, P-gp, MRP1 and MRP4 transport a broad range of substrates, including endogenous molecules involved in cellular signaling and communication, such as folic acid, cyclic nucleotides, conjugated steroids, vitamin B2 and uric acid. Interestingly, a recent study has demonstrated impaired testicular testosterone production in Mrp4-deficient mice, suggesting a physiological role for
this transporter in spermatogenesis. Based on our findings that BPA, TBBPA, PFOA and PFOS are able to inhibit the activity of one or more ABC transporters, it can be expected that this potentially influences a myriad of cellular processes that are regulated by ABC transporters and disturbs cellular homeostasis and local sex steroidogenesis.

Mammalian male sexual differentiation and functioning is entirely androgen-dependent and therefore endocrine disrupting effects on the androgen-producing Leydig cells can have profound biological consequences. Testicular steroidogenesis is regulated by a negative feedback mechanism via the hypothalamus–pituitary–testis axis but also locally via cAMP-mediated modulation of CYP17 activity. This latter mechanism was shown to be present in MA-10 cells that we used in this study. Mouse Leydig cells appear to resemble human Leydig cells with respect to interferences with local testicular steroidogenesis. Studies using fetal testis xenotransplants show that both human and mouse fetal testis, but not rat testis, are refractory to phthalate-induced inhibition of testosterone production. However, it is important to note that in men, a large portion of sex steroids and their precursor DHEA is produced in the adrenals. Circulating DHEA and its sulfate (DHEAS) provide a high level of substrates for androgen formation in peripheral tissues like the testis, which is reflected by extremely high expression levels and activity of human testicular 17β-HSD. This is in strong contrast with experimental animals, such as rat and mouse, where the secretion of sex steroids takes place exclusively in the gonads. These species-differences are mechanistically important and should be taken into account when translating effects of EDCs found in animal models for human risk assessment.

In vitro studies have demonstrated that exposure to estrogenic or anti-androgenic EDCs can affect sex steroidogenesis. For example, BPA and DEHP have been shown to induce CYP19 but not CYP17 activity in human adrenocortical H295R cells. TBBPA inhibited CYP17 activity but had no effect on CYP19 activity. Here, we also demonstrate that BPA and TBBPA can affect testosterone secretion from MA-10 Leydig cells. In line with our findings, MEHP did not affect steroidogenesis in the H295R steroidogenesis assay nor altered testosterone production in MA-10 cells. In our study, 8-Br-cAMP increased testosterone secretion by MA-10 cells, which was accompanied by a marked increase in STAR and Cyp17 gene expression. Steroidogenesis is initiated by cholesterol transport into the inner mitochondrial membrane, which is mediated by the STAR protein. However, the changes in STAR and/or Cyp17 gene expression did not concur with the changes in testosterone levels observed in media of MA-10 cells upon exposure to BPA or, even more profoundly, TBBPA. This indicates that other mechanisms are involved that can explain the concentration-dependent increase in testosterone levels upon BPA and TBBPA exposure. We also showed that TBBPA
potently inhibits P-gp transporter activity and inhibition of P-gp by the specific transporter inhibitor PSC 833 increased testosterone levels in MA-10 cells. This led us to hypothesize that transporter-mediated efflux of testosterone precursors, i.e. androstenedione or DHEA, from the MA-10 cells is inhibited by TBBPA and PSC 833 (graphically explained in figure 5). As a result, more precursors are available for testosterone production, which is reflected by increased testosterone levels measured. An in vivo study with rats showed that TBBPA exposure resulted in increased plasma testosterone levels, which correlated with an increase in testicular weight. In our study, the Mrp inhibitor MK-571 completely abolished TBBPA-mediated increase in testosterone secretion in MA-10 cells, suggesting a decrease in active transport of testosterone out of the Leydig cells. The high rate of local testosterone production that is observed in vivo implies the need for local, intratesticular transport of testosterone from Leydig cells to specific target cells, i.e. Sertoli cells that nourish and support the developing sperm cells throughout the stages of spermatogenesis. Moreover, intratesticular testosterone levels are maintained at ~100 times the level found in the systemic circulation in both humans and rodents, stressing the importance of local, active transport of sex steroids within the testis. Possibly, ABC transporters play a role in this local transport. In humans, plasma TBBPA levels are very low but maximum plasma concentrations of TBBPA-glucuronide were found to be 16 nmol/l after a single oral dose of 0.1 mg/kg TBBPA given to healthy volunteers. In our studies, the concentrations of EDCs that affected ABC transporter activity and testosterone secretion were in the nanomolar to micromolar range. However, plasma EDC levels have limited relevance for predicting effects on a membrane protein that transports molecules from inside to outside the cell. To our knowledge no data on intracellular concentrations for the selected EDCs exist. Yet, the fact that people in industrialized countries are daily exposed to these chemicals emphasizes the importance of our findings. In conclusion, our results provide a novel insight in mechanisms of male testicular toxicity by EDCs that warrants further investigation. The results from this study demonstrate that the endocrine disrupting capacity of suggested EDCs should not only be sought in direct effects on steroidogenesis upon accumulation in the testis, but can also entail indirect effects via interaction with ABC transporters in the BTB. It is, therefore, of high toxicological and clinical relevance to take the interactions between EDCs and the ABC transporters into account when performing risk assessment related to testicular toxicity.
EDCs differentially target ABC transporters in the BTB and affect Leydig cell testosterone secretion

**Figure 5.** Proposed effect of inhibition of P-gp and MRPs on testosterone production and secretion. In this study, we show that TBBPA potently inhibits P-gp. Also, TBBPA increases testosterone secretion by MA-10 Leydig cells, which suggests an increased conversion from testosterone (T-) precursors into testosterone. Since steroidogenic genes were not markedly upregulated by TBBPA, this effect is probably caused by increased availability of T-precursors due to P-gp inhibition. The same effect was provoked by P-gp inhibitor PSC 833. Subsequent inhibition of Mrps by MK-571 totally abolished TBBPA-mediated increase in testosterone secretion, indicating the potential role of Mrps to transport testosterone out of the cell.

**Acknowledgements**

The authors would like to thank A. Kulkarni and S.M. Nijmeijer for the technical assistance, J.J.M.W. van den Heuvel for preparing the baculoviruses and P.H.H. van den Broek for the LC-MS/MS measurements. The research performed at the Institute for Risk Assessment Sciences (IRAS; Utrecht University) was funded by the European Community’s Seventh Framework Program ([FP7/2010-2014]; GA244236), ChemScreen (http://www.bds.nl/chemscreen) and the Doerenkamp-Zbinden Foundation (http://www.doerenkamp.ch).
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References

EDCs differentially target ABC transporters in the BTB and affect Leydig cell testosterone secretion

Supplementary figure 1. Chemical structures of selected molecules prepared using the molecule editor ChemDraw (CambridgeSoft, Cambridge, MA, USA). These commonly suggested endocrine disruptors are widely used in the production of plastics (BPA, DEHP, and MEHP), as flame retardant (TBBPA), or surfactant (PFOA and PFOS).

Supplementary figure 2. Expression of genes of ABC transporters in MA-10 cells after a 24-h exposure to MK-571 (100 μM), PSC 833 (5 μM) or one of the EDCs (BPA, TBBPA; 10 μM). Data are represented as means ± SEM of 3 independent experiments that were performed in triplicate. Significance was assessed by means of a Student’s t-test. The dotted line indicate the reference level of the DMSO control.
EDCs differentially target ABC transporters in the BTB and affect Leydig cell testosterone secretion

Supplementary figure 3. Expression of genes involved in steroidogenesis in MA-10 cells after a 24-h exposure to MK-571 (100 μM), PSC 833 (5 μM) or one of the EDCs (DEHP, MEHP, PFOA, or PFOS; 10 μM). Data are represented as means ± SEM of 3 independent experiments that were performed in triplicate. Significance was assessed by means of a Student’s t-test. The dotted line indicate the reference level of the DMSO control.

Supplementary table 1. Sequences of primer pairs used in this study. All primers are designed for mouse genes as described in the Materials and methods section.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<td>β-actin</td>
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<td>P-gp</td>
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<tr>
<td>StAR</td>
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<td>LHr</td>
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Abbreviations: Mrp, multidrug resistance protein; P-gp, P-glycoprotein; StAR, steroidogenic acute regulatory protein; Cyp, cytochrome P450; HSD, hydroxysteroid dehydrogenase; 5αRed1, 5α-reductase type 1; LHr, luteinizing hormone receptor.
Disposition of the human BCRP substrate bisphenol A is not altered in Bcrp-deficient mice

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Manuscript in preparation
Abstract

Normal endocrine function is a prerequisite for proper development of the reproductive system and maintenance of function throughout adult life. The endocrine disruptor bisphenol A (BPA), widely used in the production of plastics, is thought to contribute to the increasing incidence of male subfertility because of its estrogenic properties. Recently we showed that the ABC transporter breast cancer resistance protein (BCRP), an efflux pump which is localized amongst others in the capillary endothelium of endocrine organs, transports BPA. In the present study, we aimed to determine the role of Bcrp in the disposition of BPA using wild type and Bcrp<sup>−/−</sup> mice. Mice were subcutaneously exposed to [³H]-BPA with or without elacridar, an inhibitor of Bcrp and another efflux pump, P-glycoprotein (P-gp), via a micro-osmotic pump. Maximum BPA plasma concentrations were reached after three to four days. After seven days, animals were sacrificed and total BPA levels were determined in plasma and several organs. In general, BPA accumulation in the testes, adrenal and pituitary glands, adipose tissue, brain, liver and kidneys of Bcrp<sup>−/−</sup> mice was not different as compared to wild type mice. Also, co-administration of elacridar did not affect BPA accumulation. Interestingly, the testis weight of unexposed Bcrp<sup>−/−</sup> mice was significantly higher as compared to wild type mice, but was not affected by BPA exposure with or without elacridar. Taken together, our results indicate that the disposition of the human BCRP substrate BPA is not altered in Bcrp-deficient mice, suggesting minor importance of the transporter in BPA handling in mice.
Introduction

Male infertility or subfertility is an increasing problem within our modern society. About 30-40% of all infertile couples can be attributed to male factors. Normal endocrine function is a prerequisite for proper development of the reproductive system and maintenance of function throughout adult life. Endocrine disrupting chemicals (EDCs), compounds that interfere with hormone biosynthesis, metabolism or action, are thought to contribute to the increasing incidence of male subfertility. The effects of EDCs on male fertility are widely investigated, especially those of high-volume chemicals that are used in plastics and food-packaging such as bisphenol A (BPA). This compound is used in the manufacture of polycarbonate plastics and epoxy resins, and the widespread inclusion of BPA in consumer products has greatly enhanced the potential for human exposure. Of particular concern is the use of BPA in plastic food and beverage packaging and medical devices. BPA can leach from these products in appreciable amounts, resulting in nearly ubiquitous daily exposure to humans. Detectable levels of BPA have been found in the urine of 93% of the US population, as reported in 2008. Health concerns regarding human exposures to BPA stem from its estrogenic properties. While BPA was initially considered to be a ‘weak’ estrogen based on a lower affinity for the nuclear estrogen receptor (ER) alpha relative to estradiol, research showed that BPA is equipotent to estradiol in its ability to activate responses via estrogen receptors associated with the cell membrane. In addition, BPA has been described to antagonistically bind the androgen receptor. Exposures to BPA have been linked to reduced sperm counts in a rodent model and a human epidemiology study. Evidence has been reported that BPA can rapidly activate membrane-initiated ER signaling at doses found in human blood. Moreover, another study showed transplacental, multigenerational reproductive and carcinogenic effects by BPA in vivo at human relevant exposure levels. Understanding the kinetics and mechanism of toxicity of BPA is thus of key importance.

The endocrine organs of the hypothalamic–pituitary–gonadal (HPG) axis are highly susceptible to BPA interference. described that the effects of BPA affects the feedback regulatory circuits in the HPG axis. At the hypothalamic-pituitary level, perinatal and postnatal BPA exposures resulted in an upregulation of the expression levels of kisspeptin (Kiss1), gonadotropin-releasing hormone (GnRH) and follicle-stimulating hormone (FSH) mRNA in male and female mice. At the gonadal level, BPA inhibited expression levels of testicular steroidogenic enzymes and the synthesis of testosterone in males. In accordance, another group also found that neonatal BPA exposure interferes with sex specific gene expression of estrogen receptor-α, -β and Kiss1 in rat anterior hypothalamus. Moreover, BPA triggered an
immediate inhibition of pituitary LH secretion in lambs, as reported by Collet et al.\textsuperscript{16} A way in which organs can protect themselves against compounds like BPA is extrusion via active transport. ATP binding cassette (ABC) transporters are involved in the efflux of a variety of molecules against steep concentration gradients at the expense of ATP. The transporters are present in various tissues, protecting the organs from harmful substances and influencing the disposition and metabolism of xenobiotics. We recently showed that BPA is a substrate for the ABC transporter breast cancer resistance protein (BCRP/ABCG2).\textsuperscript{17} Moreover, we showed that Bcrp is localized in capillary endothelium of murine pituitary and adrenal glands, adipose tissue and testes.\textsuperscript{18} These findings suggest that BCRP may be an important determinant in the distribution, disposition and, thereby, the toxicity of BPA. In the present study, we aimed at elucidating the role of Bcrp in the disposition and potential endocrine disrupting effects of BPA in mice.

**Materials and methods**

**Chemicals**

\[^{[3]}\text{H}(G)\]-BPA (80 µM; 25 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Prior to the \textit{in vivo} experiment, \[^{[3]}\text{H}\]-BPA was diluted in physiological salt solution to 8 µM (= 0.2 mCi/ml in 10% ethanol). [6,7-\(^{[3]}\text{H}(N)\)]-Estrone sulfate ([\[^{3}\text{H}\]-E\(_1\)S; 54.26 Ci/mmol) was obtained from Perkin Elmer (Groningen, the Netherlands) and \[^{[3]}\text{H}\]-N-Methylquinidine ([\[^{3}\text{H}\]-NMQ; 85 Ci/mmol) from Solvo Biotechnology (Szeged, Hungary). The P-gp and BCRP/Bcrp inhibitor elacridar (GF120918) was purchased from Sequoia Research Products Limited (Pangbourne, United Kingdom) and dissolved in DMSO. BPA-glucuronide (BPA-G) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Biodistribution of BPA in wild type and Bcrp\(^{-/-}\) mice**

Friend leukemia virus type-B (FVB) mice and FVB-Bcrp\(^{-/-}\) mice were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands)\textsuperscript{19} and bred and housed at the Central Animal Laboratory of the Radboudumc. The Animal Experimental Committee of the Radboudumc approved all procedures involving the animal experiments in this study. Male mice (13-20 weeks old) were implanted with a micro-osmotic pump (model 1007D; Alzet\textsuperscript{\textregistered}, Durect Corp, Cupertino, CA, USA), designed to release a steady exposure. The mice were anesthetized by inhalation of isoﬂurane for 5 min and the pumps were implanted subcutaneously at the back of the mice. After suturing the wound, Carprofen (Rimadyl\textsuperscript{\textregistered}; 1 µg/g body weight) was administered subcutaneously at day 1 and 2 for pain treatment.
and the mice were individually caged. Prior to implantation, the pumps were filled with a physiological salt solution containing 20 µCi [3H]-BPA (10% ethanol) with or without the P-gp and BCRP/Bcrp inhibitor elacridar (1.68 µmol in 10% dimethylsulfoxide; DMSO) under sterile conditions and weighted to confirm the absence of air bubbles in the pumps. To the solutions without elacridar, 10% DMSO was added as solvent control. Leftovers of prepared solutions were taken for reference [3H]-BPA measurements. The pumps had a mean release rate of 0.5 µl/h, resulting in the following exposure; 100 µCi [3H]-BPA/kg/day and 8 µmol elacridar/kg/day. Six mice per group were used (N=3 for the Bcrp−/− mice exposed to both BPA and elacridar). Every day, 20 µl blood was withdrawn from the tail vein. After seven days, mice were sacrificed by a single, lethal intraperitoneal injection of 144 mg/kg pentobarbital (Nembutal®, 15 mg/ml) and blood was collected by a heart puncture. Diverse organs were isolated, weighed and homogenized using micropestles in Solvable® (Perkin Elmer, Waltham, MA, USA). Radioactivity was determined by scintillation counting (Tri-Carb® 2900TR; Perkin Elmer, Waltham, MA, USA) and total BPA content in blood and tissue samples was calculated based on reference samples. Accumulation in adrenal glands, kidneys and testes was determined in one organ per animal.

Preparation of membrane vesicles and transport interaction assays
Overexpression of human BCRP and P-gp in HEK293 cells was established using baculoviruses, which were produced using the Bac-to-Bac system (Invitrogen, Breda, the Netherlands), as described previously.18,20,21 As a control, the enhanced yellow fluorescent protein (eYFP) was overexpressed using the same system. Crude membranes were isolated, resuspended in TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) and membrane vesicles were prepared as described previously.20 Total protein content was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Veenendaal, the Netherlands). Crude membrane vesicles were dispensed in aliquots, snap frozen in liquid nitrogen, and stored at -80 °C until further use. The interaction of BPA's primary metabolite BPA-G with transporter activity was assessed by adding a reaction mix consisting of TS buffer supplemented with 4 mM ATP or AMP, 10 mM MgCl₂, radiolabelled substrates (250 nM [3H]-E₁S for BCRP or 100 nM [3H]-NMQ for P-gp) and various concentrations of BPA-G to 7.5 µg of membrane vesicles, based on total protein content. After an incubation of 60 sec at 37 ºC to enable ATP-dependent uptake, the reaction was stopped by placing the samples on ice and by addition of ice-cold TS buffer. Reaction mix was removed and the vesicles were washed by means of a rapid filtration technique using glass fiber filter plates (Millipore, Etten-Leur, the Netherlands). Scintillation fluid was added to the filters and the amount of radioactivity was determined using a scintillation counter.
Reference samples were measured to calculate the amount of transported substrate. ATP-dependent transport was calculated by subtracting values measured in the presence of AMP from those measured in the presence of ATP. Net transporter-mediated transport was calculated by subtracting ATP-dependent uptake in eYFP vesicles from that of transporter-overexpressing vesicles.

Data analysis
Area under the curve (AUC) values were calculated from the plasma BPA concentration time curve. Differences in organ BPA accumulation were assessed by means of a one-way ANOVA test followed by Dunnett’s post hoc test. Membrane vesicle transport results were analyzed using a one-way ANOVA test followed by Dunnett’s post hoc test. All analyses were performed with GraphPad Prism software (version 5.02, GraphPad Software Inc., San Diego, CA, USA).

Results

Plasma BPA levels in wild type and Bcrp\textsuperscript{-/-} mice
Kinetics of the BCRP-substrate BPA were studied in male wild type and Bcrp\textsuperscript{-/-} mice, that were subcutaneously exposed to [\textsuperscript{3}H]-BPA with or without elacridar via a micro-osmotic pump for seven days. Total plasma BPA levels were determined daily (figure 1). Maximum plasma concentrations were reached three to four days after implantation of the micro-osmotic pumps and plasma levels decreased thereafter. In Bcrp\textsuperscript{-/-} mice, slightly lower plasma BPA levels were found compared to wild type mice, as supported by the calculated AUC values (figure 1A), however, this difference was not statistically significant. No significant effect of elacridar was found on plasma BPA levels in wild type mice, however, the Bcrp and P-gp inhibitor seemed to reduce the decrease in plasma levels between day 4 and 7 slightly. The AUC in Bcrp-deficient mice treated with elacridar was significantly lower than elacridar-treated wild type mice (figure 1B). However, this difference could be due to low BPA measurements in this group at day 4, since BPA levels in the days thereafter were not lower than the other groups. Interestingly, plasma BPA levels kept rising after day 4 in inhibited Bcrp\textsuperscript{-/-} mice while plasma levels of the other groups decreased after day 4.
Disposition of the human BCRP substrate bisphenol A is not altered in Bcrp-deficient mice

No effects of BPA-G on transport activity of BCRP and P-gp
The concentration of [\(^3\)H\]-BPA in plasma reflects total levels of the compound, including its primary phase II metabolite BPA-G. It was shown previously that BPA is a substrate for human BCRP\(^{17}\) and possibly for rat Bcrp and P-glycoprotein\(^{22}\), however the handling of its major metabolite was less clear. We assessed the interaction of BPA-G on transport activity of BCRP and P-gp by incubating membrane vesicles overexpressing the transporters with the substrates [\(^3\)H\]-E\(_1\)S (BCRP) and [\(^3\)H\]-NMQ (P-gp) in the presence of increasing concentrations of BPA-G.\(^{18,21}\) Figure 2 depicts the calculated net transporter-mediated substrate uptake by membrane vesicles incubated with 1, 10 and 100 µM BPA-G, expressed as percentage of maximum. Neither of these concentrations influenced BCRP and P-gp activity.

Accumulation of BPA in Bcrp-expressing organs
To investigate the role of the efflux pump in the disposition of BPA, the biodistribution of BPA was investigated in various Bcrp-expressing organs. After seven days of exposure, BPA accumulation was determined in plasma, testis, adrenal and pituitary glands, adipose tissue, brain, liver and kidney (figure 3 and 4A). Highest BPA levels per gram tissue were found in the testis and adrenal and pituitary gland and the lowest BPA concentrations were found in adipose tissue (figure 3 and 4A). Bcrp-deficiency did not lead to altered BPA concentrations in endocrine organs nor in the two main metabolizing organs, the liver and kidney. Co-exposure to elacridar resulted only in increased liver BPA levels in Bcrp\(^{-/-}\) mice in accordance
with inhibition of efflux. Yet, in the kidneys of wild type mice, elacridar-treatment resulted in increased BPA levels in wild type mice only. Interestingly, these levels were not reached by elacridar-treated Bcrp<sup>−/−</sup> mice.

Figure 2. Effect of BPA-G on transport activity of BCRP (A) and P-gp (B). Membrane vesicles overexpressing the transporters or eYFP were incubated with [3H]-labeled substrates and increasing concentrations of BPA for 60 s at 37 °C in the presence of AMP or ATP. AMP values were subtracted from ATP values. Net transporter-mediated substrate uptake was calculated by subtraction of corresponding eYFP values and expressed as a percentage of maximum uptake. Graphs represent means + SEM of triplicate measurements in a representative experiment.
Disposition of the human BCRP substrate bisphenol A is not altered in Bcrp-deficient mice

Figure 3. BPA accumulation in organs of wild type and Bcrp<sup>−/−</sup> mice after a seven day exposure to [3H]-BPA (100 µCi/kg/day) with or without BCRP and P-gp inhibitor elacridar (8 µmol/kg/day), subcutaneously via a micro-osmotic pump. Accumulation of BPA (pmol/g tissue) in indicated organs was depicted as mean + SEM of N=6 (N=3 for Bcrp<sup>−/−</sup> exposed to BPA and elacridar). Chequered bar, exposed to BPA alone; Black bar, exposed to BPA in combination with elacridar. (** P < 0.01).

BPA accumulation in the testis, the primary site of male steroidogenesis and thought to be highly susceptible for BPA-mediated endocrine disruption, is shown in figure 4. BPA accumulation expressed as pmol/g testis (figure 4A) is reduced in Bcrp<sup>−/−</sup> mice, however, absolute accumulation was not different between wild type and Bcrp<sup>−/−</sup> mice (figure 4B). The difference in relative accumulation can be explained by an increased testis weight of Bcrp<sup>−/−</sup> mice as compared to wild type mice (figure 4C). Exposure to BPA and elacridar did not affect the testicular weight. The weights of the adrenal glands, brains and kidneys, which were harvested as a whole, did not differ between the experimental groups.
In this study, we investigated the biodistribution of BPA in male wild type versus Bcrp<sup>-/-</sup> mice to elucidate the role of Bcrp in the disposition and potential endocrine disrupting effects of BPA. Our results show that the disposition of human BCRP substrate BPA is not altered by murine Bcrp or P-gp.

The role of efflux pumps in BPA disposition has recently gained interest of other investigators as well. Mazur <i>et al.</i> showed that BPA is a potential substrate for BCRP, based on an indirect ATPase activity assay. In accordance, we previously reported that BPA is indeed transported by BCRP but not by human P-glycoprotein. BPA is a lipophilic compound, which has been shown to accumulate in brain, adipose tissue and mammary glands of exposed rats since murine Bcrp is localized in the capillary endothelium of these organs (except in the mammary gland), as well as in the testes and pituitary and adrenal glands where it transports molecules towards the blood<sup>18</sup>, we hypothesized that the efflux pump influences the disposition of BPA. Accordingly, we expected BPA accumulation in these organs to be increased in Bcrp-deficient mice. Nevertheless, our results show that Bcrp does not influence the accumulation of BPA in mice. Knockout mouse models are useful to investigate the role of a specific transporter,
however, compensatory gene expression and protein synthesis may countervail the lack of the specific gene. To exclude the possibility that other, yet unknown, BPA-transporters were upregulated in Bcrp-deficient mice and counteracted the potential effects of Bcrp-deficiency, two additional groups were included, i.e. wild type and Bcrp-/- mice treated with the Bcrp and P-gp inhibitor elacridar. Except for the liver, elacridar inhibition did not significantly affect BPA accumulation in Bcrp-/- mice, therefore no effect of P-gp is suggested. The increasing effect of elacridar on BPA levels in the livers Bcrp-/- mice, would suggest an effect of P-gp, however, this was not found in wild type mice. Also, in the kidneys of wild type mice, elacridar-mediated increase in BPA levels could probably not be explained by P-gp-mediated efflux of BPA as this effect of elacridar was not found in the knockout mice.

The mice were continuously exposed to BPA to ensure measurable tissue levels. BPA was administered subcutaneously via an osmotic pump to eliminate differences in absorption between the mouse strains that could occur after oral administration. It was reported by Doerge et al. that maximum BPA plasma levels in orally-treated neonatal rats were 34 times lower than from subcutaneous injections. These results reinforce the critical role of first-pass phase II metabolism of BPA in gut and liver. The same group examined the distribution of BPA and its primary phase II metabolite BPA-G in adult rats and showed that the fraction of total BPA present as the parent compound was >90% in adipose, muscle and brain tissues, three half-lives after intravenous injection. This indicates that BPA-G does not accumulate in tissues and is immediately excreted after formation. In our study, using radiolabelled BPA, total BPA levels were determined and, thus, we were not able to distinguish between BPA and its metabolites. However, based on the findings of Doerge et al., we assumed that the levels found in the tissues could be assigned to BPA itself. As BPA-G did not influence BCRP and P-gp activity in vitro, even at high concentrations (100 µM) and also Mazur et al. did not find an interaction between BPA-G and BCRP or P-gp, it can be assumed that the small fraction of BPA-G present in total BPA levels did not affect BPA accumulation in wild type and Bcrp-/- mice.

Furthermore, we describe for the first time that the testicular weight of Bcrp-/- mice (either treated or not) was 40% higher than that of wild type mice. Generally, it is believed that testicular weight increases to compensate a decreased testicular function, i.e. steroidogenesis and spermatogenesis. BCRP/Bcrp have been correlated with steroid hormones before. Several steroids, including estradiol, testosterone, progesterone and androstenedione, can inhibit BCRP function. Besides, the localization of BCRP in the plasma membrane is shown to be sex specific, which is in males suggested to be due to the inductive effect of testosterone. N.B. We observed that Bcrp-/- mice exhibit reduced fertility with a reduced
number of pregnancies and smaller litter of pups as compared to wild type animals (7.2 ± 0.5 versus 9.5 ± 0.2 pups, respectively; N=10). The localization of the transporter in the plasma membrane is shown to be sex specific and the sex steroids estradiol, progesterone, and testosterone have been shown to impact \( \text{BCRP} \) gene expression. \(^{30-34} \) However, the role of BCRP/Bcrp in steroid hormone metabolism is far from elucidated. Our results suggest that Bcrp could be involved in proper testicular function. We, however, did not examine steroid production and germ cell count, hence, we cannot confirm that testicular function was impaired in Bcrp-deficient mice. Clearly, further research is required to elucidate why testicular weight is increased in Bcrp-deficient mice.

BPA was efficiently transported by human BCRP, as was shown in BCRP-overexpressing MDCKII cells loaded with 40 nM BPA with or without elacridar. \(^{17} \) Our current results argue that the murine orthologue Bcrp is probably not involved in the disposition of BPA. Significant interspecies differences between human and rodents in terms of BCRP/Bcrp mRNA and protein expression have long been recognized. \(^{35-37} \) However, delineating differences in substrate affinities between BCRP and Bcrp have been much less reported. Zhang \( et \ al. \) \(^{38} \) described the interactions of the flavonoids chrysin and benzoflavone with human BCRP, which were not found with rat or mouse Bcrp. Moreover, Mazur \( et \ al. \) \(^{22} \) showed interspecies differences in the efflux transporter specificities for BPA between rat and human ABC transporters, including BCRP. BPA-mediated increase in ATPase activity was clearly found for human BCRP but not for rat Bcrp. Possibly, this species difference can explain the different outcomes in our experiments with human and mouse BCRP/Bcrp. No interaction of BPA with murine Bcrp has been described before. These differences between human and rodent ABC transporters need further confirmation, for example by studying BCRP/Bcrp from different species transfected in the same cell line. These results may have significant implications for interspecies extrapolation used in risk assessment. In humans, BCRP may be a potential determinant in BPA-mediated endocrine disruption, because increased BPA exposure of endocrine producing cells due to lack of BCRP activity can potentially increase the endocrine disrupting effects of BPA.

In summary, we show for the first time that Bcrp\(^{-/-} \) mice display increased testicular weight, which could indicate a physiological role for Bcrp in testicular homeostasis and male fertility. Further, our results indicate that the disposition of BPA, a substrate for human BCRP, is not altered in Bcrp-deficient mice. Possibly, the EDC is not a substrate for the efflux transporter in mice. Although this interspecies difference needs further confirmation, it is an important phenomenon that should be taken into account when interpreting results obtained from rodent animal models, as the disposition and thus potentially the mechanism of toxicity may be different in humans as compared to rodents.
References

Chapter 4


Disposition of the human BCRP substrate bisphenol A is not altered in Bcrp-deficient mice
Heme oxygenase-1 and breast cancer resistance protein protect against heme-induced toxicity

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Chapter 5

Abstract

Heme is the functional group of diverse hemoproteins and crucial for many cellular processes. However, heme is increasingly recognized as a culprit for a wide variety of pathologies, including sepsis, malaria, and kidney failure. Excess of free heme can be detrimental to tissues by mediating oxidative and inflammatory injury. Protective mechanisms against free heme are therefore pivotal for cellular survival. We postulated that overexpression of heme oxygenase-1 (HO-1) and breast cancer resistance protein (BCRP) would protect against heme-induced cytotoxicity. HO-1 is a heme-degrading enzyme generating carbon monoxide, iron, and biliverdin/bilirubin, while BCRP is a heme efflux transporter. Human embryonic kidney cells were transduced using a baculovirus system as a novel strategy to efficiently overexpress HO-1 and BCRP. Exposing cells to heme resulted in a dose-dependent increase in reactive oxygen species formation, DNA damage and cell death. Heme-induced cell death was significantly attenuated when cells overexpressed HO-1, BCRP, or both. The protective effects of HO-1-overexpression were most pronounced, while co-treatment with the HO-activity inhibitor tin mesoporphyrin reversed these protective effects. Also cells treated with the anti-oxidants N-acetylcysteine or HO-effector molecule bilirubin showed protection against heme insults, which may explain the increased protection by HO-1 compared to BCRP. In conclusion, both HO-1 and BCRP protect against heme-induced toxicity and may thus form novel therapeutic targets for heme-mediated pathologies.
Introduction

Heme is a complex of free iron and a porphyrin ring and is synthesized in every nucleated cell. Heme possesses dual biological effects. It is crucial as the functional group of a variety of hemoproteins involved in oxygen transport and storage, electron transfer, and signal transduction. However, free heme catalyzes also the formation of reactive oxygen species (ROS) through the Fenton reaction resulting in tissue injury. Free heme not only promotes oxidative stress, but also possesses proinflammatory properties both in vitro and in vivo. Heme induces vascular adhesion molecule expression, causes vascular permeability, and promotes leukocyte recruitment and the induction of inflammatory cytokines. Large amounts of free heme can be a major culprit in the etiology of a wide variety of pathological conditions and diseases.

Heme can also exacerbate disease processes as exemplified for malaria and sepsis. In an experimental setup in which mice infected with Plasmodium Berghei ANKA developed malaria but no experimental cerebral malaria. Administration of free heme to these mice promoted oxidative and inflammatory insults resulting in blood-brain barrier dysfunction. This allowed the entrance of inflammatory mediators capable of exacerbating disease towards the brain. Similarly, in a murine model of sepsis, it was found that administration of heme severely aggravated sepsis by worsening organ injury. It is therefore pivotal to protect cells from heme-induced oxidative and inflammatory insults. Rhabdomyolysis, intravascular hemolysis and cell injury are characterized by the release of large amounts of heme. The heme- and hemoglobin (Hb)-scavengers hemopexin and haptoglobin act as a first line of defense against the injurious actions of heme. They have been demonstrated to protect against heme- and Hb-mediated insults such as vascular adhesion, lipid peroxidation, and organ failure. However, these heme-scavengers may be overwhelmed when exposed to large amounts of heme. Down-stream, several putative defense systems against intracellular heme are present, such as heme oxygenase-1 (HO-1) and the efflux pump breast cancer resistance protein (BCRP).

Heme-mediated pathologies can be attenuated by activation of the heme oxygenase system (HO) system. HO breaks down heme into biliverdin, iron, and carbon monoxide. Biliverdin is then rapidly converted into the antioxidant bilirubin by biliverdin reductase, while iron is rendered inactive by co-induction of its scavenger ferritin. Two isoforms of HO have been identified in humans: HO-1 and HO-2. The inducible isoform, HO-1, shows low basal expression but can be highly induced by a variety of stimuli, including cytokines and oxidative stress, while HO-2 is thought to be constitutively expressed. Another putative determinant...
in heme-related toxicity is a member of the ATP-binding cassette (ABC) transporter family localized at the plasma membrane known as the breast cancer resistance protein (BCRP: ABCG2).\textsuperscript{23} This efflux pump modulates erythroid maturation by decreasing cellular heme levels\textsuperscript{24,25} and by protecting cells and tissues from heme accumulation following hypoxia.\textsuperscript{26} The possible protection by BCRP against heme is further strengthened by the observation that heme accumulates in tissues of BCRP-knockout mice.\textsuperscript{26-28} Since both HO and BCRP have the potential to clear heme from the cells, we postulated that the proteins BCRP and HO-1 protect against heme-induced toxicity. To test this, we overexpressed BCRP and HO-1, and combinations to investigate the possible protective roles of both proteins and their combination in heme-induced cytotoxicity in human embryonic kidney (HEK293) cells.

**Materials and methods**

*Culture of HEK293 cells*

Human embryonic kidney (HEK293) cells were cultured in T75 flasks in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose levels, containing 10% fetal calf serum (FCS) (MP Biomedicals, Uden, the Netherlands) at 37°C in a 5% CO\textsubscript{2} atmosphere. Cells were passed (1:5) using Trypsin/EDTA when reaching approximately 90% of confluency.

*Preparation of porphyrin and bilirubin solutions*

Heme (FePP), the HO-activity blocker tin mesoporphyrin (SnMP) and the antioxidant bilirubin (all obtained from Frontier Scientific, Carnforth, UK) were freshly prepared as previously described.\textsuperscript{5} In short, the substances were dissolved together with Trizma base in a 0.1 M NaOH solution and diluted in H\textsubscript{2}O. The obtained solution (pH 11 to 12) was adjusted to pH 8 with HCl. The solution was then filter-sterilized, protected from light and directly used.

*Measurement of reactive oxygen species (ROS)*

The ROS-specific labeling dye 2',7'-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCFDA) (Invitrogen, Breda, The Netherlands) was used to measure heme-induced ROS formation. HEK293 cells were seeded and grown till 70% confluency in DMEM without phenol red with 10% FCS in collagen pre-coated 96-wells plates, suitable for fluorescence measurement (ThermoFischer Scientific, Vantaa, Finland) as described previously.\textsuperscript{29} Cells were washed once with Hanks’ Balanced Salt Solution (HBSS, Invitrogen) prior to labelling with 10 µM H\textsubscript{2}DCFDA in HBSS for 20 min at 37°C. Afterwards, cells were washed twice with HBSS and exposed to
Heme oxygenase-1 and breast cancer resistance protein protect against heme-induced toxicity

Heme (0-12.5 µM) for 90 min in the presence or absence of 6 mM N-acetylcysteine (NAC; glutathione precursor) or 30 µM bilirubin in DMEM without phenol red and 1% FCS. Unlabeled cells were used as negative control and H₂O₂-treated cells (200 µM) were used as positive control. Fluorescence, representing ROS formation, was determined using a FluoStar Galaxy fluorometer at excitation 485 nm and emission 520 nm (BMG Lab Technologies, Offenburg, Germany).

Detection of DNA damage
The comet-assay (single cell gel electrophoresis) was used to detect DNA damage after heme-treatment. HEK293 cells were cultured until 40-50% confluency in 6-wells plates. Subsequently, cells were treated with 200 µM heme in presence or absence of 6 mM NAC or 30 µM bilirubin for 48h. Cells were harvested and the amount of cells/mL was determined. In addition, 6000 cells were resuspended in 75 µL LM-Agarose (Trevigen, Gaithersburg, Germany) and immediately applied to the comet slides.Slides were allowed to solidify for 30 min at 4°C and subsequently submerged in lysis buffer (Trevigen) for 60 min at 4°C. After cell lysis, the slides were placed on a horizontal electrophoresis system tray (Owl separation system) containing alkaline electrophoresis buffer (pH>13; 300 mM NaOH, 1 mM EDTA in H₂O) and electrophoresis was performed at 25 V for 30 min to allow DNA unwinding. Slides were then neutralized by three times washing with neutralization buffer (0.4 M Tris-HCl pH 7.5), fixed in 100% ethanol during 10 min and air dried. After neutralization, samples were stained with 50 µL ethidium bromide solution (1:75,000 in H₂O) and enclosed with VectaShield (Vector Laboratories Inc, Burlingame, CA). All steps were performed at dimmed light conditions to avoid DNA damage by UV light. Slides were examined (40x magnification) using a LeicaDM fluorescent microscope with a TRITC-filter. The images of 20 randomly chosen nuclei per slide were blindly scored. Each nucleus was visually scored from undamaged DNA stage 0 to maximally damaged DNA stage 4.30

Preparation of baculovirus constructs
To study the role of HO-1 and BCRP in the protection of heme-induced toxicity more extensively, we generated baculoviruses, suitable for transduction of human cells to overexpress the proteins, as described previously.31,32 In short, for BCRP-overexpression the pDONR-221-hBCRP clone (HsCD00044371; Harvard Institute of Proteomics, Harvard Medical School, Cambridge, MA, USA) was used and cloned into pFB-VSV-CMV-DEST. For HO-1-overexpression the pFastBac-VSV-CMV-DEST hHO-1 WT construct was used. This construct was made as follows: the human HO-1 gene was amplified using the following primers:
forward primer 5’-ggggacaagtttgtcaaaaagacagcccttaacaccatggagcgtccgcaacccga and reverse primer 5’-ggggaccactttgtacagaaagctgggtctcacatggcataaagcccta. Then hHO-1 was cloned into the entry vector pDONR201 (Invitrogen, Breda, the Netherlands) using the attB flanked PCR product by the BP clonase reaction. Subsequently, the hHO-1 gene was cloned into the destination vector pFB-VSV-CMV-DEST by the LR clonase reaction. DH10Bac competent cells were transformed with the destination vector and bacmide DNA was isolated. Baculoviruses were produced as described in the Bac-to-Bac manual. Enhanced yellow fluorescent protein (EYFP) cloned into pFB-VSV-CMV DEST was used as a control (Invitrogen). Sf9 insect cells were transfected with the recombinant bacmide DNA and viruses were harvested. P3 viruses were used for transduction.

**EYFP/EYFP, BCRP/EYFP, HO-1/EYFP and BCRP/HO-1 baculovirus-mediated double transductions**

Human Embryonic Kidney (HEK293) cells were cultured in T75 flasks in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose levels, containing 10% fetal calf serum (FCS) (MP Biomedicals, Uden, the Netherlands) at 37°C in a 5% CO₂ atmosphere. Cells were passed (1:5) when reaching approximately 90% of confluency. Before double-transductions were performed, HEK293 cells were cultured in T75 flasks until 40% confluency. To overexpress BCRP and/or HO-1 proteins, the medium was replaced by equal volumes (650 µL baculovirus of each) and completed to 3.3 mL with medium. In all cases, double-transductions were performed to correct for the amount of virus. After 15-60 min of incubation at 37°C, medium was added to a final volume of 10 mL. Sodium butyrate (5 mM) was added 24 h after transduction to realize an up-regulation of protein expression.

**Western blot analysis for HO-1 and BCRP protein expression**

To determine the HO-1- and BCRP-overexpression, cells were harvested, 48 h, 72 h and 96 h after transduction, and pelleted by 5 min of centrifugation (500 g). For HO-1 protein determination, pellets were lysed for 10 min on ice using lysis buffer containing protease inhibitors (1 mM EDTA, 0.5% Triton-X-100, 25 µg/ml leupeptin, 25 µg/ml pepstatin, 100 µM phenylmethylsulphonyl fluoride, 3 µg/ml aprotinin in PBS, pH 7.2, all Sigma-Aldrich). Subsequently, cell lysates were centrifuged (20,000 g) for 2 min at 4°C. For the determination of BCRP expression, membrane fractions were prepared. To this end, pellets of transduced cells were resuspended in PBS with added protease inhibitors (Roche, Mannheim, Germany). Afterwards, the lysates were snap-frozen in liquid nitrogen and defrosted at 37°C three times. Samples were centrifuged (20,000 g) for 10 min. The pellet was resuspended in 40 µL PBS. Samples were prepared for gel electrophoresis by incubation with Laemmli sample.
buffer (consisting of 0.5 M tris-HCl pH 6.8, 8% sodium dodecyl sulfate (SDS), 40% glycerol (100%), H₂O, 0.08% bromophenol blue and 0.4 M β-mercapto-ethanol) for 5 min at 95°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and blotted onto a nitrocellulose membrane using a BioRad wet blotting system. The membrane was blocked for 1 h at room RT using Odyssey Blocking Buffer (Westburg BV, Leusden, the Netherlands). The blot was incubated overnight at 4°C with rabbit-anti-HO-1 Ab (rat) (SPA-895; 1:5.000; Stressgen/ITK, Uithoorn, The Netherlands) or mouse-anti-BCRP (BXP-21, 1:200, Kamiya Biomedical Company, Seattle, USA). Mouse-anti-β-actin Ab (human) was simultaneously incubated as a protein loading control (1:100.000, Sigma-Aldrich). Afterwards, the membrane was rinsed three times with PBS followed by washing three times 10 min with PBS containing 0.1% Tween-20. Both the HO-1 blot and the BCRP blot were incubated with the secondary antibodies goat-anti-mouse IRDye 800 (1:20.000, Rockland, Heerhugowaard, the Netherlands) and goat-anti-rabbit Alexa Fluor 680 (1:20.000 Sigma-Aldrich) in Odyssey Blocking Buffer containing 0.1% Tween-20 and 0.01% SDS for 1 h at RT protected from light. After thorough washing, expression of β-actin and BCRP was assessed with the Odyssey Infrared Imaging System using channel 800 while HO-1 expression was determined using channel 700 (LI-COR Biosciences). Intensity of the bands was determined using the Odyssey application software. The ratios of HO-1/BCRP and β-actin intensities were calculated and protein levels were normalized to the expression in EYFP/EYFP transduced cells.

**BCRP activity determination using flow cytometric analysis of Hoechst33342 accumulation in BCRP transduced HEK293 cells**

Fumitremorgin C (FTC) is used as a BCRP-mediated transport inhibitor. To assess whether FTC actually inhibits the efflux pump in our BCRP-overexpressing HEK293 cells, the cells were incubated with 10 µM FTC or DMSO control, three days after transduction. After 20 min, the fluorescent BCRP substrate Hoechst33342 (10 µM) was added and incubated for 45 min at 37°C. Subsequently, cells were washed with HBSS/Hepes (pH 7.4) and fixed with 4% PFA. Hoechst accumulation was assessed with a Beckman Coulter Altra flow cytometer, using a Coherent Enterprice II laser (621-CL).

**Determination of cell viability**

The MTT-assay was used to determine cell survival after heme treatment. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is reduced to the purple formazan in the mitochondria of living cells which was used as a measure for cell viability. HO-1 and BCRP transduced HEK293 cells were harvested (36 h after transduction), and
seeded on collagen pre-coated 96-wells plates at a density of 2.5*10^4 cells/well. After 12 h, cells were treated for 48 h with increasing doses of heme (0-500 µM) in the presence or absence of 10 µM FTC, 10 µM SnMP, 6 mM NAC or 10 µM or 30 µM bilirubin in culture medium. A concentration of 500 µM heme was used as positive control for cytotoxicity. After heme treatment, medium was removed and 20 µL MTT solution (5 mg/mL in PBS; Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the wells and incubated for 4 h at 37°C. Dimethyl sulfoxide (DMSO) was added to dissolve the water insoluble formazan. After 1 min of shaking, the absorbance of this colored solution was measured spectrophotometrically at a wavelength of 560 nm (Biorad, benchmark plus microplate spectrophotometer, UK). Cell viability of treated cells is expressed as a percentage compared to untreated cells.

Data analysis
Data were analyzed by means of GraphPad Prism (version 5.01 for Windows; Graph Pad Software, San Diego, Ca, USA). The effect of heme treatment on cell survival was assessed by non-linear regression (sigmoidal dose response with variable slope). Results of ROS determination were statistically tested by use of a one-way ANOVA test followed by post hoc testing (Dunnett or Bonferroni). For the comet assay, a Kruskal-Wallis test was performed. The results of the FACS analysis were statistically evaluated using an unpaired T-test. Groups were considered to be significantly different when \( P < 0.05 \).

Results

Heme induces cell death in HEK293 cells
To evaluate the toxicity of heme, HEK293 cell survival was determined using the MTT-assay after 48 h of heme treatment (0-500 µM). Figure 1 shows that concentrations higher than 10 µM heme cause cell death in HEK293 cells. The concentration that resulted in 50% reduction in cell survival (IC\(_{50}\) value) was 143 ± 1 µM. No cell survival was observed at concentrations of 250 µM heme or higher.

The effects on cell morphology of heme treatment, and the protective effect of the cellular antioxidant NAC, was visualized using light microscopy with 200x magnification (figure 1B). A 200 µM heme treatment resulted in rounding and detachment of the cells, while the cells were protected by NAC for this effect.
**Heme oxygenase-1 and breast cancer resistance protein protect against heme-induced toxicity**

**Figure 1.** Assessment of cell survival in HEK293 cells after heme treatment. (A) Cells were treated for 48 h with heme (0-500 µM) and cell survival was determined using the MTT assay. Data are expressed as the mean ± SEM (N=6-8). Results were analyzed by non-linear regression (sigmoidal dose-response with variable slope). (B) Changes in cell morphology after 48 hours of heme (200 µM) treatment and the protective effect of co-incubation with the cellular antioxidant NAC (6 mM) is visualized by light microscopy with 200x magnification.

**Determination of heme-induced reactive oxygen species**

Exposing HEK293 cells to heme results in a dose-dependent ROS production (figure 2). After addition of the cellular antioxidant NAC, a significant decline in the amount of ROS produced by 3.0, 6.0, and 12.5 µM heme exposure was observed. Concentrations of heme above 12.5 µM resulted in quenching of the ROS sensitive probe. By simultaneous addition of heme and the strong cellular antioxidant, bilirubin, a considerable attenuation in intracellular ROS production was observed at all heme concentrations. This indicates that bilirubin is a more powerful antioxidant than NAC.
Figure 2. Determination of intracellular ROS accumulation (after 30 min) in HEK293 cells directly after heme treatment. Cells were labeled with H$_2$DCFDA prior to heme treatment (0-12.5 µM). 200 µM H$_2$O$_2$ was used as a positive control to induce ROS production. ROS levels are presented as arbitrary fluorescence units. Data represent mean + SD of two independent replications, each condition was assayed in quadruplicate within an experiment. *** compared to medium (P<0.001); b compared to control within the same heme treatment group (P<0.01); c compared to control within the same heme treatment group (P<0.001).

**Detection of oxidant-induced DNA damage**

In view of the oxidative properties of heme, the comet assay was used to evaluate oxidative DNA damage in HEK293 cells (figure 3). Therefore, cells were treated for 48 h with heme (200 µM) in the absence or presence of NAC (6 mM) or bilirubin (30 µM). Figure 3B shows an induction of comet formation, indicating DNA damage, compared to the untreated group (P<0.05; Kruskal Wallis; n=20). The antioxidants NAC and bilirubin protected against heme-induced DNA damage. Notably, however, the differences between the heme-treated group with and without NAC or bilirubin were rather small. This is in contradiction with results as presented in figure 2, where we observed that heme-induced ROS formation can be substantially reduced by NAC and bilirubin.
Figure 3. Heme-induced DNA damage assessed by the comet-assay. HEK293 cells were treated for 48 h with heme in the presence or absence of 6 mM NAC or 30 μM bilirubin. Cells were examined from undamaged DNA stage 0 to maximally damaged DNA stage 4. Panel A reflects representative comets for DNA damage stage 0-2 observed in this assay. Panel B represents individual DNA damage scores. * compared to control (P<0.05).

Western blotting to assess expression of BCRP and HO-1 after baculovirus-mediated transductions

We examined the protein expression of HO-1 and BCRP in all baculovirus-mediated transduced HEK293 cells, 48 h, 72 h and 96 h after transduction. Treatment of HEK293 cells with baculovirus resulted in an induction of endogenous HO-1, independent of the type of baculovirus. This is shown in figure 4 where in EYFP/EYFP- and BCRP/EYFP-transduced cells the presence of HO-1 protein was detectable after 48 h. However, this figure also shows considerably higher HO-1 expression in the HO-1/EYFP- and BCRP/HO-1-transduced cells compared to the EYFP/EYFP- and BCRP/EYFP-transduced cells after 48 and 72 h. There is a substantial decrease in HO-1 expression over time in all groups to similar levels as the EYFP/EYFP-transduced cells 96 h after transduction (figure 4A+B). Figure 5 shows the presence of
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BCRP proteins in BCRP/EYFP- or BCRP/HO-1-transduced cells. Hardly no expression of BCRP was seen in EYFP/EYFP- and HO-1/EYFP-transduced cells. Baculovirus treatment resulted in a transient transduction of the cells with highest BCRP expression at 48 h after treatment, after which the expression decreased in time. After 96 h, the BCRP expression had decreased to approximately 50% of the level at 48 h, but was still high compared to EYFP/EYFP control (figure 5).

Figure 4. Effect of EYFP/EYFP, BCRP/EYFP, HO-1/EYFP and BCRP/HO-1 baculovirus transductions on the expression of HO-1 protein in HEK293 cells. (A) Whole cell lysates were obtained after 48, 72 and 96 h of transduction to assess the protein expression of HO-1 and β-actin (housekeeping protein). One representative blot is shown for HO-1 and β-actin expression. (B) HO-1 expression was corrected for β-actin expression and compared to EYFP/EYFP. Data represent mean + SEM of 3 (48 h) or 4 (72 and 96 h) independent experiments.
Heme oxygenase-1 and breast cancer resistance protein protect against heme-induced toxicity

Figure 5. Effect of EYFP/EYFP, BCRP/EYFP, HO-1/EYFP and BCRP/HO-1 virus transductions on expression of BCRP proteins in HEK293 cells. (A) Total membrane fractions were harvested after 48, 72 and 96h of transduction to assess the protein expression of BCRP and β-actin. One representative blot is shown for BCRP and β-actin expression. (B) BCRP expression was corrected for β-actin expression and compared to EYFP/EYFP. Data represent the mean ± SEM of 2 (48 h) or 3 (72 and 96 h) independent experiments.

FTC increases the Hoechst33342 accumulation in BCRP-transduced HEK293 cells

To assess BCRP function in overexpressing HEK293 cells, the cells were incubated with 10 µM of the fluorescent BCRP substrate Hoechst33342 in the presence or absence of the BCRP activity inhibitor FTC (10 µM). Subsequently, the Hoechst accumulation was assessed by flow cytometry. Figure 6A demonstrates the percentage of Hoechst negative cells, indicative of dye extrusion. When cells were incubated with FTC, 14.2 ± 1.9% of the cells were Hoechst negative (figure 6B), which is significantly less than cell samples that were not incubated with FTC (66.8 ± 2.8%). These results indicate that BCRP-mediated Hoechst33342 efflux could be largely inhibited by FTC.
Effect of FTC on Hoechst33342 accumulation of BCRP-transduced HEK293 cells analyzed by flow cytometry. Three days after transduction, the cells were incubated with 10 µM Hoechst33342 for 45 min at 37°C. Prior to this, the cells were pre-incubated with the BCRP activity inhibitor FTC (10 µM) for 20 min. The percentage of Hoechst negative cells was determined as a measure of BCRP efflux activity. One representative experiment is shown in panel A; the experiment was performed in quadruplicate and depicted as the percentage of Hoechst negative cells + SD in panel B. An unpaired T-test was used for statistical testing; *** p<0.001.

Figure 7 demonstrates a significantly higher cell survival due to overexpression of BCRP, HO-1, or a combination of both proteins. Further, HO-1 shows to be more important in reducing heme-induced cytotoxicity than BCRP. Nevertheless, overexpression of both HO-1 and BCRP resulted in the highest protection against heme treatment, but there was only a slight difference (P<0.05) with HO-1-overexpression solely.

After treating cells with a concentration range of heme (0-500 µM), the 50% reduction in cell survival (IC$_{50}$ values) was determined by curve fitting, and is presented in Table 1. The IC$_{50}$ values confirm the protective role of BCRP and HO-1 against heme treatment. Fitted curves are presented in supplementary figure 1.
Figure 7. Assessment of cell survival in transduced HEK293 cells after heme treatment. EYFP/EYFP, BCRP/EYFP, HO-1/EYFP or BCRP/HO-1 proteins were overexpressed in HEK293 cells via baculovirus-mediated transduction. 48 h after transduction, cells were treated with heme (200 μM) for 48 h and cell survival was assessed using the MTT assay. Cell survival is expressed in percentages, relative to untreated cells. Data are presented as mean ± SEM (N=3-4). # non-transduced cells compared to transduced cells (P<0.01); *** EYFP/EYFP transduced cells compared to all other transduced conditions (P<0.001). Comparisons between the specific combinations: * P<0.05; *** p<0.001

Table 1. Assessment of cell survival in baculovirus-transduced HEK293 cells after treatment with heme.

<table>
<thead>
<tr>
<th>Condition</th>
<th>IC_{50} (µM)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>No transduction</td>
<td>143 ± 1</td>
<td></td>
</tr>
<tr>
<td>EYFP/EYFP</td>
<td>165 ± 7</td>
<td></td>
</tr>
<tr>
<td>BCRP/EYFP</td>
<td>198 ± 10</td>
<td>**</td>
</tr>
<tr>
<td>HO-1/EYFP</td>
<td>234 ± 22</td>
<td>***</td>
</tr>
<tr>
<td>BCRP/HO-1</td>
<td>262 ± 52</td>
<td>***</td>
</tr>
</tbody>
</table>

*Cells were transduced with EYFP/EYFP, BCRP/EYFP, HO-1/EYFP or BCRP/HO-1. 48 h after transduction, cells were treated with heme (0-500 µM) for 48 h and cell survival was assessed by using the MTT assay. The IC_{50} values (mean ± SEM, N=3-4) were calculated using non-linear regression analysis. ANOVA was used for statistical testing followed by Bonferroni’s multiple comparison: compared to no transduction ** (P<0.01), *** (P<0.001); compared to EYFP transduction *** (P<0.001).
Influence of BCRP and HO-1 activity inhibitors and the antioxidant NAC on heme-induced cell death

To test whether the protective effects of BCRP and HO-1 on heme-induced cytotoxicity could be diminished by specific inhibitors, FTC and SnMP were used to inhibit BCRP and HO-activity, respectively. Figure 8 shows the effect of the treatments on cell survival for each transduction group. SnMP or the combination of SnMP with FTC resulted in significantly lower cell survival rates in the cells transduced with BCRP/EYFP, HO-1/EYFP and BCRP/HO-1. Surprisingly, FTC alone did not reduce cell survival in the BCRP/EYFP and BCRP/HO-1 transduced cells. NAC co-treatment demonstrated protection in all the baculovirus treatments.

**Figure 8.** At 48 h after transduction, cells were treated for another 48 h followed by cell survival assessment using the MTT assay. Effect of co-exposure to 10 µM FTC, 10 µM SnMP, 6 mM NAC or the combination of 10 µM FTC and 10 µM SnMP on survival of BCRP and/or HO-1-overexpressing HEK293 cells after treatment with 200 µM heme was measured. Cells were transduced with EYFP/EYFP, BCRP/EYFP, HO-1/EYFP or BCRP/HO-1. Data represent mean ± SEM (N=2-6). * P<0.05; ** P<0.01; *** P<0.001 compared to heme treatment solely.
Discussion

This study demonstrates that heme promotes ROS formation, DNA damage, and cell death in a dose-dependent manner in HEK293 cells. Heme-mediated toxic effects were attenuated when cells overexpressed the heme-degrading enzyme, HO-1, the heme efflux transporter, BCRP, or combinations thereof. The protective effect of HO-1 was most pronounced, and co-treatment with an HO-activity inhibitor reversed this protective effect. In addition, exposure to antioxidants NAC or bilirubin protected cells from heme.

Heme has been found to be the causative and detrimental factor in the etiology of a wide variety of conditions, including hemolytic diseases, hemoglobinopathies, trauma, wound healing, sepsis, malaria, pregnancy complications, and renal failure. The relatively high concentrations of heme used in the current study (0-500 µM) are within the range of those observed in an HO-1-deficient patient, with a reported serum heme concentration of 0.5 mM, the concentrations in blood clots (0.4 mM), and in experimental models of heme overload. Accumulating heme concentrations resulted in ROS formation and cellular injury, in agreement with previous investigations, however, whether excessive heme also induces DNA damage is less well understood. Using the comet assay, we observed significant DNA damage after treatment of the cells with 200 µM heme. This is in agreement with an in vitro study that revealed nuclear damage after exposure to 50 µM heme in kidney cells. Furthermore, we showed that the antioxidants NAC and bilirubin attenuated this heme-induced DNA damage.

Successful overexpression of BCRP, HO-1 or both was achieved using the baculovirus expression system, which is widely used for the functional expression of proteins in vitro, but its use in overexpressing HO-1 was not demonstrated earlier. Transduction of HEK293 cells resulted in a very high functional expression of HO-1 and BCRP, which was suitable to address our research questions.

Since vital organs, such as the kidney, brain and liver, are highly sensitive to increased intracellular heme levels, it is important that the intracellular heme concentration is carefully regulated. Heme-binding proteins have been shown to lower intracellular free heme levels by scavenging heme with high affinity, but the current study provides evidence for both BCRP and HO-1 as critical regulators of intracellular heme concentration.

HO-1-overexpression protected against heme-induced cell death in HEK293 cells. We observed a significant increase in cell survival percentage in heme-treated HO-1/EYFP-transduced HEK293 cells compared to heme-treated EYFP/EYFP-transduced (control) cells. Exposure to 200 µM heme increased the cell survival from approximately 25% (EYFP/EYFP-
control) to 60% in HO-1-transduced HEK293 cells. Additionally, by inhibiting HO-activity using SnMP, this cell survival decreased significantly. This is in good agreement with other studies in which the protective role of HO-1 was demonstrated, such as in HO-1 knock-out mice and in a glycerol model in rats.\textsuperscript{18,19,50,51} In this glycerol model, rhabdomyolysis results in the release of a large amount of heme-proteins which accumulate in the kidney causing severe heme-induced damage.\textsuperscript{20}

The present study shows for the first time the protective effects of overexpression of BCRP against the injurious actions of heme. BCRP is expressed in a variety of cells and tissues with a barrier function.\textsuperscript{52,53} Previously, we found BCRP expressed in the apical membrane of human kidney proximal tubules where it likely contributes to the urinary excretion of a diverse range of endogenous and exogenous xenobiotics and their metabolites.\textsuperscript{53,54}

Heme-induced cell death was significantly attenuated in BCRP/EYFP-transduced HEK293 cells compared to EYFP/EYFP (control)-transduced cells. After an exposure to 200 μM heme, cell survival increased from approximately 25% (EYFP/EYFP) to 50% in BCRP/EYFP-transduced cells. In addition, other studies demonstrated a role for BCRP in decreasing heme levels in erythroid maturation\textsuperscript{25} and decreasing heme accumulation under hypoxic conditions\textsuperscript{26}. Surprisingly, FTC, which inhibits the activity of BCRP, did not affect cell survival, arguing against a prominent role for BCRP. However, when BCRP-mediated heme efflux may have hampered, BCRP could have functioned as a ‘heme-sink’ by shielding it and thereby rendering it inactive, similar to other heme-binding proteins.\textsuperscript{4,39}

Although the effect of transduction itself (EYFP/EYFP transduction-induced endogenous HO-1) seemed small as judged from the IC\textsubscript{50} values (165 ± 7 versus 143 ± 1; Table 1) and cell survival (figure 7), it may have larger effects in combination with other protective systems. In fact, figure 8 shows that BCRP and HO-1 can work together, since inhibition of HO-activity using SnMP in the setting with high BCRP levels demonstrates a significant decrease in cell rescue. Thus, under circumstances where baculovirus-induced endogenous HO-1 does not have to tackle the heme at its own, it is no longer overwhelmed by the high heme levels, but is able to significantly contribute to the BCRP-mediated protection against heme.

Moreover, when HEK293 cells overexpressed both BCRP and HO-1 proteins we observed an additive effect of BCRP in protecting against heme-induced cell death compared to HO-1-transduced cells alone. Again, suggesting a prominent role for both HO-1 and BCRP. But cell survival in the BCRP/HO-1-transduced cells was diminished by inhibition of HO-activity only and not by FTC treatment. In addition, in parallel experiments, we found that SnMP did not affect BCRP transport activity, indicating that the reduced cell survival after SnMP treatment of BCRP/HO-1-transduced cells is due to HO activity inhibition solely (data not shown). The
more dominant role for HO may be explained by its dual protection against heme-mediated
damage. First, by the degradation of the pro-oxidant heme and secondly by the generation of
the antioxidant bilirubin, the gasotransmitter carbon monoxide and the co-induction of the
iron-scavenger ferritin.\textsuperscript{39}

Heme-induced ROS production, causes directly or indirectly (via the formation of DNA
damage) cell death. When the redox balance in the cell is completely skewed by the ROS
produced by the high levels of heme this will result in an environment where hardly survival
is possible. Also mild induction of HO-1 levels is in that case not able to rescue (figure 8).
However, when there are more players to restore the skewed redox balance than these
cytoprotective proteins act together to fight the heme insult.

The importance of restoring the redox balance is further underscored by a decrease in heme-
induced ROS formation by NAC. In baculovirus-transduced cells (EYFP/EYFP, BCRP/EYFP, HO-1/
EYFP and BCRP/HO-1), the addition of NAC resulted in significantly higher cell survival (figure
8). Probably, cell survival increased due to attenuated ROS levels that were induced via heme-
mediated catalysis of the Fenton reaction. Additionally, simultaneous treatment of the cells
with heme and NAC showed protection on the level of morphology and cell survival of HEK293
cells. Furthermore, this protective effect of NAC could have clinical relevance in protecting
against heme-induced cytotoxicity, although \textit{in vivo}, NAC failed to protect against renal
function impairment in the rat glycerol model.\textsuperscript{55} Interestingly, we recently demonstrated that
experimentally-elevated bilirubin levels using atazanavir treatment caused both amelioration
of vascular function and an improved redox status in type 2 diabetes mellitus patients\textsuperscript{56}
This suggests that bilirubin treatment may also protect against heme-induced toxicity and
improve the redox status in a clinical setting.

In conclusion, both HO-1 and BCRP-overexpression protect cells from damage caused by
heme, which may lead to novel therapeutic strategies for heme-mediated pathologies.

\textbf{Acknowledgements}

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(Grant # 09.110).
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Supplemental figure 1. Assessment of cell survival in transduced HEK293 cells after heme treatment. Cells were transduced with EYFP/EYFP, BCRP/EYFP, HO-1/EYFP or BCRP/HO-1. 48 h after transduction, cells were treated with heme (0-500 μM) for 48 h and cell survival was assessed using the MTT assay. Results were analyzed by non-linear regression and IC50 values were calculated and are summarized in Table 1. Data are presented as mean ± SEM (N=3-4).
Breast cancer resistance protein influences brain energy metabolism by regulating kynurenic acid levels

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Manuscript in preparation
Abstract

Little is known about the transport of metabolites from the brain towards cerebrospinal fluid (CSF). We found the efflux transporter breast cancer resistance protein (BCRP) to be localized in the choroidal cell layer of the brain-CSF interface of murine brain. Next, we investigated the potential role of BCRP in regulating CSF composition. CSF of Bcrp⁻/⁻ mice was compared to that of wild types by ¹H-NMR analysis. Metabolites that differed in abundance were assessed for their effects on BCRP function. Furthermore, levels of the neuroactive compound kynurenic acid (KynA) was determined in plasma and brain homogenates and its effect on glucose uptake in malignant neuroblastoma (N2a) cells was determined. ¹H-NMR analysis revealed that the concentration of glucose, glycine, creatine, alanine, and glutamine was altered in CSF in Bcrp⁻/⁻ mice. Glucose was most discriminative and its concentration was significantly lower in the CSF compared to wild type mice. However, as neither glucose nor any of the other metabolites affected BCRP function, it seemed more likely that absence of the efflux transporter indirectly affected murine brain energy metabolism. KynA has been associated with energy metabolism disturbances and several neurological disorders. We identified KynA as a new BCRP substrate and elevated levels were found in plasma and brain homogenates of Bcrp-deficient mice. Moreover, KynA was shown to increase glucose uptake into N2a cells in a concentration-dependent fashion. These findings provide evidence for a link between BCRP-mediated KynA transport and brain energy metabolism, suggesting that the efflux transporter could be a novel therapeutic target to modulate cerebral glucose and KynA levels.
Introduction

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ATP Binding Cassette (ABC) transporter superfamily and is involved in the active efflux of small molecules at the expense of ATP. BCRP is present in various tissues with a barrier function, including kidney, liver and brain, and is known to act as a transporter of several drugs and endogenous compounds, such as folate, riboflavin, urate and heme. In the brain, BCRP is localized in the blood-brain barrier (BBB) and in the choroid plexus (CP), the so-called blood-cerebrospinal fluid (CSF) barrier.¹

Research on the role of BCRP in the brain has been predominantly focused on the restriction of drug penetration.¹² Yet, little is known about the transport of substrates towards the CSF. The CSF provides a highly controlled and stable environment for the central nervous system and allows for homeostatic regulation by removal of metabolic waste, distribution of neuroendocrine factors and the exchange of fluids, electrolytes and nutrients between the CSF and brain interstitial fluid (ISF) at the ependymal interface.³ The subcommissural organ (SCO), one of the circumventricular tissues, represents the interface between the CSF and ISF. The SCO consists of choroidal cells that line part of the third ventricle and facilitates regulatory processes such as detection of monoamine and neuropeptide levels in the CSF. Moreover, the choroidal cells secrete glycoproteins into the CSF, which aggregate to form Reissner’s fibers and enable CSF flow.⁴ It is not known, as of yet, whether the SCO secretes other molecules into the CSF, nor has the presence of ABC transporters in the SCO been described.

Transporters present at the blood-CSF and ISF-CSF barrier, may have a role either in the transport of nutrients from the blood into the CSF in order to provide the brain with its necessary fuel or in protecting the brain from waste products by transporting them from the ISF towards the CSF. So far, the role of Bcrp in the transport of endogenous substrates towards the CSF is yet unknown. In the present study, we assessed the localization of Bcrp at these barriers in murine brain slices. Furthermore, we aimed at investigating the potential role of BCRP in regulating CSF composition, which was explored by using CSF obtained from wild type and from a Bcrp-deficient mouse strain and comparative ¹H-NMR analysis. Finally, murine neuroblastoma (N2a) cells were used to investigate the metabolic effects of the BCRP substrate KynA, which is potentially involved in brain energy metabolism disturbances. Our data suggest that BCRP may be a novel therapeutic target to modulate cerebral glucose and KynA levels.
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Materials and methods

Immunohistochemistry

All procedures involving animals were approved by the Animal Experimental Committee of RUNMC. The wild type Friend leukemia virus B (FVB) and Bcrp−/− mice were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands) and bred and housed at the Central Animal Laboratory of RUNMC. Localization of Bcrp in the circumventricular organs was assessed by immunohistochemistry as previously described, as was the quality and specificity of the used antibody which was assessed using tissues from Bcrp−/− mice. In brief, perfusion-fixed, paraffin embedded brain-sections of male wild type mice were incubated with a primary antibody against mouse Bcrp (BXP-9). Diaminobenzidin was used to visualize Bcrp localization. The sections were prepared and structures were localized using the Mouse Brain Library. For P-glycoprotein (P-gp) staining, we used the mouse monoclonal antibody JSB-1 (Abcam ab3366; diluted 1:20). Sections were incubated for 1h at room temperature, followed by incubation with a biotin conjugated secondary antibody (rabbit anti-mouse IgG, Agrisera AS09 609; diluted 1:500) for 1h at room temperature. Detection was carried out with the streptavidin-biotin immunoperoxidase method and diaminobenzidin substrate for visualization. After counterstaining with hematoxylin, the slides were mounted. For negative control, the primary antibody was omitted.

CSF sampling, sample preparation and data acquisition

The composition of the CSF from male wild type mice was compared to that of male Bcrp−/− mice by 1H-nuclear magnetic resonance (1H-NMR) spectroscopy. CSF samples were taken from the cisterna magna, using a method described elsewhere. The mice (N=8) were anesthetized with an intraperitoneal injection of 144 mg/kg pentobarbital (Nembutal®, 15 mg/ml) and fixed in a stereotaxic instrument. After opening the skin, the subcutaneous tissue and muscles were separated. The dura mater was penetrated with a glass capillary and CSF was withdrawn. The mice were sacrificed by cervical dislocation and, subsequently, blood and relevant organs were collected, snap frozen in liquid nitrogen and stored at -80 °C. CSF samples were prepared for 1H-NMR measurement by addition of 3 µl 10 mM TSP-d4 (sodium-3-(trimethylsilyl)propionate-2,2,3,3-d4) in heavy water (D2O) and 327 µl 100 mM potassium phosphate buffer (pH 7.4) in D2O. 250 µl was transferred into a 5 mm symmetrical microtube (Shigemi Inc.) susceptibility-matched to D2O. 1H-NMR spectra were acquired at 298K on an Avance-III spectrometer (Bruker BioSpin, Billerica, MA, USA) operating at 14.1 T (599.76 MHz 1H Larmor frequency) equipped with a 5 mm CP-TCI probe with Z-gradient,
an automatic tuning-matching unit and sample changer. Samples were kept for 300 s inside the probe-head for temperature equilibration. One dimensional NOESY-presat (Bruker pulse sequence noesygpppr1d), to suppress the solvent resonance, were recorded with 512 scans, 10 ms mixing time and 4 s relaxation delay. The spectra were recorded using a 64K size FID and a spectral width of 12 ppm, resulting in an acquisition time of 4.6 s. Data was zero-filled and multiplied with an exponential window function prior to Fourier transformation. After data acquisition, the samples were stored at 4 °C until further analysis.

**Preprocessing of ¹H-NMR spectra and data analysis**

Sixteen spectra were pre-processed. Due to blood contamination four spectra were not suitable for data analysis, leaving N=6 per strain. Baseline-correction was performed by applying Asymmetric Least Squares method. Spectra were aligned by using Correlation Optimizing Warping (COW) method and binned to reduce the number of variables by adaptive intelligent binning in the chemical shift range Δ 0.75 – 4.15. To facilitate the comparison between different samples, integral normalization and autoscaling were applied. In order to focus on the significant variables of the numerically complex NMR data, we applied the cross model validation (CMV) procedure. Principal Component Analysis (PCA) was then applied to the set of significant variables to extract and display the systematic variation in the data. PCA converts the multidimensional data space into a low-dimensional model plane and expresses most of the variance within a dataset using a smaller number of factors, so called principal components (PCs). Each PC is orthogonal and therefore independent of other PCs.

**Metabolite identification**

Metabolites were identified using the 600 MHz library of metabolite spectra from the Chenomx NMR Suite 7.0, which is predicted based on a database of pure compound spectra acquired using particular pulse sequence and acquisition parameters. The software fits the spectral signatures of a compound from an internal database of reference spectra to the experimental spectrum. To avoid misidentification, the resonance assignments were further checked against literatures spectra and spectra in the Human Metabolome Database.

**Preparation of membrane vesicle, inhibition assays and KynA uptake experiments**

The methods for the overexpression of human BCRP in human embryonic kidney (HEK293) cells using baculoviruses (produced using the Bac-to-Bac system; Invitrogen, Breda, the Netherlands), the preparation of membrane vesicles and inhibition of BCRP-mediated ³H-estrone sulfate (³H-E₁S; 250 nM) uptake into vesicles were described earlier.
KynA into the membrane vesicles was determined by incubating membrane vesicles with 100 µM KynA for 5 min at 37 °C in the presence of AMP or ATP. Vesicles were washed by means of a rapid filtration technique using 96-well polyvinylidene difluoride filter plates (Millipore, Etten-Leur, the Netherlands). KynA uptake into the vesicles was determined by LC-MS/MS.

**KynA measurements by LC-MS/MS**

After the completion of the vesicle uptake experiments, KynA was extracted from the filters in 3.3 % perchloric acid. Diluted CSF samples were used after 1H-NMR analysis and plasma samples were diluted in H2O (1:1) prior to LC-MS/MS measurements. The brains were cut longitudinally and one half was homogenized in H2O using a micropestle. All samples were deproteinized with perchloric acid (final concentration 3.3 %) and centrifuged at 12,000 x g for 3 min. Deuterated KynA was added as an internal standard for quantification. Clear supernatant was injected into the LC-MS/MS system and KynA measurements were performed as published earlier.17

**Plasma glucose measurements**

Plasma glucose levels were measured using a glucose oxidation method (Beckman Glucose Analyser II, Beckman Instruments, Fullerton, CA, USA).

**Energy metabolism experiments**

Murine neuro-2a (N2a) malignant neuroblastoma cells (ATCC #ccl-131, Manassas, VA, US) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose containing 10 % fetal calf serum (FCS; MP Biomedicals, Uden, the Netherlands). For experiments, cells were seeded at 50 % confluency in a 96-well plate. After 6 h, medium was replaced with low glucose (5 mM) DMEM, supplemented with 10 % dialyzed FCS and incubated overnight. Subsequently, cells were exposed to KynA (0-2000 µM) in low glucose DMEM. After 3 h, glucose concentrations in collected media were determined using the Amplex Red glucose oxidase assay kit (Invitrogen, Breda, the Netherlands).
Results

Localization of Bcrp in murine brain

Immunohistochemistry on wild type brain sections was performed to reveal localization of Bcrp (figure 1). Figure 1a shows a frontal section of the brain with Bcrp positive staining of the CP, SCO and arachnoid barrier (the negative control for antibody specificity is shown in figure 1g). In the CP, Bcrp is located at the ventricular side of ependymal cells facing the CSF (figure 1b,d). The choroidal cells that face the CSF in murine brain SCO also showed clear staining associated with the apical membrane of the ependymal cell surface (figure 1c,e). Besides, there was staining associated with the basal and lateral membranes of these cells, which was clearly not observed in the CP (figure 1e). Other circumventricular organs, not containing an ependymal interface between brain and CSF (i.e. area postrema, pineal gland, subfornical organ, median eminence and organum vasculosum lamina terminalis) were negative for Bcrp staining (see supplementary figure 1). Besides, significant Bcrp staining was found in brain capillaries forming the BBB (figure 1b-c, depicted with arrows). As recently reported by Yasuda et al., the arachnoid barrier also showed clear Bcrp expression (figure 1f), as well as of the related ABC transporter P-glycoprotein (P-gp; ABCB1; figure 2). P-gp staining was further confirmed in CP (figure 2a versus 2b negative control) and BBB (figure 2d), but not in SCO (figure 2c). These data suggest that Bcrp could play a prominent role in CSF homeostasis.
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Figure 1. Bcrp expression in the murine brain. Immunochemical staining using an anti-Bcrp antibody shows expression in a frontal section of the murine brain (a) with Bcrp-positive staining in choroid plexus, SCO and arachnoid barriers. Bcrp is localized to the ventricular side of the ependymal cells of the CP (b, d) in the choroidal cells that face the CSF (*) of the SCO (c, e), and in arachnoid barrier cells (f). The corresponding negative control for Bcrp-specific SCO staining was obtained after incubating sections with the secondary antibody solely (e). Arrows show capillaries. Bars represent 500 µm (a), 200 µm (b,c), 20 µm (d,e) or 100 µm (f,g).
Bcrp influences brain energy metabolism by regulating kynurenic acid levels

Figure 2. P-gp expression in the murine brain. Immunochemical staining using an anti-P-gp antibody shows expression of P-gp in CP (a), BBB and arachnoid barrier (AB) (d), but not in SCO (c). The corresponding negative control for P-gp-specific CP staining (b) was obtained after incubating sections with the secondary antibody solely. Bars represent 20 µm (a, b, d) or 50 µm (c).

Comparison of CSF composition between wild type and Bcrp−/− mice
To investigate the role of Bcrp in the transport of endogenous substrates towards the CSF, we compared CSF composition of wild type and Bcrp−/− mice. CSF samples were withdrawn from the cisterna magna (figure 3a), with a yield of 2-11 µl clear fluid per mouse, and analyzed by 1H-NMR spectroscopy. The spectra obtained were divided into 237 bins, containing resonances of approximately 30 identified metabolites. A representative spectrum is shown in figure 3b. One metabolite can be represented by several resonances (peaks) in the spectrum. The most
informative variables corresponded to twenty-five NMR resonances. The resulting PCA biplot, defined by PC1 and PC2, shows each resonance as represented by a line drawn from the origin towards the corresponding PC1/PC2 level (figure 3c). Based on the position of the individual mice, we assessed whether the specific resonance was different between the two groups. The direction of the presented lines indicated whether the metabolites, represented by the specific resonances, were either decreased or increased in CSF of the wild type compared to Bcrp\(^{-/-}\) mice. A clear separation between the two groups was found, mostly along PC1 which explained 69.7 % of total variance in the data, indicating differences in concentrations of metabolites involved in cellular energy metabolism. The resonances pointing towards negative PC1 levels indicated lower peak intensities and, thus, lower concentrations of the metabolites in wild type compared to Bcrp\(^{-/-}\) mice and correspond to glycine, creatine and alanine. The opposite applied for another set of resonances, corresponding to glucose and glutamine. A boxplot was established per metabolite, from either one (figure 3d,f,h) or multiple (figure 3e,g) corresponding resonances. Our data revealed that glucose was most discriminative between the two strains; its concentration was significantly lower in the CSF of Bcrp\(^{-/-}\) mice, compared to wild types (P=0.007).

**Effects of identified metabolites on BCRP activity**

To verify the interaction between the metabolites identified by \(^1\)H-NMR analysis and BCRP transport function, we used a previously established method in which the uptake of BCRP substrate E\(_1\)S in membrane vesicles overexpressing the transporter was measured (figure 4a). As shown in figure 4b, none of the metabolites tested inhibited BCRP-mediated E\(_1\)S uptake, suggesting that these metabolites do not interact with the efflux transporter. These results indicate that Bcrp alters glucose levels in the brain potentially via an indirect mechanism involving other substrates. A likely candidate is KynA, an end product of tryptophan metabolism, which has been correlated with brain energy metabolism disturbances and recently shown to inhibit BCRP activity\(^{17}\).
Bcrp influences brain energy metabolism by regulating kynurenic acid levels.

Figure 3. Comparison between CSF of Bcrp$^{-/-}$ and wild type mice by $^1$H-NMR spectroscopy. CSF samples were withdrawn from the cisterna magna (a) and analyzed by $^1$H-NMR spectroscopy (N=6). A representative 599.76 MHz-spectrum (b) and a PCA biplot, defined by PC1 and PC2 in which each resonance is represented by a line (c; wild type) are depicted. Based on the position of the individual mice (diamonds and triangles) it was assessed whether the resonances were different between the two groups. Peak intensities of the resonances were either higher (#) or lower (##) in Bcrp$^{-/-}$ mice. A boxplot per metabolite was established from one (d,f,h) and multiple (e,g) corresponding resonances and expressed as (mean) peak intensity. The significance of the differences was tested by analysis of variance (ANOVA) with Bonferroni correction (α-level 0.05).
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Figure 4. Effects of metabolites on BCRP transport activity. BCRP transport activity was assessed after incubating mock or BCRP-overexpressing membrane vesicles with 250 nM \(^{3}\)H-estrone sulfate (E\(_{1}\)S) for 5 minutes in the presence of AMP or ATP (a). Indicated metabolites did not affect net BCRP-mediated E\(_{1}\)S uptake, expressed as percentage of negative control (b). One-way ANOVA followed by Dunnett’s post hoc test was used for statistical analysis. Bars represent means + SEM of triplicate experiments.

KynA and brain glucose uptake

The decreased CSF glucose concentrations observed in the CSF of Bcrp\(^{-/-}\) mice did not coincide with a difference in plasma glucose levels (figure 5a), suggesting that the difference in CSF glucose concentrations might originate from the brain. To determine a role for KynA in glucose metabolism, we first determined its levels in plasma and brain homogenates using LC-MS/MS analysis. KynA levels were significantly elevated in plasma of Bcrp-deficient mice, 150 ± 20 nM versus 86 ± 12 in wild types (figure 5b). Elevated KynA levels were also observed in brain homogenates of Bcrp\(^{-/-}\) mice (4.2 ± 0.6 versus 2.7 ± 0.2 in wild types; figure 5c). Unfortunately, we were not able to detect KynA in murine CSF samples because the diluted samples that were necessary for the \(^{3}\)H-NMR spectroscopy did not exceed the KynA detection limit of the LC-MS/MS analysis.

To assess whether KynA is a substrate for BCRP, membrane vesicles overexpressing the transporter were incubated with KynA and uptake was, subsequently, determined by LC-MS/MS analysis. We clearly observed ATP-dependent transport for KynA, which was significantly different in vesicles incubated with KynA in presence of ATP from vesicles incubated in absence of ATP and from controls (mock transduced; figure 5d). Together, these data confirmed that KynA is an endogenous substrate for BCRP.
Next, we determined whether KynA could also influence brain glucose homeostasis. The effects of KynA on glucose uptake were assessed using malignant neuroblastoma (N2a) cells. After 3 h of exposure to KynA in low glucose medium, a clear increase in glucose uptake by these cells was observed (figure 5e). This effect was dependent on KynA concentration with 200 and 2000 µM causing a more than 100 % increase of glucose uptake compared to unexposed cells. Thus, increased Kyna levels in absence of Bcrp are a likely explanation for the disturbed glucose homeostasis in CSF of transporter-deficient mice.

**Discussion**

We describe an important putative new role for the efflux transporter BCRP in the brain, i.e. regulating KynA levels and thereby influencing key neuronal processes. Moreover, we are the first to show specific Bcrp expression at the brain-CSF interface, in the SCO. Bcrp was mainly located at the ventricular side of ependymal cells facing the CSF, indicating a potential role for the transporter in the maintenance of a stable environment for the central nervous system. In the SCO, Bcrp was also expressed at the basolateral and lateral membranes of the choroidal cells. Therefore, Bcrp could potentially be responsible for a two-directional transport towards the CSF as well as towards the ISF, however, Bcrp expression at the basolateral and
lateral membranes was much lower than at the ventricular side. The presence of any ABC transporter in the SCO has never been described and we also did not observe P-gp-specific staining, suggesting a specific but yet unknown function for Bcrp. Circumventricular organs, such as the SCO, are located in the walls of the lateral 3rd or 4th cerebral ventricles and are the most vascularized tissues in the brain that allow bidirectional movement of (polar) molecules between blood and CSF. Localization of Bcrp in the BBB and in the CP, representing the blood-CSF barrier, has been demonstrated here and/or previously. Furthermore, the presence of Bcrp was recently shown at the arachnoid barrier (AB), another part of the blood-CSF barrier. Interestingly, its direction of transport is different in both barriers. In the BBB, the luminally-located Bcrp facilitates efflux of substrates into blood, while Bcrp transports substrates from blood towards CSF in the CP and AB cell layers. Considering its localization in the brain, Bcrp potentially influences the central nervous system’s homeostatic environment, however, nothing is known about its role in the transport of endogenous substrates towards CSF. Therefore, we compared the CSF of wild type and Bcrp−/− mice by 1H-NMR spectroscopy. This technique is very suitable to analyze and compare biological samples when there is no specific lead. We aimed to compare CSF samples based on a broad range of molecules, since Bcrp has a wide variety of substrates. The drawback of using murine CSF samples, obviously, is the limited volume obtained from one animal and, as a consequence, we were only able to detect molecules that were present in the µmolar range. Moreover, the high dilution of the CSF samples, needed to allow spectroscopy, may have been a limitation for some Bcrp substrates to be detected. Our results did reveal different concentrations of metabolites involved in cellular energy metabolism. Glucose was most discriminative and its concentration was significantly lower in CSF of Bcrp−/− mice. However, it is unlikely that glucose is a substrate based upon archetypical Bcrp-substrate-specific characteristics. Moreover, glucose is efficiently transported towards the ISF by the glucose transporter-1 (GLUT1), which is abundantly present in the BBB. Indeed, glucose did not inhibit vesicular BCRP transport activity nor did the other metabolites identified by 1H-NMR analysis, suggesting that these metabolites are not transported by the efflux transporter. Hence, we propose that Bcrp alters glucose levels in the brain via an indirect mechanism.

Glucose is the principal energy source for the mammalian brain and a continuous supply is essential to maintain cerebral function and, therefore, it is dynamically exchanged between blood, CSF and ISF. The decreased CSF glucose concentrations observed in Bcrp−/− mice suggest increased glucose utilization by the brain since plasma glucose levels were not changed. A likely candidate, which has been correlated with energy metabolism disturbances and found to inhibit BCRP activity, is KynA, an end product of tryptophan.
metabolism. In the mammalian brain, astrocytes facilitate the conversion of kynurenine into KynA via kynurenine aminotransferase-II. Once synthesized, KynA is promptly released into the ISF where it affects its postsynaptic neuronal targets, therefore, KynA levels in brain homogenates mainly represent KynA levels in the ISF.\(^6\) We found significantly elevated KynA levels in plasma and brain homogenates of Bcrp-deficient mice. Using a well-established membrane vesicle uptake assay, we confirmed that KynA is a substrate for human BCRP. Consequently, the elevated KynA plasma levels observed in mice can be partially explained by the lack of Bcrp-mediated renal clearance\(^{26}\), however, the elevated levels of this metabolite in the brain must be due to the absence of Bcrp in this organ. Remarkably, no change in brain/plasma ratio was found between wild type and Bcrp-deficient mice, indicating that also other transporters are involved in the regulation of KynA distribution. A potential other KynA transporter is MRP4, for which an interaction with KynA has been described\(^{17,27}\) and which was found to be present in the BBB and CP\(^{28}\). Nevertheless, Bcrp is the major KynA efflux transporter described thus far and we demonstrated that it is capable of influencing KynA distribution in vivo, which makes it an interesting target in influencing several neuronal processes. As mentioned previously, KynA has been correlated with energy metabolism disturbances in heart mitochondria\(^{23,29}\) and rat cerebral cortex\(^{24}\). We evaluated whether KynA could affect glucose uptake using malignant neuroblastoma cells. After 3 h of exposure to KynA in low glucose medium, a clear concentration-dependent increase in glucose uptake was observed, indicating increased glucose utilization by these cells. In accordance, Schuck et al.\(^{24}\) reported increased glucose uptake after exposing rat cerebral cortex to KynA in vitro. They proposed that KynA interferes with the citric acid cycle, leading to increased glucose utilization (i.e. glycolysis) to compensate for the energy deficit, and our results support this hypothesis. On the other hand, Hodgkins et al.\(^{30}\) found a link between the decrease in KynA levels during hypoglycaemia and cellular energy metabolism in rat cortical tissue and showed that this decrease could be effectively reversed by lactate or pyruvate. Further investigation is needed to gain more insight into the mechanism by which KynA triggers glycolysis in the brain.

Besides the effect on energy metabolism, multiple actions of KynA in the brain have been described, including interferences with cholinergic, glutamatergic and dopaminergic neurotransmission by antagonizing the α7-nicotinic-acetylcholine-(α7nAch-) and the N-methyl-d-aspartate-(NMDA-) receptor.\(^{31,32}\) KynA suppresses the presynaptic release of glutamate by binding to the α7nAch-receptor and it counteracts glutamate-mediated the NMDA-receptor activation.\(^{33}\) Neuronal disorders, as observed with Alzheimer’s disease and schizophrenia, are associated with increased KynA levels in the brain. In these cases, KynA...
leads to a hypoglumatamergic state which induces many of the cardinal symptoms.\textsuperscript{34,35} Under physiological conditions, mammalian astrocytes facilitate the conversion of kynurenine into KynA via kynurenine aminotransferase-II (KATII). Elevation in brain KynA concentration can result in cognitive impairment\textsuperscript{34,36}, however, under some pathological conditions, KynA has neuroprotective properties by counteracting glutamate excitotoxicity, for example in Huntington’s and Alzheimer’s disease. Because of the potent antiglutamatergic features of KynA, it has earned greater interest as an attractive pharmacological target to treat neurological disorders. In this respect, and because of its ability to influence brain energy metabolism, tight regulation of this metabolite is of key importance and our results indicate that BCRP/Bcrp could play a role in this. Bcrp-deficient mice are viable and besides sensitivity to the dietary chlorophyll-breakdown product phenophorbide a\textsuperscript{37}, no severe abnormalities linked to developmental or neurological disorders have been described. Nevertheless, this does not imply that Bcrp-deficiency could not lead to subtle neurological effects, which, to our knowledge, have never been tested in these mice. Still, one should not neglect the complex consequences related to transporter deficiency in knockout animals. Besides, the link between brain glucose levels, KynA and Bcrp warrants further research to demonstrate a direct relation.

In conclusion, we describe a possible new role for BCRP/Bcrp in the brain, \textit{i.e.} regulating KynA levels and thereby influencing key neuronal processes. We suggest that BCRP could be a potential novel therapeutic target to modulate KynA-mediated pathologies associated with cerebral glucose homeostasis.

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References


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Supplementary figure 1. Circumventricular organs negative for Bcrp staining. These organs do not contain an ependymal interface between brain and CSF, and include the area postrema (a), pineal gland (b), median eminence (c), subfornical organ (d) and organum vasculosum lamina terminalis (e). Bars represent 100 µm (a) or 200 µm (b-e).
Hyperuricemia influences tryptophan metabolism via inhibition of multidrug resistance protein 4 and breast cancer resistance protein

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Abstract

Hyperuricemia is related to a variety of pathologies, including chronic kidney disease (CKD). However, the pathophysiological mechanisms underlying disease development are not yet fully elucidated. Here, we studied the effect of hyperuricemia on tryptophan metabolism and the potential role herein of two important uric acid efflux transporters, multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP). Hyperuricemia was induced in mice by treatment with the uricase inhibitor oxonic acid, confirmed by the presence of urate crystals in the urine of treated animals. A transport assay, using membrane vesicles of cells overexpressing the transporters, revealed that uric acid inhibited substrate-specific transport by BCRP at clinically relevant concentrations (calculated IC_{50} value: 365 ± 13 µM), as was previously reported for MRP4. Moreover, we identified kynurenic acid as a novel substrate for MRP4 and BCRP. This finding was corroborated by increased plasma levels of kynurenic acid observed in Mrp4^{−/−} (107 ± 19 nM; P=0.145) and Bcrp^{−/−} mice (133 ± 10 nM; P=0.0007) compared to wild type animals (71 ± 11 nM). Hyperuricemia was associated with >1.5 fold increase in plasma kynurenine levels in all strains. Moreover, hyperuricemia led to elevated plasma kynurenic acid levels (128 ± 13 nM, P=0.005) in wild type mice but did not further increase kynurenic acid levels in knockout mice. Based on our results, we postulate that elevated uric acid levels hamper MRP4 and BCRP functioning, thereby promoting the retention of other potentially toxic substrates, including kynurenic acid, which could contribute to the development of CKD.
Introduction

Uric acid is a weak organic acid and the end product of purine nucleotides degradation in humans. One of the enzymes involved in this process is xanthine oxidoreductase, which enables the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. During this reaction, reactive oxygen species are generated as by-product. Therefore, uric acid is recognized as a marker for oxidative stress. However, the molecule itself has antioxidant properties and can act as a free radical scavenger and chelator of transitional metal ions which are converted into poorly reactive forms. Hyperuricemia, i.e. elevated plasma uric acid levels (≥ 360 µM), is related to a variety of pathologies, including gout, cardiovascular disease and chronic kidney disease (CKD). Gout is the most common form of inflammatory arthritis caused by sodium uric acid crystal precipitation, which is followed by phagocytosis of the crystals by neutrophils and macrophages and activation of acute inflammation and tissue injury. Epidemiological studies show that prevalence and incidence are still increasing. Formation of uric acid crystals is also the cause of nephrolithiasis, i.e. kidney stones, which is significantly more common among patients diagnosed with metabolic syndrome, obesity and type 2 diabetes. Hyperuricemia also correlates with the development and progression of cardiovascular diseases, potentially via interfering with nitric oxide function. In animal models, it has been shown that mild hyperuricemia contributes to the development of hypertension as a result of endothelial dysfunction and reduction of nitric oxide levels. Recently, hyperuricemia has received attention as a possible risk factor for CKD, which affects approximately 10% of the adult population in developed countries. Hyperuricemia has been associated with a hazard ratio of 2.1 and 1.3 for men and women for developing CKD, respectively. Several mechanisms were proposed via which uric acid could contribute to the development of CKD, including uric acid-induced glomerular hypertrophy and endothelial dysfunction. However, the pathophysiological mechanism has as of yet not been fully elucidated.

In healthy individuals, two-thirds of uric acid is excreted by the kidney and one-third by the intestine due to breakdown of urate by gut bacteria. Purine ingestion, endogenous synthesis of purines from nonpurine precursors and reutilization of preformed purine compounds are the sources of uric acid production, a process that, under steady-state conditions, is in balance with the uric acid disposal. Hyperuricemia can develop due to overproduction or a diminished excretion of uric acid. Maintaining uric acid homeostasis is highly dependent on kidney function and regulated by a number of transporters, including the urate transporter 1 (URAT1: SLC22A12) - responsible for up to 99% of uric acid reabsorption after glomerular
filtration - the facilitated glucose transporter (solute carrier family 2 member 9 (SLC2A9))\(^{19}\), several organic anion transporters including OAT1 (SLC22A6) and OAT3 (SLC22A8)\(^{6}\), and the ATP-dependent urate efflux transporters multidrug resistance protein 4 (MRP4: ABCC4)\(^{20}\) and breast cancer resistance protein (BCRP: ABCG2)\(^{21-23}\).

As uric acid is one of the important factors in a variety of pathologies, tight regulation of this metabolite is of key importance. The vital role of transporters in uric acid homeostasis can clearly be observed in patients suffering from hyperuricemia due to single nucleotide polymorphisms (SNPs) that render the transporters inactive, such as the common SNP C421A encoding the Q141K mutation of BCRP\(^{21,22,24}\) and several genetic variants for SLC2A9\(^{19}\). Next to genetic factors, high plasma levels of uric acid might also result in a reduced transporter activity.\(^{20}\) Since these transporters are also involved in the excretion of a wide variety of other compounds, changes in transport efficacy could result in metabolic disturbances. This hypothesis is corroborated by two recent studies showing that high uric acid levels in patients with acute gout were associated with altered tryptophan concentrations in plasma and urine.\(^{25,26}\) Therefore, the aim of our study was to investigate the effect of hyperuricemia on tryptophan metabolism and the potential role herein of two important uric acid efflux transporters, MRP4 and BCRP. Both transporters are expressed in the apical membrane of renal proximal tubule cells, amongst other tissues, and are involved in the urinary excretion of a multitude of endogenous compounds and drugs.\(^{27}\) Using Mrp4\(^{-/-}\) and Bcrp\(^{-/-}\) mice, we show that hyperuricemia is associated with the accumulation of tryptophan and associated metabolites, most likely due to transporter dysfunction.

**Material and methods**

*Transduction of Human Embryonic Kidney cells and preparation of membrane vesicles*

Overexpression of MRP4 and BCRP in human embryonic kidney cells (HEK293: American Type Culture Collection, Manassas, VA) was established using baculoviruses, which were produced using the Bac-to-Bac and the Gateway system (Invitrogen, the Netherlands), as described previously.\(^{28,29}\) As a control, the enhanced yellow fluorescent protein (eYFP) was introduced as mock protein into the baculovirus expression system. Crude membranes of HEK293-MRP4,-BCRP and -mock cells were isolated, resuspended in TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) and membrane vesicles were prepared according to a previously described method\(^{28}\) by means of ultracentrifugation. Crude membrane vesicles were dispensed in aliquots, snap frozen in liquid nitrogen, and stored at -80 °C until further use.
Membrane vesicle inhibition and uptake assays
The effects of uric acid and oxonic acid on MRP4 and/or BCRP activity were assessed by a well-established assay in our laboratory.28-31 In brief, a reaction mix consisting of TS buffer supplemented with 4 mM ATP/AMP, 10 mM MgCl₂, and 250 nM [³H]-methotrexate (MTX for MRP4) or [³H]-estrone sulfate (E₁S for BCRP) at pH 7.4 was added to 7.5 µg of membrane vesicles (based on total protein content). After incubation at 37 ºC to enable ATP-dependent uptake, the reaction was stopped by placing the samples on ice and by addition of ice-cold TS buffer. Reaction mix was removed and the vesicles were washed by means of a rapid filtration technique using filter plates (Millipore, Etten-Leur, the Netherlands). Scintillation fluid was added to the filters and the amount of radioactivity was determined using a scintillation counter (Tri-Carb® 2900TR: Perkin Elmer, Waltham, MA, USA). Reference samples were measured to calculate the amount of transported MTX and E₁S. ATP-dependent transport was calculated by subtracting values measured in the presence of AMP from those measured in the presence of ATP. Net transporter-mediated substrate uptake was calculated by subtracting ATP-dependent uptake in HEK293-mock vesicles from that of HEK293-transporter vesicles. Uptake of kynurenic acid into MRP4-overexpressing membrane vesicles was established using the same assay. Vesicles were incubated with 0.1 mM kynurenic acid in the presence of AMP or ATP. After the described washing step, kynurenic acid was determined by LC-MS/MS.

Oxonic acid-mediated induction of hyperuricemia in mice
All experiments were approved by the local Animal Welfare Committee of the Radboudumc (RU-DEC 2012-018), in accordance with the directive for animal experiments (2010/63/EU) of the European Parliament. The effects of hyperuricemia in vivo were examined in wild type Friend leukemia virus B (FVB) mice as well as Mrp4⁻/⁻ and Bcrp⁻/⁻ mice (both FVB background). The wild type FVB and Bcrp⁻/⁻ mice were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands) and the Mrp4⁻/⁻ mice by Dr. J. Schuetz (St. Jude Children’s Research Hospital, Memphis, TN, USA) and Dr. P. Borst (Netherlands Cancer Institute, Amsterdam, the Netherlands), all animals were bred and housed at the Central Animal Laboratory of the RUNMC. The animals (N=9) received the uricase inhibitor oxonic acid (2% w/v: pH 7) via their drinking water, ad libitum, to induce hyperuricemia.32 The animals were individually caged and housed under controlled conditions. Parallel control groups were also individually caged and received normal tap water at equal pH. After 14 days, mice were placed individually in metabolic cages (Techniplast, Germany GmbH) to collect 24 h urine samples, with access to water (with or without oxonic acid 2% w/v) and pulverized standard chow ad libitum. Next, blood was collected from the orbital sinus in lithium-heparin tubes via
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a terminal procedure performed under isoflurane anesthesia and centrifuged for 15 min at 3,000 x g to obtain plasma. Animals were sacrificed by cervical dislocation. Isolated kidneys, plasma and urine were immediately snap frozen in liquid nitrogen and stored at -80 °C until further analysis. Biochemical parameters were determined by routine clinical chemistry.

Energy-dispersive X-ray (EDX) microanalysis
Transmission electron microscopy and EDX were performed for identification of the ultrastructure and composition of the insoluble crystals found in the urine samples of oxonic acid-treated mice. Urine samples were spotted onto copper grids (100 mesh) coated with a support film, air dried, negative stained with uranyl acetate and examined using a Jeol 1200 EX II. For EDX measurements, the grids were examined using an Jeol 1200/STEM in combination with a Thermo Noran microanalysis six system. Accelerated voltage of 60 KeV was used for X-ray microanalysis. X-ray spectra for element distribution were acquired. In each sample, 3-5 measuring points were selected.

RNA isolation and quantitative PCR
Effects of hyperuricemia on kidney injury was evaluated by determining mRNA expression levels of early renal injury markers kidney injury molecule-1 (Kim-1) and neutrophil gelatinase-associated lipocalin (Ngal) in kidneys of treated and control mice. Frozen kidneys were homogenized using a Mikro-dismembrator U (Sartorius B. Braun Biotech Int., Melsungen, Germany). Subsequently, total RNA was isolated using a NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer’s instructions. Immediately, a reverse transcriptase reaction was performed with 250 ng RNA using random primers (Invitrogen, Breda, the Netherlands) and an Omniscript® RT kit (Qiagen, Hilden, Germany), following manufacturer’s recommendations. Synthesized cDNA was used for quantitative PCR, performed in a StepOnePlus™ Real-Time PCR system by means of the TaqMan® protocol (Applied Biosystems, Warrington, UK). Kim-1 and Ngal mRNA concentrations were normalized to the mRNA concentration of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The primer-probe sets were obtained from Applied Biosystems (Gapdh: Mm999999915_g1, Kim-1: havcr1 Mm00506686_m1, Ngal: lcn2 Mm01324470_m1).

Tryptophan, kynurenine and kynurenic acid measurements by LC-MS/MS
After the vesicle uptake experiments, kynurenic acid was extracted from the filters in 3.3% perchloric acid. Plasma samples were diluted in H2O (1:1) prior to LC-MS/MS measurements.
and deproteinized with perchloric acid (final concentration 3.3%). Samples were centrifuged at 12,000 x g for 3 min. Clear supernatant was injected into the LC-MS/MS system that consisted of an Accela HPLC system (Thermo scientific, Breda, the Netherlands) equipped with a C18 HPLC column (VisionHT C18 B 10062 mm, 1.5 mm: Grace). Tryptophan, kynurenine and kynurenic acid were measured in the same run. Deuterated kynurenic acid was added as an internal standard for quantification. Measurements were performed as published earlier.30

**Kinetic analysis and statistics**

Statistics were performed using GraphPad Prism 5.02 via one-way analysis of variance (ANOVA) followed by the Dunnett’s Multiple Comparison Test to study differences between all groups as well as an unpaired Student’s t-test to study the impact of oxonic acid treatment within each strain. Differences between groups were considered to be statistically significant when p < 0.05. GraphPad Prism was also used to perform non-linear regression analysis.

**Results**

**Oxonic acid induces hyperuricemia**

To study the impact of hyperuricemia on murine physiology, wild type, Mrp4−/− and Bcrp−/− mice were treated with the uricase inhibitor oxonic acid as described before.32 Two weeks of oxonic acid treatment did neither affect overall weight of the mice, nor plasma levels of urea, sodium and calcium (table 1). Interestingly, water intake was strongly increased after oxonic acid treatment in all strains investigated, with an increase up to four times that of controls in Bcrp-deficient mice. This was accompanied by a two-fold increase in urine flow in treated wild type animals; whereas no significant changes were observed in knockout animals. Yet baseline urine flow of Bcrp−/− mice tended to be increased as compared to wild type animals. Following oxonic acid treatment, the urine collected from all animals was turbid due to presence of crystals (figure 1A-D). Energy-dispersive X-ray microanalysis (EDX) revealed that the major constituents of the urinary crystals were sodium, potassium, phosphorus and calcium; the copper signals arose from the sample grid used in the analysis (figure 1E). Two types of crystals could be distinguished: first, crystals that showed an archetypical peak pattern corresponding to uric acid crystals, which consisted primarily of sodium, calcium and phosphorus (figure 1F). Second, crystals that mainly contained calcium and potassium (figure 1G), which was likely due to the treatment with oxonic acid potassium salt. These data are in accordance with the development of hyperuricemia in oxonic acid-treated mice.
### Table 1. General characteristics and serum and urine biochemistry of experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT + oxonic acid</th>
<th>Mrp4−/−</th>
<th>Mrp4−/− + oxonic acid</th>
<th>Bcrp−/−</th>
<th>Bcrp−/− + oxonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight d.0 (g)</td>
<td>22.4 ± 0.8</td>
<td>25.3 ± 1.1</td>
<td>23.4 ± 1.4</td>
<td>25.0 ± 1.5</td>
<td>23.8 ± 1.1</td>
<td>25.7 ± 1.0</td>
</tr>
<tr>
<td>Weight d.14 (g)</td>
<td>24.6 ± 1.0</td>
<td>24.9 ± 1.2</td>
<td>23.5 ± 0.9</td>
<td>24.6 ± 0.9</td>
<td>24.5 ± 0.7</td>
<td>24.6 ± 0.6</td>
</tr>
<tr>
<td>Water intake (ml/24h)</td>
<td>1.6 ± 0.4</td>
<td>5.5 ± 0.8***</td>
<td>2.9 ± 0.6</td>
<td>5.5 ± 1.0*</td>
<td>2.1 ± 0.7</td>
<td>8.5 ± 0.5***</td>
</tr>
<tr>
<td>Urine flow (ml/24h)</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.1*</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mM)</td>
<td>12.5 ± 0.8</td>
<td>11.8 ± 0.7</td>
<td>11.4 ± 0.4</td>
<td>10.5 ± 0.5</td>
<td>12.5 ± 0.5</td>
<td>11.7 ± 0.6</td>
</tr>
<tr>
<td>Sodium (mM)</td>
<td>149.7 ± 1.1</td>
<td>151.1 ± 1.4</td>
<td>147.6 ± 1.8</td>
<td>151.7 ± 0.8</td>
<td>149.4 ± 0.9</td>
<td>148.0 ± 1.0</td>
</tr>
<tr>
<td>Calcium (mM)</td>
<td>2.3 ± 0.02</td>
<td>2.4 ± 0.03</td>
<td>2.2 ± 0.02</td>
<td>2.3 ± 0.02</td>
<td>2.3 ± 0.01</td>
<td>2.3 ± 0.02</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (μmol/24h)</td>
<td>1.4 ± 0.6</td>
<td>1.6 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>2.4 ± 0.8</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Sodium (μmol/24h)</td>
<td>139.7 ± 63.1</td>
<td>126.3 ± 14.1</td>
<td>93.4 ± 17.4</td>
<td>150.8 ± 42.7</td>
<td>196.0 ± 49.2</td>
<td>138.8 ± 21.4</td>
</tr>
</tbody>
</table>

Individually caged mice were treated with oxonic acid via their drinking water for 14 days. Mice were weighed before and after treatment period. Water intake, urine flow and urine content were determined after a 24h-period in metabolic cages at day 14. Plasma was collected at day 14. Data represent means ± SEM of 9 mice per group. *P<0.05 and ***P<0.001 compared to untreated mice from the same strain using a Student’s t-test. Differences between the strains were not significant (one-way ANOVA).
Hyperuricemia influences tryptophan metabolism via inhibition of MRP4 and BCRP

Uric acid inhibits BCRP activity

Plasma urate levels are controlled by an interplay of transporters expressed in the kidney proximal tubules. MRP4 and BCRP have been associated with active urinary urate efflux, and MRP4 was also sensitive to inhibition by urate.\textsuperscript{20} We determined the inhibitory properties of uric acid on BCRP activity using membrane vesicles prepared from BCRP-overexpressing HEK293 cells. Uric acid dose-dependently inhibited BCRP-mediated uptake of the substrate E\textsubscript{1}S (figure 2A) with a calculated half maximal inhibitory concentration (IC\textsubscript{50}) value of 365 ± 13 µM. Complete inhibition of BCRP activity was found at the highest uric acid concentration used (\textit{i.e.} 1 mM). These findings indicate that uric acid can reduce transport activity of two important efflux pumps at concentrations demonstrated in patients with hyperuricemia (≥ 360 µM).
Chapter 7

MRP4-mediated transport of kynurenic acid
There is some evidence linking hyperuricemia to changes in tryptophan levels. In addition, we recently reported that kynurenic acid, a tryptophan derivative, can interact with MRP4 and BCRP, and we identified kynurenic acid as a BCRP substrate (Dankers et al., submitted for publication). Here, we investigated whether kynurenic acid is also a substrate for MRP4. Figure 2B shows that the ATP-dependent uptake of kynurenic acid in MRP4-overexpressing vesicles is 7-fold higher as compared to controls with an average rate of 21.6 pmol/mg*min⁻¹. Furthermore, figure 2B demonstrates that non-specific, AMP-dependent, uptake is very low. These results indicate that kynurenic acid is indeed transported by MRP4.

Figure 2. Concentration-dependent inhibition of net BCRP-mediated [³H]-estrone sulfate (E⁴S) uptake by uric acid and MRP4-mediated kynurenic acid uptake into membrane vesicles. (A) Membrane vesicles were incubated with 250 nM E⁴S and increasing concentrations of uric acid in the presence of AMP or ATP, for 60 sec at 37 ºC. Net BCRP-mediated E⁴S uptake was calculated by subtraction of corresponding mock values. Curve fitting was performed by non-linear regression analysis using GraphPad Prism software (version 5.02, GraphPad Software Inc., San Diego, CA, USA). Graph represents means ± SEM of three independent experiments. (B) Kynurenic acid uptake was assessed by LC-MS/MS analysis after incubating membrane vesicles with 0.1 mM kynurenic acid in the presence of AMP or ATP, for five min at 37 ºC. Bars represent means ± SEM of three independent experiments. ***P<0.001 compared to other bars by one-way ANOVA followed by Dunnett’s post hoc test.

Retention of tryptophan and its metabolites during hyperuricemia
As uric acid can inhibit MRP4 and BCRP-mediated transport and kynurenic acid is a substrate for both pumps, we determined the effects of hyperuricemia on tryptophan metabolism and the role of the efflux pumps herein. Plasma tryptophan levels were similar in all untreated...
groups, but oxonic acid treatment led to increased tryptophan levels in Mrp4\(^{-/-}\) mice (125 \(\mu\)M) as compared to untreated animals (93 \(\mu\)M: figure 3). Baseline plasma levels of the intermediate tryptophan metabolite, kynurenine, were similar in knockout animals as compared to wild type animals. And oxonic acid treatment led to a >1.5 fold increase in kynurenine plasma levels in all three strains, without differences between strains. In contrast, baseline plasma kynurenic acid levels of knockout mice were elevated compared to wild type mice, which was significant for Bcrp\(^{-/-}\) mice. Hyperuricemia did not further increase plasma kynurenic acid levels in knockout mice, but led to significantly elevated levels in wild type animals to the levels of untreated knockout mice. Furthermore, IDO activity, represented by the ratio between tryptophan and kynurenine, was similar in untreated strains, but significantly increased after oxonic acid treatment in Bcrp\(^{-/-}\) mice (0.5 vs 0.7). Thus, hyperuricemia clearly affects tryptophan metabolism.

**Figure 3.** Plasma tryptophan metabolism in wild type (WT), Mrp4\(^{-/-}\) en Bcrp\(^{-/-}\) mice. Plasma tryptophan, kynurenine and kynurenic acid levels and IDO activity of untreated (grey) and oxonic acid-treated (black) wild type, Mrp4\(^{-/-}\) en Bcrp\(^{-/-}\) mice after 14 days of treatment via drinking water, determined by LC-MS/MS analysis. IDO activity is expressed as the ratio between kynurenine and tryptophan *100. Bars represent means + SEM of 9 mice per group. Statistical analysis was performed using both one-way ANOVA followed by the Dunnett’s Multiple Comparison Test and an unpaired Student’s t-test. \(^*P<0.05\) and \(^{**}P<0.01\) by Student’s t-test and \#\(P<0.001\) by one-way ANOVA.
**Oxonic acid does not influence MRP4 and BCRP activity**

To exclude the possibility that oxonic acid itself inhibited the efflux pumps resulting in metabolite retention, we investigated the effect of oxonic acid on MRP4 and BCRP transport activity. Membrane vesicle uptake studies revealed that oxonic acid itself did not affect MRP4-mediated MTX uptake and BCRP-mediated E$_S$ uptake with more than 15% in a concentration range of 1 µM to 1000 µM (figure 4).

![Figure 4](image_url)

**Figure 4.** Oxonic acid does not interfere with MRP4-mediated MTX uptake and BCRP-mediated E$_S$ uptake. Membrane vesicles were incubated with 250 nM [3H]-MTX or [3H]-E$_S$ and indicated concentrations of oxonic acid for 5 min at 37 °C in the presence of AMP or ATP. AMP values were subtracted from ATP values. Net transporter-mediated uptake was expressed as means + SEM of triplicate measurements in a representative experiment. Results were analyzed by one-way ANOVA followed by Dunnett’s post hoc test.

**Hyperuricemia induces expression of the early kidney injury marker Ngal**

Finally, we studied whether hyperuricemia and changes in tryptophan metabolism coincided with an amelioration of kidney function. Renal damage was evaluated by assessing mRNA expression of the early renal injury markers Kim-1 and Ngal in kidneys of treated and control mice. As shown in figure 5A, Kim-1 was not differentially expressed in oxonic acid-treated mice compared to untreated mice. In contrast, renal Ngal expression (figure 5B) was increased after oxonic acid-induced hyperuricemia. These increases were significant for both knockout strains, and in Mrp4$^{-/-}$ mice expression levels rose up to 2.3 times that of untreated mice. With regard to kidney function, no significant effect was observed on creatinine and sodium excretion (table 1), suggesting the absence of overt kidney damage which was confirmed by histology. Light microscopic evaluation of HE-stained kidney slices (figure 5C) revealed intact...
brush borders and absence of casts or destroyed tubules in exposed animals. Taken together, these results indicate that two weeks of oxonic acid-induced hyperuricemia reveals, at most, early signs of kidney damage.

Figure 5. Expression of kidney injury markers and histology after oxonic acid treatment. Relative mRNA expression levels of kidney injury markers Kim-1 (A) and Ngal (B) in kidney tissue of untreated (grey) and oxonic acid-treated (black) mice obtained by qPCR. Animals were exposed to oxonic acid via their drinking water for 14 days. Cycle threshold (Ct) values were normalized for the endogenous reference gene Gapdh and expressed as mean fold difference from untreated animals + SEM (N=9). *P<0.05 by Student’s t-test. Both genes were not differentially expressed in untreated animals of the different strains. Panel C depicts a representative micrograph of a oxonic acid-treated wild type mice and shows that no kidney damage was observed in oxonic acid-treated mice (HE staining), which was comparable in treated knockout mice.
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Discussion

This study reports for the first time that hyperuricemia is associated with disturbances in tryptophan metabolism, most likely due to uric acid-induced dysfunction of the renal efflux pumps MRP4 and BCRP. High levels of uric acid are associated with an increased risk for the development of various diseases and the common mode of action is the formation of crystals (e.g. gout and nephrolithiasis) or by negatively influencing the endothelium (e.g. hypertension). Here, we hypothesized that hyperuricemia could also contribute to disease development in an indirect manner by promoting the retention of other potentially toxic metabolites. Our results revealed that oxonic acid-induced hyperuricemia resulted in elevated plasma levels of tryptophan, kynurenine and kynurenic acid in vivo. The findings are in agreement with two recent studies by Liu et al.\textsuperscript{25,26}, who reported that plasma levels of tryptophan were increased in patients with acute gout while their urinary concentrations were decreased. Hence, there appears to be a link between hyperuricemia and disturbances in tryptophan metabolism.

The efflux transporters MRP4 and BCRP are important in regulating uric acid levels, but also essential for the clearance of many exogenous and endogenous waste products. Recently, we demonstrated that kynurenic acid could interact with MRP4 and BCRP activity in the membrane vesicle transport assay, suggesting that the metabolite is a possible substrate for both transporters.\textsuperscript{30} Using the same assay, we have shown that kynurenic acid is indeed transported by BCRP (Dankers et al., submitted for publication) and here we report that kynurenic acid is also a substrate for MRP4. These findings are in agreement with the observed increase in plasma kynurenic acid levels in Mrp4\textsuperscript{-/-} and Bcrp\textsuperscript{-/-} mice. Therefore, MRP4 and BCRP could be potential novel therapeutic targets for the regulation of kynurenic acid levels in a variety of diseases.

Kynurenic acid is a widely studied antagonist of the N-methyl-D-aspartate-receptor and the α7-nicotinic acetylcholine receptors, and elevated levels of kynurenic acid are related to several neurological disorders.\textsuperscript{33} Another target of kynurenic acid is the orphan G-protein-coupled receptor GPR35, of which kynurenic acid is one of the most potent endogenous agonists currently known. The receptor is highly expressed in the intestine and in several immune cells, including monocytes and T cells. Kynurenic acid also alters the release of multiple growth factors such as nerve growth factor and fibroblast growth factor-1.\textsuperscript{34} Thus, perturbations in kynurenic acid levels can result in marked effects on receptor activation and changes in growth factors. With regard to pathophysiological effects, classic experiments have demonstrated that kynurenic acid inhibited pro-insulin synthesis in isolated rat
Hyperuricemia influences tryptophan metabolism via inhibition of MRP4 and BCRP

pancreatic islets and increased the release of insulin in rats, suggesting a role in diabetes.\textsuperscript{35,36} Furthermore, in CKD patients, kynurenic acid accumulates\textsuperscript{30} and increased levels correlate positively to multiple markers of endothelial dysfunction, namely von Willebrand factor, thrombomodulin and soluble adhesion molecules (sICAM-1, sVCAM-1).\textsuperscript{37,38} In addition, kynurenic acid is suggested to be an important early mediator of leukocyte recruitment.\textsuperscript{39} Moreover, kynurenic acid reduces glucuronidation activity of UDP-glucuronosyltransferases, as shown in proximal tubule cells, thereby affecting the metabolic capacity of the kidney.\textsuperscript{40} Hence, elevated levels of kynurenic acid induced by hyperuricemia, as observed in our study, might play a pivotal role in the pathophysiological effects currently attributed to uric acid.

Our results further demonstrated that kynurenine levels were similar in untreated wild type and knockout animals, suggesting that the levels of this metabolite are not influenced by MRP4 and BCRP activity. Following induction of hyperuricemia, kynurenine levels were markedly increased in all groups. The observed increase might be due to a reduced activity of a uric acid transporter other than MRP4 and BCRP, elevated tryptophan levels, as seen in Mrp4\textsuperscript{-/-} mice, and/or increased activity of indoleamine 2,3-dioxygenase (IDO), as observed in Bcrp\textsuperscript{-/-} mice. Interestingly, IDO is involved in immune regulation and enzyme activity is higher during chronic inflammation and in CKD patients.\textsuperscript{41} The exact interaction between hyperuricemia and IDO requires further investigation. For a long time, kynurenine was regarded as an intermediate of tryptophan metabolism with little biological activity. Yet Opitz et al.\textsuperscript{42} recently demonstrated that this metabolite is a ligand for the aryl hydrocarbon receptor (AHR) and can promote tumor cell survival and suppress antitumor immune responses. The AHR signaling pathway is involved in a myriad of cellular processes, including embryogenesis, inflammation and phase I and phase II metabolism. Fascinatingly, another tryptophan metabolite, indoxyl sulfate, is also reported to activate the AHR in primary human hepatocytes and human umbilical vein endothelial cells.\textsuperscript{43,44} Thus, by disturbing tryptophan metabolism, hyperuricemia could indirectly be involved in AHR activation and subsequent pathologies.

Several polymorphisms in transporter genes are associated with elevated serum uric acid levels. For instance, Woodward et al.\textsuperscript{22}, described that the common single nucleotide polymorphism (SNP) rs2231142 encoding the Q141K mutation of BCRP caused hyperuricemia-based gout. Also in a Japanese population, Q141K was shown to be a common dysfunctional form of BCRP causing gout.\textsuperscript{21} Another transporter recently implicated in uric acid metabolism is SLC2A9. Using Xenopus oocytes, Vitart et al.\textsuperscript{19} demonstrated that SLC2A9-mediated uptake of uric acid was sevenfold higher in SLC2A9-expressing oocytes compared to URAT1-expressing oocytes. These findings have been confirmed by others.\textsuperscript{30,45} Moreover, multiple
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genome-wide association studies reported a relationship between uric acid levels and SNPs in several transporters including SLC17A3, SLC22A11 and SLC22A12.\textsuperscript{23,46,47} Taken together, when studying the link between uric acid and disease progression it is important to take into consideration the presence of possible polymorphisms in transporter genes as well as changes in metabolite levels of transporter-specific substrates.

Two weeks of hyperuricemia induced early signs of kidney damage, as observed by an increased mRNA expression of Ngal in knockout animals. In contrast, no signs of renal damage were seen in wild type animals. These findings suggest that knockout animals were more prone to the development of renal failure. Since, kynurenic acid levels were already elevated in Mrp4\textsuperscript{--} and Bcrp\textsuperscript{--} animals before the induction of hyperuricemia, one could argue that other tryptophan metabolites are responsible for induction of Ngal observed in knockout mice. To better understand the effect of hyperuricemia on the development of CKD, future studies should include longer treatment periods (> 2 wk) and also determine the levels of other intermediates and end products of tryptophan metabolism.

In conclusion, our results showed that uric acid dose-dependently inhibited BCRP activity and inhibition occurred at physiologically relevant levels as was reported previously for MRP4.\textsuperscript{20} Moreover, we demonstrated that Mrp4- and Bcrp-deficiency as well as hyperuricemia are associated with alterations in tryptophan metabolism and the retention of kynurenine and kynurenic acid, two metabolites with a broad array of biological activities. Therefore, we postulate that elevated uric acid levels hamper MRP4 and BCRP functioning, thereby promoting the retention of other potentially toxic substrates, including kynurenic acid, which could contribute to the development of CKD. These finding underline the complex relation between hyperuricemia and linked pathologies, which should be taken into account when interpreting results obtained using \textit{in vivo} hyperuricemia models.

Acknowledgements

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Hyperuricemia influences tryptophan metabolism via inhibition of MRP4 and BCRP

References

Chapter 7


General discussion
Introduction

BCRP is a versatile efflux transporter with a myriad of xenobiotic as well as endogenous molecules as substrates. Besides its protective function against the toxic effects of xenobiotics, it becomes increasingly clear that the protein also has a physiological function. In this thesis, the role of BCRP in endocrine and metabolic processes was investigated. Different *in vitro* and *in vivo* techniques were used to elucidate the involvement of efflux pump in the transport of steroids, the protective function of BCRP on heme-induced cellular injury, and the role of BCRP in hyperuricemia and in endocrine disruption. In this chapter, the relevance of these results is being discussed. Furthermore, the effects of decreased BCRP function, the pitfalls which might be encountered while studying the role of BCRP or other efflux transporters, and the possibility to target BCRP for clinical applications are considered.

The role of BCRP in endocrine processes

The research described in chapters 2, 3 and 4 of this thesis was focused on the role of BCRP in endocrine processes. The presence of BCRP in various tissues, including placenta, prostate, small intestine, brain, colon, liver, mammary gland and kidney has been shown previously, however, investigations on BCRP expression in other endocrine organs received much less attention and localization of the protein in these organs has not been clearly described. We investigated the expression and possible role of BCRP in endocrine organs in chapter 2. In addition to adrenal gland, pancreas and testis, we show for the first time localization of Bcrp in murine ovary, pituitary gland and adipose tissues. Except in the adipose tissues, were it is expressed only in the plasma membrane, Bcrp was located mainly in the capillaries of both endocrine and non-endocrine organs. Bcrp is most likely not involved in steroid hormone secretion as we did not find expression in most endocrine cells. The only steroid-secreting cells expressing Bcrp were found in the adrenal gland, *i.e.* cells lining the zona glomerulosa and zona reticularis, which secrete mineralocorticosteroids and androgens, respectively, and in adipose tissue, a major site for metabolism of sex steroids and glucocorticoids. Whether Bcrp/BCRP actually has a role in the excretion of the produced steroids and whether steroid hormones influence BCRP function, was investigated further. Also in chapter 2, it is shown that several steroid hormones, including estradiol, testosterone, progesterone and androstenedione, can inhibit BCRP function, however, at supraphysiological (plasma) concentrations. To predict the clinical relevance of the interaction between steroids and BCRP, knowledge on intracellular steroid concentrations is important, which so far appeared
to be difficult to determine. One can imagine that intracellular concentrations in organs responsible for the production of hormones may be much higher than plasma levels and, thereby, could potentially influence BCRP function. Besides, the actual steroid concentrations in the experiments were not determined and steroids are known to easily stick to plastic tubes and/or wells, so their effects on BCRP function could have been significantly underestimated. Moreover, high concentrations required to cause inhibitory effects do not necessarily mean that the molecules are not transported by BCRP. Nevertheless, we have undertaken studies to measure transport of steroids in HEK293-BCRP membrane vesicles and BCRP-overexpressing MDCKII cells, but we did not find clear evidence of BCRP involvement in the transport of steroid hormones in these models. The question whether steroids are substrates for BCRP has been addressed before and contradictory findings have been reported. In 2003, Janvilisri et al. demonstrated transport of estradiol in membrane vesicles from Lactococcus lactis containing functional human BCRP. However, this has not been repeated so far. Most studies support the conclusion that steroids interact but are not substrates of BCRP, which is in agreement with our findings.

Steroids, however, are clearly involved in the regulation of BCRP expression. The localization of BCRP/Bcrp in the plasma membrane is shown to be gender specific, as demonstrated in rodent liver, kidney and brain capillaries as well as in human breast, ovarian and placental cancer cell lines and as also confirmed in chapter 2. This gender-dependent BCRP/Bcrp expression is suggested to be due to the suppressive effect of estradiol and the inductive effect of testosterone, as was demonstrated for example in ovariectomized or castrated mice and rats with or without sex steroid treatment. Also, progesterone has been demonstrated to regulate BCRP transcription, as was shown in cancer cell lines. In human placental BeWo cells, progesterone and estradiol significantly increased and decreased BCRP mRNA levels, respectively. Estradiol likely downregulates BCRP through an estrogen receptor (ER), while progesterone upregulates BCRP via a non-progesterone receptor-mediated mechanism. Imai et al. found that estrogens post-transcriptionally downregulated BCRP in estrogen-responsive cancer cells. This effect could be counteracted by gene silencing of estrogen receptor-α (ERα), indicating that ERα is necessary for the suppression of the BCRP protein. In agreement, Hartz et al. described that estradiol signals through ERβ and ERα to initiate Bcrp internalization and acts via ERβ to stimulate proteosomal degradation of Bcrp in murine brain capillaries. In contrast, Ee et al. found that estradiol enhanced Bcrp mRNA levels in cells stably expressing ERα at estradiol concentrations similar to those used by Hartz et al.

By means of homology modelling, Mares-Sámano et al. generated a three-dimensional model of the nucleotide binding domain (NBD) of BCRP and its interaction with a series of
steroidal ligands were investigated using an in silico docking approach. All steroids, including progesterone, testosterone, estradiol and corticosterone, were predicted to bind in the vicinity of the P-Loop within the NBD of BCRP. By binding the NBD, ATP-binding is thought to be hindered, resulting in a decrease in BCRP activity. Eventually, decreased function might result in an increase in ABGC2 transcription and expression as a compensatory mechanism. This hypothesis might explain the inductive effect found for testosterone, but not the suppressive effect of estradiol.

Besides the steroid-dependent regulation of BCRP expression, we found another relationship between BCRP and the endocrine system. In chapter 4, we describe that the testicular weight of Bcrp⁻/⁻ mice was 40% higher than of wild type mice. Generally, it is believed that testicular weight increases to compensate a decreased testicular function.¹¹,¹² Our results indicate that Bcrp may be necessary for a proper testicular functioning in mice. However, since we did not examine steroid production and the number of Sertoli and total germ cells, we cannot conclude that testicular function was indeed impaired in Bcrp-deficient mice. Though, this interesting hypothesis requires further research to elucidate the underlying mechanism.

This thesis also presents work performed with other efflux transporters expressed in the blood-testis barrier, like P-gp, MRP1 and MRP4, which were found to be present in a murine Leydig (MA-10) cell line. In chapter 3, one of the endocrine disruptors tested, TBBPA, increased Leydig cell testosterone secretion concentration-dependently without altering expression levels of androgen-producing enzymes. This led us to hypothesize that transporter-mediated (probably P-gp-mediated) efflux of testosterone precursors, e.g. androstenedione or DHEA, from the cells is inhibited by TBBPA. As a result, more precursors are available for testosterone production, which is reflected by increased testosterone levels. In addition, TBBPA-mediated increase in testosterone secretion was completely abolished by the Mrp inhibitor MK-571 suggesting a decrease in active transport of testosterone out of the Leydig cells. Neither P-gp-mediated transport of testosterone precursors nor testosterone transport by MRPs/Mrps have been described before, so further experimental support is needed to confirm this hypothesis.

**BCRP and metabolic processes**

Besides steroids, other endogenous molecules, like heme, vitamin B2, folic acid and uric acid also interact with BCRP.¹³-¹⁹ In chapters 5, 6 and 7, we aimed to investigate further the role of BCRP in a number of metabolic processes in which these substrates are involved. We found that BCRP protects against the injurious actions of heme, next to the heme-degrading enzyme.
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heme oxygenase-1 (HO-1) (chapter 5). Heme is the functional group of various hemoproteins and crucial for many cellular processes. However, heme is increasingly recognized as a culprit for a wide variety of pathologies, including sepsis, malaria, and kidney failure. Excess of free heme can be detrimental to tissues by mediating oxidative and inflammatory injury. Protective mechanisms against free heme, like BCRP and HO-1 expression, are therefore pivotal for cellular survival. Furthermore, the BCRP-substrate uric acid, the end product of purine nucleotide degradation, is related to a variety of pathologies, including gout, nephrolithiasis, cardiovascular disease and chronic kidney disease (CKD) (chapter 7). The importance of BCRP in the regulation of uric acid levels is endorsed by several reports on the correlation of a common single nucleotide polymorphism (SNP) C421A, encoding the Q141K mutation of BCRP, with elevated serum uric acid levels (hyperuricemia). Woodward et al. described that the Q141K mutation caused hyperuricemia-based gout, which was confirmed in a Japanese population. Several studies of SNPs in the ABCG2 gene have been published and over two dozen sequence variations of ABCG2 have been reported. Of these, the C421A nucleotide change, resulting in a glutamine to lysine substitution in the translated protein, has received the most attention. The SNP renders the transporter inactive, causing substrates to accumulate in tissues.

Next to genetic factors, inhibitory effects can also result in reduced transporter activity. Nowadays, many inhibitors of BCRP are known. Some of them inhibit BCRP-mediated efflux by competition for the same substrate binding site, others are pure inhibitors without being transported themselves. Molecules that interact with BCRP include xenobiotics, such as chemotherapeutic agents, tyrosine kinase inhibitors, antivirals, HMG-CoA reductase inhibitors, statins and flavonoids, as well as endogenous molecules including steroids, BPA and uric acid (chapter 7). In addition, we showed that several steroid hormones are able to inhibit BCRP activity (chapter 2). Decreased BCRP functioning can promote the retention of other BCRP substrates. In chapter 7, we indeed show that uric acid-induced dysfunction of Bcrp caused disturbances in tryptophan metabolism in mice, including the retention of the novel identified substrate for the transporter, kynurenic acid. Elevated kynurenic acid levels were also found in plasma and brain homogenates of Bcrp-deficient mice (chapter 6). Kynurenic acid is a widely studied antagonist of the N-methyl-D-aspartate-receptor and the α7-nicotinic acetylcholine receptor, and elevated levels of kynurenic acid are related to several neurological disorders. It may also alter the release of multiple growth factors such as nerve growth factor and fibroblast growth factor-1. Furthermore, in patients with disturbed kidney function, kynurenic acid accumulates and the increased levels correlate positively with multiple markers of endothelial dysfunction. These findings suggest that changes
in transport efficacy of molecules involved in cellular signaling and communication, caused by hampered BCRP function in which BCRP is impaired as well, might potentially influence a myriad of cellular processes that are regulated by BCRP and disturb cellular homeostasis.

**Animal models and extrapolation to humans**

When studying the role of ABC transporters, often transporter knockout animals are used. Authors that used Bcrp-knockout animals often state that the possibility of compensatory gene expression and protein synthesis of other transporters may counteract the lack of the Bcrp gene. In the kidneys of Bcrp<sup>−/−</sup> mice, mRNA expression of two sterol transporter genes, *Abcg5* and *Abcg8*, was strongly increased, however, none of the Bcrp-substrates are known to be transported by either *Abcg5* or *Abcg8*. Agarwal et al. studied compensatory changes in brain capillary endothelial cells of Bcrp knockout mice and found no significant differences in the expression of any of the measured proteins, including 12 ATP-binding cassette transporters, 10 solute carrier transporters, five transmembrane receptors, and two housekeeping proteins. Besides, Zamek-Gliszczynski et al. demonstrated very modest compensatory changes in Bcrp knockout rats, which do not preclude their general application to study for example transporter-mediated pharmacokinetics. Though, they did find significant upregulation of intestinal catechol-O-methyltransferase in these rats, which potentially influences the metabolism of compounds containing a free catechol group. Obviously, this only accounts for a small fraction of drugs and can be readily identified based on chemical structure. Yet, in the liver of Bcrp knockout mice, carboxylesterase enzymes appeared to be highly upregulated. This might have significant consequences for drugs hydrolyzed by these enzymes but, apparently, also for compounds that are not hydrolyzed, like the anticancer drug everolimus. Blood pharmacokinetics of this drug was altered in Bcrp<sup>−/−</sup> mice by increased binding to carboxylesterase enzymes.

In general, a series of laboratory techniques is used to study the role of ABC transporters, including *in vitro* and *in vivo* models of animal or human material. Some studies present both laboratory animal and human data. However, one should take into account that expression levels and activity of ABC transporters are different between animal species compared to humans. For BCRP, Li et al. observed significant elimination of phaeophorbide A (PhA), an exclusive BCRP/Bcrp substrate, by human and dog hepatocytes, while Bcrp-mediated PhA transport in monkey and rat hepatocytes was limited. In agreement, low Bcrp expression in rat hepatocytes has been reported before, although this was determined on mRNA levels.
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Absolute rat Bcrp protein levels in liver tissue were slightly higher compared to human and monkey BCRP/Bcrp, as was determined by LC-MS/MS analysis. The highest Bcrp levels were found in dog livers. In the BBB, BCRP expression is lower in humans compared to rat, murine and bovine BBB. In rodent kidney, Bcrp mRNA levels were clearly higher as compared to human kidney, with highest expression in the mouse. Also for the placenta, differences in placental structure and transport to the fetus between species are described. Human, mouse and rat BCRP/Bcrp is located mainly in the apical membrane of syncytiotrophoblasts and also on the apical membrane of fetal capillary endothelium. Nevertheless, the number of cell layers between maternal and fetal circulations varies from one species to another, and, thus, the role of membrane-bound transporters may differ as well. Variances in BCRP/Bcrp expression levels between placentas of humans and laboratory animals are not yet known.

In addition, genes coding for receptors and metabolic enzymes are differentially expressed in animals compared to humans. Species differences can also be a result of different specificity or affinity of a molecule for human transporters than for animal transporters. Although it was recently reported that substrate specificity of human and mouse BCRP is very similar, Zhang et al. described the interaction of the flavonoids chrysin and benzoflavone with human BCRP. This interaction was not found with rat or mouse Bcrp. Moreover, Mazur et al. showed interspecies differences in the efflux transporter specificities for BPA between rat and human ABC transporters, including BCRP. BPA-mediated increase in ATPase activity was clearly found for human BCRP, but not for rat Bcrp. This might explain the different responses we found in chapter 4 in which we reported that BPA was well transported by human BCRP but in mice, Bcrp did not affect the disposition of BPA. Possibly, BPA is not a substrate for murine and/or rat Bcrp, though, we did not study this.

Chapters 6 and 7 describe in vivo mouse studies on the role of Bcrp and Mrp4 in metabolic processes. Reasons to start these experiments originate from results obtained from in vitro studies using human transporter proteins. In chapter 6, we found Bcrp expression in the choroidal cell layer of the subcommissural organ (representing the brain-CSF interface) at the ventricular side of ependymal cells facing the CSF in the murine brain, which made us curious to investigate the role of Bcrp in regulating CSF composition. By comparing Bcrp and wild type mice for several physiological parameters involved in energy or tryptophan metabolism, and studying murine neuroblastoma cells in vitro, we showed that Bcrp might influence brain energy metabolism by regulating kynurenine acid levels. Also in chapter 7, tryptophan metabolism was studied in mice, this time after inducing hyperuricemia. The results showed that elevated uric acid levels hamper MRP4 and BCRP functioning, thereby promoting the
retention of other potentially toxic substrates, including kynurenic acid. Although the \textit{in vivo} data implied that kynurenic acid is probably a substrate of murine Bcrp and Mrp4, again, we only confirmed transport of the molecule by human BCRP and MRP4. All together, these differences result in deviated kinetics and dynamics of molecules between humans and laboratory animals and, obviously, lead to difficulties in the extrapolation of animal results to humans. In the end, we want to clarify physiological processes in human. Since it is obviously difficult to study certain processes in human \textit{in vivo}, probably the easiest way to add relevant data (at least regarding the research presented in this thesis) is to perform \textit{in vitro} studies in both animal and human material. When human and murine \textit{in vitro} data are similar, the extrapolation of the murine \textit{in vivo} data to the human \textit{in vivo} situation is somewhat more legitimate. Other possibilities to investigate human transporters are \textit{ex vivo} human organ perfusion models or humanized animal models. The advantage of using organ and tissue material, \textit{e.g.} placenta or liver tissue, to study transporter function is that they retain tissue structure and the polarized nature of endothelial cells, often expressing efflux transporters like BCRP.\textsuperscript{52-57} Also freshly isolated human brain capillaries have been successfully used to study transporter function and its dynamic regulation.\textsuperscript{58,59} Applications of these systems to date are limited by the availability of appropriate tissue samples and the complexity of the technique. Several humanized animals models exist. Chimeric mice are engrafted with human cells, \textit{e.g.} bone marrow cells or hepatocytes. The advantage of humanized liver chimeric mice is that the entire liver is human, so the interplay between human transporters and metabolizing enzymes can be studied.\textsuperscript{60} Nevertheless, all other organs are murine. Consequently, circulating murine metabolites reach the humanized liver and bind the human proteins so the model does not fully reflect the human situation. Obviously, these are complex, laborious and costly models, which are mainly used to study metabolic pathways and drug-induced liver injury (\textit{e.g.} in pharmaceutical development)\textsuperscript{60,61}, and not often to study exclusively one specific transporter. To this end, mice models expressing specific human transporters have been generated. Once a stable genetic mouse model is generated, the mice can easily be bred and readily be used, whereas the chimeric mice have to be generated every experiment. Recently, van der Steeg \textit{et al}.\textsuperscript{62,63} generated transgenic mouse models with specific expression of human OATP1B1, -1B3, or -1A2 in the liver and proved functionality by showing transport of the anti-cancer drugs methotrexate and paclitaxel. The only model expressing a human efflux transporter, as of yet, is the human MRP2 mouse model, generated by Scheer \textit{et al}.\textsuperscript{64}. This was achieved by a knock-in strategy allowing the expression of the human transporter under control of the corresponding mouse promoter. Therefore, the expression of human MRP2 was not restricted to one organ but the protein was ubiquitiously expressed.
Chapter 8

In silico modeling and multiple BCRP binding sites

In numerous areas of molecular research, such as research focusing on the understanding of the mechanisms in physiology and diseases as well as drug development and environmental toxicology, efflux transporters are gaining momentum. Therefore, it is more and more important to understand which molecules interact with which transporters and to determine the kinetic characteristics of transport. Computational in silico modeling is a powerful cost-effective tool often employed to predict structural requirements of transporter substrates and inhibitors prior to in vitro and in vivo studies.65 Many different in silico modeling techniques have been developed that describe the (quantitative) structure-activity-relationship (SAR/QSAR) of compounds with BCRP.66,67 Besides, computational homology models of BCRP that can be used for docking calculations and interpretation of biochemical data, have recently been developed and await further experimental validation.68-70 Several SAR and QSAR studies indicate that lipophilicity and molecular polarizability of ligands are good predictors of BCRP inhibition67,71,72, nevertheless, other studies argue that these properties are not significant70,71,73,74. In general, high substrate prediction accuracy of the existing computational models has not yet been proven. Since they cannot give a decisive answer as to whether a compound is a substrate for BCRP, transport activity should be verified using experimental models. To date, many inhibitor datasets are available for BCRP modeling, while very few substrate datasets exist. The latter might be due to lack of a high resolution crystal structure of mammalian BCRP, which precludes direct examination of the substrate binding site.75 Evidence coming from both computational studies76 as well as from experimental studies77-81 on for example BCRP mutants or inhibitory effects of different substrates, suggests that BCRP may have multiple binding sites. Different pharmacophore models, which describe the chemical properties of a compound that determine interaction with a binding site, should be developed for the different binding sites to predict the wide range of substrates. However, also evidence against the existence of more than one binding site has been raised.71 Caution is also warranted if the conclusion about the existence of multiple BCRP binding sites is solely based on experiments using different substrates. A compound can inhibit BCRP-mediated transport in several ways, for example by binding BCRP close to the substrate binding site, which might impede binding of some but not all substrates, or binding to the substrate itself, which hinders the substrate to bind to the transporter, and, thus, active transport. The existence of multiple binding sites on transport proteins could add to the complexity in identifying substrate and inhibitor molecules in in vitro assay systems, leading to incorrect interpretation of substrate-inhibitor interaction studies.
In chapter 2, we demonstrated the inhibitory effects of several steroid hormones on BCRP function. The mode of inhibition was determined using Dixon’s method combined with linear regression analysis, which revealed that estradiol, testosterone, progesterone and androstenedione non-competitively attenuated BCRP-mediated E\textsubscript{1S} transport. Based on the presence of a single BCRP binding site, non-competitive (allosteric) modulators are not likely to be substrates. However, the effect of steroid hormones on BCRP-mediated transport of other substrates is still to be determined. Similar inhibition studies, using E\textsubscript{1S} as a BCRP-substrate, were used in chapters 3, 6 and 7, however, the mode of inhibition was not determined. We used the assay as a positive selection for compounds that are able to interact with the transporter. For three of them (BPA, TBBPA and PFOA), we furthermore determined direct transport. For the negative compounds DEHP and MEHP, we cannot completely rule out the possibility that they are inhibitors of BCRP-mediated transport of other substrates or that they are transported by BCRP themselves. For uric acid (chapter 7), which was already known as a BCRP-substrate, we only determined the ability to inhibit transport of other substrates. We concluded that uric acid was able to inhibit BCRP function and that therefore hyperuricemia could promote the retention of other potentially toxic substrates. Strictly speaking, knowledge of the binding site of uric acid compared to the binding site of the particular (toxic) substrate is necessary to confirm this. So far, our in vivo results confirmed the effect of uric acid on the retention of kynurenic acid.

Whether BCRP indeed exhibits multiple binding sites, as well as the exact locations of these putative sites and their interactions with the known substrates and inhibitors, still needs to be elucidated. Nevertheless, if BCRP contains multiple binding sites, then inhibitory effects should be determined using substrates that interact at different sites of the transporter before it can be concluded that a compound is only an inhibitor and not a substrate of BCRP. For example, rhodamine and daunomycin are described to interact at pharmacologically distinct sites on the protein\textsuperscript{28,78,82} as well as pheophorbide A and imatinib\textsuperscript{79}. Also mitoxantrone and Hoechst\textsuperscript{33342} are thought to interact with another BCRP binding site than daunomycin.\textsuperscript{78}

**Targeting BCRP for clinical applications**

The presence of BCRP in many organs and tissues in the body and its role in clinical multidrug resistance makes the protein an interesting target for potential clinical applications. A strategy of BCRP inhibition could be employed to increase oral bioavailability of drugs, increase brain penetration, or overcome cancer cell resistance.\textsuperscript{83} Concerning oral bioavailability, a limited number of clinical trials have already demonstrated the feasibility of coadministering dual
BCRP/P-gp inhibitors with oral chemotherapy.\textsuperscript{84,85} This strategy is only useful for drugs that do not inhibit transporter activity at high concentrations. It has been shown that several orally administered drugs, \textit{i.e.} gefitinib, nilotinib, and imatinib, are BCRP substrates at low concentrations and behave as inhibitors at higher concentrations.\textsuperscript{86,87} Concerns of high local substrate concentrations do not apply to the blood-brain barrier where very high endothelial BCRP levels have been observed. Inhibitors of BCRP alone or both BCRP and P-gp, such as elacridar, tariquidar and dofequidar, will increase brain penetration of drugs that need to act on the CNS. This has been described repeatedly in rodent models, however, unfortunately such proof in humans is lacking.\textsuperscript{83} A reason for this includes the lack of a validated probe for BCRP functioning in the human blood-brain barrier, which makes it difficult to interpret the results of clinical trials that combined a CNS-targeting drug with a BCRP inhibitor.

The problem of accumulating potentially toxic substrates, in situations of undesirably hampered BCRP function, \textit{e.g.} in the presence of the Q141K SNP or when BCRP is inhibited, is much more difficult to challenge. In the case of BCRP-inhibition, stimulating BCRP upregulation might be a good therapeutic approach. Several drugs have been described to upregulate the protein, such as mitoxantrone.\textsuperscript{88,89} However, it is probably not feasible to use mitoxantrone for BCRP upregulation in a clinical setting, considering the fact that mitoxantrone is metabolized relatively fast and has many other unwanted effects. The same holds true for testosterone. The dose of testosterone needed to cause an induction of BCRP expression will elicit too many side effects. Regulating BCRP expression levels at its transcriptional level is a theoretical goal for clinical applications. Expression of the \textit{ABCG2} gene is controlled by two functional \textit{cis} elements in the \textit{ABCG2} promoter, the estrogen\textsuperscript{4} and hypoxia\textsuperscript{15} response elements, and a peroxisome proliferator-activated receptor-\gamma response element upstream of the \textit{ABCG2} gene.\textsuperscript{90,91} Besides, \textit{ABCG2} expression can be upregulated by the aryl hydrocarbon receptor (AhR).\textsuperscript{92,93} Some dietary flavonoids (\textit{i.e.} quercetin, chrysin, and flavone), acting as AhR agonists, were shown to induce BCRP expression.\textsuperscript{92,94} Obviously, interfering with these transcription pathways affects many more processes than only \textit{ABCG2} expression and are therefore not feasible as pharmacological targets to increase BCRP function. To date, the ideal compound that is suitable for BCRP induction without affecting other receptors and disturbing physiological pathways, is not yet known.

Genotyping of BCRP in a clinical setting might become important in the near future for personalized medicine. As mentioned above, the common dysfunctional form of BCRP, Q141K, renders the transporter inactive. Besides, other polymorphisms of \textit{ABCG2} have been identified.\textsuperscript{68} Patients with reduced or no BCRP function may suffer from, for example, disturbed metabolic processes or accumulating uremic toxins because of lack of removal of...
these molecules. Besides, the removal of drugs can be severely postponed in these patients, which could lead to toxicity if the dosage is not adjusted. Determining the genotype of ABCG2 and other important transporters could contribute to the safety of drug-using patients and/or make them aware of the risk factors of developing diseases like gout or chronic kidney disease.

**Conclusion**

In this thesis, the role of the efflux transporter BCRP in endocrine and metabolic processes was investigated. Our data show clear inhibition of BCRP by steroid hormones, however, BCRP is not involved in the transport of steroids. Besides, we identified a role for BCRP in several metabolic processes, using both *in vitro* and animal models. Our results indicate that BCRP protects against heme-induced cellular injury and potentially also against BPA-, TBBPA-, PFOA-, and PFOS-induced endocrine disruption. Moreover, we found that hyperuricemia influences tryptophan metabolism via inhibition of BCRP. Furthermore, we state that caution is warranted when translating data obtained in laboratory animals to the human situation, since interspecies differences are associated with substantial differences in kinetics and dynamics of xenobiotics.

Altogether, based on our results, we postulate that decreased BCRP function, either by transport inhibition or because of an inactive single nucleotide polymorphism, could promote the retention of potentially toxic substrates, including endocrine disruptors, heme and kynurenic acid. This influences a myriad of cellular processes that are regulated by BCRP and disturbs cellular homeostasis. Therefore, this protein could be an interesting target for potential clinical applications, such as its BCRP-induction to prevent accumulation of BCRP substrates. However, more research is needed to determine the feasibility of influencing BCRP in patients.
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Introduction

ATP-binding cassette (ABC) transporters are membrane-associated proteins that directly use ATP to transport a variety of molecules across the membrane against steep concentration gradients. Nearly all ABC proteins are efflux transporters, many of which have an important role in tissue defense through the removal of harmful molecules. The research described in this thesis was mainly focused on one of these ABC transporters, i.e. breast cancer resistance protein (BCRP), which is encoded by the ABCG2 gene. This gene was discovered in 1998 in a mitoxantrone-resistant subline of breast cancer cells. BCRP is expressed in many organs and tissues in the body, including epithelia of intestine, liver and kidney, as well as tissues with a tight barrier function like the blood-brain barrier (BBB), the blood-placenta barrier and the choroid plexus as part of the blood-cerebrospinal fluid (CSF) barrier. BCRP is recognized as a xenobiotic transporter that plays a major role in multidrug resistance and a myriad of BCRP substrates have been described, among them a variety of xenobiotics including chemotherapeutic agents, tyrosine kinase inhibitors, antivirals, HMG-CoA reductase inhibitors, and flavonoids. Research on the role of BCRP in most organs has until now been predominantly focused on the restriction of drug penetration and its protective function against the toxic effects of exogenous compounds. Yet, accumulating evidence has shown that BCRP also transports various endogenous molecules, such as uric acid, folic acid, porphyrins, riboflavin (vitamin B2) and conjugated steroid hormones. Besides, steroid hormones have been associated with BCRP function, expression and degradation, however, the molecular bases of these protein-steroid interactions are not yet elucidated. The aim of this thesis was to study the role of BCRP in endocrine and metabolic processes.

The role of BCRP in endocrine organs

The expression of BCRP in the plasma membrane is higher in males than in females. This is suggested to be due to the suppressive effect of estradiol and the inductive effect of testosterone. Moreover, involvment of BCRP in the transport of androgens and estradiol was suggested, indicating that the efflux pump might play a role in steroid action. Localization of BCRP was previously described in testis and adrenal gland, but for other organs involved in steroid hormone production or action BCRP, expression was not yet clear. In chapter 2, we investigated the presence and localization of Bcrp in endocrine organs of wild type mice.
Quantitative PCR revealed Bcrp mRNA in the pituitary and adrenal glands, pancreas, ovary, testis and adipose tissue and that mRNA levels of Bcrp in these tissues were slightly higher in male than in female mice. Immunohistochemistry was used to assess the localization of Bcrp in murine organs. Except for the fat pads, Bcrp was located mainly in the capillaries of both endocrine and non-endocrine organs. Although endocrine organs have a significant secretory function, we did not find expression of Bcrp in most of the endocrine cells, indicating that the efflux pump is not involved in the excretion of steroids. The only steroid secreting cells expressing Bcrp were found in the adrenal gland, i.e. cells of the zona glomerulosa and zona reticularis which secrete mineralocorticosteroids and androgens, and in the plasma membrane of adipocytes, a major site for metabolism of sex steroids and glucocorticoids.

The interaction between BCRP and twelve steroid hormones was studied using membrane vesicles of BCRP-overexpressing HEK293 cells. Estradiol, testosterone, progesterone and androstenedione inhibited BCRP-mediated uptake of $^3$H-estrone sulfate (E$_1$S) most potently. BCRP function was inhibited non-competitively, which implies an allosteric interaction of these steroids with BCRP-mediated E$_1$S transport. We have undertaken studies to measure transport of steroids in HEK293-BCRP membrane vesicles and BCRP-overexpressing MDCKII cells, but we did not found clear evidence for the involvement of BCRP in steroid secretion. This is in line with previously reported findings, although conflicting data regarding this matter has been published as well.

**Efflux transporters present in the blood-testis barrier are targeted by endocrine disruptors**

Epidemiological trends show an increase in male subfertility in Western society. This is not only a consequence of lifestyle factors or side effects of therapeutic agents, but is also suggested to be a result of environmental exposures. Endocrine disrupting chemicals (EDCs) are considered to cause testicular toxicity primarily via interference with steroid hormone function. The testicular microenvironment is pivotal for mammalian steroidogenesis and spermatogenesis. The blood-testis barrier (BTB) physically divides the seminiferous epithelium into a basal and an apical compartment, whereas, efflux transporters actively protect the organ from internal exposure to harmful substances. In addition to BCRP, the most abundant efflux transporters in the BTB include P-glycoprotein (P-gp) and multidrug resistance protein 1 and 4 (MRP1 and -4). Chapter 3 describes the interaction of six selected EDCs with the transport activity of these human efflux transporters, using the aforementioned vesicle transport assay. Bis(2-ethylhexyl) phthalate (DEHP) and mono(2-ethylhexyl) phthalate (MEHP) did not inhibit activity of the transporters studied. Bisphenol A (BPA) solely inhibited
BCRP activity, whereas tetrabromobisphenol A (TBBPA), perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) inhibited all transporters tested. An accumulation assay using transporter-overexpressing MDCKII cells revealed that BPA and PFOA, but not TBBPA, are transported by BCRP, and none of these compounds were transported by P-gp.

In adult males, interstitial cells of Leydig are responsible for steroidogenesis. Local testicular steroidogenesis, i.e. production of androgens and estrogens, is essential in regulating spermatogenesis and involves multiple cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes. In chapter 3, we further investigated the toxicological implications of transporter inhibition by the EDCs in murine Leydig (MA-10) cells. This P-gp, Mrp1- and Mrp4-expressing cell line exhibits a de novo steroid synthesis pathway. Bcrp expression was undetectable in these cells. Only BPA and TBBPA concentration-dependently increased testosterone secretion by MA-10 cells to 6- and 46-fold of control levels, respectively. Inhibition of the Mrp's by MK-571 completely blocked testosterone secretion elicited by TBBPA, which could not be explained by coinciding changes in expression of steroidogenic genes. This led us to hypothesize that transporter-mediated (probably P-gp-mediated) efflux of testosterone precursors, e.g. androstenedione or DHEA, from the cells is inhibited by TBBPA. As a result, more precursors are available for testosterone production, which is reflected by increased testosterone levels. The data described in this chapter suggest that EDCs might affect male fertility by acting on ABC transporters present in the BTB and influence steroidogenesis, and endorse the toxicological and clinical relevance of ABC transporters in EDC risk assessment related to testicular toxicity.

BPA is widely used in the production of plastics and its widespread inclusion in consumer products has greatly enhanced the potential for human exposure. Detectable levels of BPA have been found in the urine of 93% of the US population, as reported in 2008. Health concerns regarding human exposures to BPA stem from its estrogenic properties. Since we showed that BPA is a substrate for BCRP, we hypothesized that BCRP may be an important determinant in the distribution, disposition and, thereby, the toxicity of BPA. In chapter 4, we aimed at elucidating the role of Bcrp in the disposition and potential endocrine disrupting effects of BPA in mice. Both wild type as well as homozygous Bcrp-knockout (Bcrp⁻/⁻) mice were subcutaneously exposed, via a micro-osmotic pump, to [³H]-BPA with or without elacridar, an inhibitor of Bcrp and P-gp. BPA accumulation in the testes, adrenal and pituitary glands, adipose tissue, brain, liver and kidneys of Bcrp⁻/⁻ mice was not different as compared to wild type mice. Also, co-administration of elacridar did not affect BPA accumulation. Taken together, our results indicate that the disposition of the human BCRP substrate BPA is not altered in Bcrp-deficient mice, suggesting minor importance of the transporter in BPA
handling in mice. Possibly, BPA is not a substrate for murine Bcrp. Although this interspecies difference needs further confirmation, it is important to realize that the disposition and mechanism of toxicity of BPA or other xenobiotics may be different in humans as compared to rodents.

**The role of BCRP in metabolic processes**

The endogenous BCRP substrates heme, kynurenic acid and uric acid can negatively influence physiological processes at high concentrations. Heme is the functional group of hemoproteins and crucial for many cellular processes, such as oxygen transport and storage, electron transfer, and signal transduction. However, excess of free heme can be detrimental to tissues by mediating oxidative and inflammatory injury. Kynurenic acid is a widely studied antagonist of the N-methyl-D-aspartate receptor and the α7-nicotinic acetylcholine receptor, and elevated levels are related to several neurological disorders. In addition, elevated serum uric acid levels (hyperuricemia) are related to a variety of pathologies, including gout, nephrolithiasis, cardiovascular disease and chronic kidney disease.

In chapter 5, we postulated that overexpression of heme oxygenase-1 (HO-1) and BCRP would protect against heme-induced cytotoxicity. To this end, HEK293 cells were transduced using a baculovirus system to efficiently overexpress HO-1 and/or BCRP. Exposing cells to heme resulted in a dose-dependent increase in reactive oxygen species formation, DNA damage and cell death. Heme-induced cell death was significantly attenuated when cells overexpressed HO-1, BCRP, or both, thus, their protective mechanisms against free heme are pivotal for cell survival.

Little is known about the transport of metabolites from brain towards cerebrospinal fluid (CSF). We found Bcrp to be localized in the choroidal cell layer of the murine brain-CSF interface, as described in chapter 6. We investigated the potential role of BCRP in regulating CSF composition by comparing CSF of Bcrp<sup>−/−</sup> mice to that of wild types by 1H-NMR analysis. The concentration of glucose, glycine, creatine, alanine, and glutamine was altered in CSF of Bcrp<sup>−/−</sup> mice. Glucose was most discriminative and its concentration was significantly lower in CSF of Bcrp<sup>−/−</sup> mice compared to that of wild type mice. However, as neither glucose nor any of the other metabolites, differentially present in CSF, affected BCRP function, it seemed more likely that absence of the efflux transporter indirectly affected murine brain energy metabolism. Kynurenic acid has been associated with energy metabolism disturbances and several neurological disorders. Using the membrane vesicle assay we identified kynurenic acid as a new BCRP substrate and elevated levels were found in plasma and brain homogenates.
of Bcrp-deficient mice. Moreover, kynurenic acid increased glucose uptake into malignant neuroblastoma (N2a) cells in a concentration-dependent fashion. From this, we conclude that increased intracellular kynurenic acid concentrations as a result of a decreased kynurenic acid-efflux, may result in increased glucose uptake and, therefore, CSF may contain less glucose.

Kynurenic acid was also studied in oxonic acid-mediated hyperuricemic mice. Hyperuricemia is related to a variety of pathologies, however, the pathophysiological mechanisms underlying disease development are not yet fully elucidated. In chapter 7, we studied the effect of hyperuricemia on tryptophan metabolism and the potential role herein of two important uric acid efflux transporters MRP4 and BCRP. Uric acid inhibited both MRP4 as well as BCRP activity. Besides, the membrane vesicle assay revealed that, next to BCRP, MRP4 also transports kynurenic acid. Increased plasma levels of kynurenic acid observed in Mrp4⁻/⁻ and Bcrp⁻/⁻ mice corroborated this finding. Hyperuricemia was associated with elevated plasma kynurenic acid levels in wild type mice, but they did not further increase in Bcrp- or Mrp4-knockout mice. Based on these results, we postulate that elevated uric acid levels hamper MRP4 and BCRP functioning, thereby promoting the retention of other potentially toxic substrates, including kynurenic acid, which could contribute to the development of chronic kidney disease.

Conclusions and future perspectives

In this thesis, the role of the efflux transporter BCRP in endocrine and metabolic processes was investigated. Our data show clear inhibition of BCRP by steroid hormones, however, BCRP is not involved in the transport of steroids. Based on our results, we postulate that decreased BCRP function, either by transport inhibition or because of an inactive single nucleotide polymorphism, could promote the retention of potentially toxic substrates, including endocrine disruptors, heme and kynurenic acid. This influences a myriad of cellular processes that are regulated by BCRP and disturbs cellular homeostasis. Therefore, this protein could be an interesting target for potential clinical applications, such as its induction to prevent accumulation of BCRP substrates. However, more research is needed to determine the feasibility of influencing BCRP in patients.
Nederlandse samenvatting

Introductie

ATP-binding cassette (ABC) transporters zijn membraangebonden eiwitten die de energie die vrijkomt na hydrolyse van ATP gebruiken om verschillende moleculen tegen een concentratiegradiënt over de celmembraan te transporteren. Bijna alle ABC-transporters zijn effluxtransporters, waarvan vele een belangrijke rol spelen bij de verdediging van weefsels tegen schadelijke moleculen, door deze te verwijderen. Het in dit proefschrift beschreven onderzoek was vooral gericht op een van deze ABC-transporters, namelijk het borstkankerresistentie-eiwit (breast cancer resistance protein: BCRP) dat wordt gecodeerd door het \textit{ABCG2}-gen. Het gen werd in 1998 ontdekt in een sublijn van borstkankercellen die resistent bleek voor het cytostaticum mitoxantrone. Sindsdien heeft men ontdekt dat BCRP tot expressie wordt gebracht in vele organen en weefsels in het lichaam, waaronder epitheel van de darm, lever en nieren, alsmede weefsels met een barrièrefunctie zoals de bloed-hersenbarrière, bloed-placentabarrière en plexus choroideus, als onderdeel van de bloed-hersenvochtbarrière. BCRP wordt gezien als een xenobioticatransporter die een belangrijke rol speelt bij resistentie tegen verschillende geneesmiddelen. Talloze BCRP-substraten zijn reeds beschreven, waaronder chemotherapeutische middelen, tyrosinekinaseremmers, antivirale middelen, HMG-CoA-reductaseremmers en flavonoïden. Onderzoek naar BCRP heeft zich tot nu toe voornamelijk gericht op haar beperkende rol in de opname van geneesmiddelen in bepaalde organen en de beschermende functie tegen schadelijke exogene stoffen. Er is in toenemende mate bewijs gevonden dat BCRP ook endogene moleculen, zoals urinezuur, foliumzuur, porfyrinen, riboflavine (vitamine B2) en geconjugeerde steroidhormonen transporteert. Daarnaast worden steroidhormonen in verband gebracht met BCRP-functie, -expressie en -afbraak. Echter, de moleculaire onderbouwing van deze BCRP-steroidhormooninteracties is nog niet opgehelderd. Het doel van dit proefschrift was om de rol van BCRP in endocriene en metabole processen te bestuderen.

De rol van BCRP in endocriene organen

De plasmamembranexpressie van BCRP is hoger bij mannen dan bij vrouwen. Er wordt gedacht dat dit komt door de remmende werking van estradiol en het stimulerende effect van testosteron. Bovendien is betrokkenheid van BCRP in het transport van androgenen en estradiol gesuggereerd, wat aangeeft dat de effluxpomp een rol zou kunnen spelen in het hormonale
systeem. De aanwezigheid van BCRP in de testis en de bijnier is reeds eerder beschreven, maar lokalisatie in andere organen die betrokken zijn bij de steroidhormoonproductie of -werking is nog niet bekend. In hoofdstuk 2 onderzochten we de aanwezigheid en lokalisatie van Bcrp in endocriene organen van controlemuizen. Uit gen-analyse bleek dat Bcrp tot expressie komt in de hypofyse, bijnieren, alvleesklier, eierstokken, testes en vetweefsel. Daarnaast vonden we dat Bcrp-expressie in deze organen bij mannelijke muizen hoger was dan bij vrouwelijke muizen. De lokalisa\n\ntie van Bcrp in de muizenorganen werd met behulp van immunohistochemie beoordeeld. Bcrp werd voornamelijk gevonden in de capillairen van zowel endocriene als niet-endocriene organen. Hoewel endocriene organen een belangrijke secretiefunctie hebben, hebben we in de meeste endocriene cellen geen Bcrp-expressie gevonden, wat aangeeft dat Bcrp niet betrokken is bij de uitscheiding van steroidhormonen. De enige cellen die steroidhormonen uitscheiden en Bcrp tot expressie brengen werden gevonden in de bijnier, namelijk cellen gelegen in de zona glomerulosa en zona reticularis die verantwoordelijk zijn voor de productie en uitscheiding van mineralocorticosteroïden en androgenen. Bcrp werd ook gelokaliseerd in de plasmamembraan van adipocyten (vetcellen), waar onder andere de omzetting van estrogenen en glucocorticoiden plaatsvindt.

De interactie tussen BCRP en twaalf steroidhormonen werd bestudeerd met behulp van membraanblaasjes (vesikels), gemaakt van HEK293-cellen die BCRP tot overexpressie brengen. Estradiol, testosteron, progesteron en androsteendion remden de BCRP-gemedieerde opname van radioactief gelabeld \[3\]H]-estronsulfaat het sterkst. Verder onderzoek wees uit dat er sprake was van non-competitieve remming, dus een allostere interactie tussen BCRP en de steroidhormonen. We hebben het transport van steroidhormonen in membraanvesikels en BCRP-overexpresserende MDCKII-cellen onderzocht maar hebben geen aanwijzingen gevonden voor een rol van BCRP bij de uitscheiding van steroidhormonen. Deze conclusie wordt ondersteund door verschillende publicaties, hoewel eveneens tegenstrijdige resultaten beschreven staan in de literatuur.

**Effluxtransporters in de bloed-testisbarrière als doelwit voor hormoonverstorende stoffen**

Epidemiologische trends laten een toename van subfertiliteit in de westere samenleving zien. Dit is niet alleen het gevolg van leefstijlfactoren of een neveneffect van geneesmiddelgebruik, maar wordt waarschijnlijk ook veroorzaakt door een toenemende blootstelling aan milieuvervuilende stoffen. Er wordt verondersteld dat sommige van deze stoffen een hormoonverstorende werking hebben die bij mannen kan resulteren in verminderde steroidogenese (de productie van androgenen en estrogenen) en/of spermatoogene\ns (de productie van spermacellen) in de testis.
De bloed-testisbarrière (BTB) verdeelt de testis in een basaal compartiment en een apicaal compartiment, waarin de spermatogenese plaatsvindt. Transporters in de BTB beschermen het apicale compartiment tegen blootstelling aan schadelijke stoffen. De meest voorkomende transporters in de BTB zijn, naast BCRP, P-glycoproteïne (P-gp) en multidrugresistentie-eiwit1 en-4 (MRP1 en-4). Hoofdstuk 3 beschrijft membraanvesikelexperimenten waarin de interactie van zes mogelijk hormoonverstorende stoffen met de bovengenoemde transporters is onderzocht. Bis(2-ethylhexyl)ftalaat (DEHP) en mono(2-ethylhexyl)ftalaat (MEHP) remden de activiteit van de transporters niet. Bisfenol A (BPA) remde uitsluitend BCRP-activiteit, terwijl tetrabroombisfenol A (TBBPA), perfluoroctaanzuur (PFOA) en perfluoroctaansulfonaat (PFOS) de activiteit van alle vier transporters remden. Vervolgens deden we een accumulatiestudie met BCRP- en P-gp-overexpresserende MDCKII-cellen waaruit bleek dat BPA en PFOA, maar niet TBBPA, worden getransporteerd door BCRP en dat geen van deze stoffen door P-gp werd getransporteerd.

De interstitiële cellen van Leydig zijn bij volwassen mannen verantwoordelijk voor de steroidogenese en zijn daarom essentieel voor spermatogenese. Verschillende cytochrome P450 (CYP)-enzymen en hydroxysteroiddehydrogenase (HSD)-enzymen zijn betrokken bij de lokale steroidogenese. In hoofdstuk 3 hebben we de consequenties van transportremming door de hormoonverstorende stoffen verder onderzocht in de muizen-Leydigcellijn, MA-10. In deze cellen komen P-gp, Mrp1 en Mrp4, maar niet Bcrp, tot expressie en de cellijn vertoont de novo-steroidhormoonsynthese. De hoeveelheid testosteron die uitgescheiden werd door de MA-10-cellen werd concentratieafhankelijk verhoogd door BPA en TBBPA (respectievelijk 6- en 46-keer hoger dan controlewaarden). De TBBPA-gemedieerde verhoging van testosteronuitscheiding kon worden voorkomen door de Mrp’s te remmen. Uit genexpressieonderzoek (qPCR-analyse) bleek dat dit effect niet kan worden verklaard door een verandering in de expressie van enzymen die verantwoordelijk zijn voor de synthese van steroidhormonen. Dit leidde tot de hypothese dat transportergemedieerde (waarschijnlijk P-gp-gemedieerde) uitscheiding van testosteronprecursors, bijvoorbeeld androsteendion of DHEA, uit de cellen wordt geremd door TBBPA. Daardoor zijn er intracellulair meer testosteronprecursors beschikbaar voor omzetting naar testosteron, hetgeen wordt weerspiegeld door een verhoogde testosteronuitscheiding. De resultaten die in dit hoofdstuk zijn beschreven suggereren dat hormoonverstorende stoffen de mannelijke fertiliteit kunnen beïnvloeden door aan te grijpen op de ABC-transporters in de BTB. Dit onderstreept de toxicologische en klinische relevantie van ABC-transporters in de risicobeoordeling van testistoxiciteit door hormoonverstorende stoffen.
BPA wordt veel gebruikt in de productie van plastics zoals voedseverpakkingen en drinkflessen. Hierdoor is de blootstelling van de mens aan BPA vrij hoog. In een Amerikaans onderzoek uit 2008 werden meetbare hoeveelheden van BPA gevonden in de urine van 93% van de proefpersonen. Bezorgdheid over de schadelijke effecten van BPA voor de volksgezondheid komt voort uit de kennis dat BPA estrogene eigenschappen bezit. Omdat we hadden aangetoond dat BPA een substraat is voor BCRP, veronderstelden we dat BCRP een belangrijke speler is in de blootstelling en dus ook de toxiciteit van BPA in het lichaam. In hoofdstuk 4 vergeleken we controlemuizen met homozygote Bcrp-knockout (Bcrp\(^{-/-}\))-muizen, die subcutaan via een micro-osmotische pomp werden blootgesteld aan \(^{3}H\)-BPA met of zonder elacridar, een potente remmer van P-gp en Bcrp. BPA-accumulatie in de testes, bijnieren, hypofyse, vetweefsel, hersenen, lever en nieren van Bcrp\(^{-/-}\)-muizen was niet verschillend in vergelijking met controlemuizen. Ook toediening van elacridar had geen invloed op de BPA-accumulatie. Samenvattend tonen onze resultaten aan dat de verdeling van het humane BCRP-substraat BPA niet is veranderd in Bcrp\(^{-/-}\)-muizen. Dit suggereert dat Bcrp geen of een geringe rol speelt bij BPA-blootstelling van muizen. Mogelijk is BPA geen substraat voor het muizen-Bcrp. Hoewel dit interspeciesverschil verder onderzocht moet worden, is het belangrijk om te realiseren dat de verdeling en het mechanisme van toxiciteit van bepaalde stoffen anders zijn in dieren dan in mensen.

**De rol van BCRP in metabole processen**

De endogene BCRP-substraten heem, kynureninezuur en urinezuur kunnen bij hoge concentraties fysiologische processen verstoren. Heem is de functionele groep van hemoproteïnen en is van cruciaal belang voor vele cellulaire processen, zoals zuurstoftransport en -opslag, elektronenoverdracht en signaaltransductie. Echter, grote hoeveelheden vrij heem kunnen schadelijk zijn via inductie van oxidatieve stress en ontstekingsmediatoren. Kynureninezuur is een antagonist van de N-methyl-D-aspartaatreceptor en α7-nicotinéacetylcholinereceptor en verhoogde kynureninezuurwaarden zijn gerelateerd aan verschillende neurologische aandoeningen. Daarnaast is verhoogde urinezuurspiegels in het bloed (hyperuricemie) gecorreleerd met het optreden van jicht, nierstenen, cardiovasculaire ziekten en chronische nierziekten.

In hoofdstuk 5 stelden we dat een overexpressie van heemoxygenase-1 (HO-1: het enzym dat heem afbreekt) en/of BCRP bescherming zou bieden tegen heemgedemiederde cytotoxiciteit. Om deze hypothese te testen hebben we HEK293-cellen met een overexpressie van één of beide eiwitten blootgesteld aan heem. Dit resulteerde in een concentratieafhankelijke
verhoging van de vorming van reactieve zuurstofdeeltjes en de daarmee gepaard gaande DNA-schade en celdood. Heemgemedieerde celdood was significant verminderd in cellen met overexpressie van één of beide eiwitten. Hiermee werd aangetoond dat beide eiwitten bescherming bieden tegen de schadelijke effecten van vrij heem.

Er is weinig bekend over het transport van metabolieten vanuit de hersenen richting het hersenvocht (cerebrospinal fluid: CSF). We ontdekten dat Bcrp gelokaliseerd is in de cellaag die zich bevindt in de hersen-CSF-interface van het brein van de muis, zoals beschreven in hoofdstuk 6. Vervolgens onderzochten we de mogelijke rol van BCRP in de regulatie van de CSF-samenstelling. Hiervoor vergeleken we de samenstelling van het CSF van Bcrp<sup>-/-</sup>-muizen met die van controlemuizen met behulp van <sup>1</sup>H-NMR-analyse. De concentraties van glucose, glycine, creatine, alanine en glutamine waren veranderd in het CSF van Bcrp<sup>-/-</sup>-muizen. Het verschil in glucosewaarden was het grootst waarbij de glucoseconcentratie in het CSF van Bcrp<sup>-/-</sup>-muizen was verlaagd ten opzichte van de controlemuizen. Echter, omdat we vonden dat glucose noch een van de andere metabolieten de activiteit van BCRP beïnvloedde, is het waarschijnlijk dat de afwezigheid van Bcrp een indirect effect op het energiemetabolisme van muizen hersenen had. Kynureninezuur was al eerder in verband gebracht met verstoringen in het energiemetabolisme en diverse neurologische aandoeningen. Door middel van de eerdergenoemde membraanvesikelopnamesstudie konden we kynureninezuur identificeren als een nieuw BCRP-substraat en verhoogde waarden van deze stof werden gevonden in plasma en hersenhomogenaten van Bcrp<sup>-/-</sup>-muizen. Bovendien zorgde blootstelling van maligne neuroblastoma (N2a)-cellen aan kynureninezuur voor een concentratieafhankelijke verhoging van de glucoseopname door de cellen. We concluderen hieruit dat de verhoogde intracellulaire kynureninezuurconcentraties, door verminderde kynureninezuuruitscheiding, een verhoogde glucoseopname in de hersenen tot gevolg kunnen hebben, waardoor het CSF minder glucose bevat.

Kynureninezuur werd ook bestudeerd in hyperuricemische muizen. Hyperuricemie wordt gerelateerd aan een verscheidenheid van ziekten, echter, de pathofysiologische mechanismen die ten grondslag liggen aan de ontwikkeling van die ziekten zijn nog niet volledig opgehelderd. In hoofdstuk 7 onderzochten we het effect van hyperuricemie op tryptofaanmetabolisme en de mogelijke rol van twee belangrijke urinezuurtransporters MRP4 en BCRP hierbij. Urinezuur remt zowel de activiteit van MRP4 als van BCRP, zoals bleek uit membraanvesikelexperimenten. Daarnaast werd gevonden dat naast BCRP ook MRP4 kynureninezuur transporteert. Deze bevinding werd bevestigd door verhoogde plasmaspiegels van kynureninezuur in Mrp4<sup>-/-</sup>-en Bcrp<sup>-/-</sup>-muizen in vergelijking met controle dieren. Hyperuricemie ging gepaard met verhoogde plasmakynureninezuurconcentraties in controlemuizen, maar deze stegen niet
verder in Mrp4<sup>−/−</sup>- of Bcrp<sup>−/−</sup>-muizen. Op basis van deze resultaten stellen wij dat verhoogde urinezuurwaarden kunnen leiden tot een verminderde functie van MRP4 en BCRP, waardoor potentieel toxische substraten, zoals kynureninezuur, kunnen ophopen en zo bijdragen aan de ontwikkeling van ziekten, waaronder chronische nierziekte.

**Conclusies en toekomstperspectieven**

In dit proefschrift werd de rol van de effluxtransporter BCRP in endocriene en metabole processen onderzocht. Onze resultaten laten een duidelijke remming zien van BCRP functie door steroidhormonen, echter, BCRP is niet betrokken bij het transport van deze hormonen. Op basis van onze resultaten stellen wij dat een verminderde BCRP-functie, hetzij door remming hetzij door de aanwezigheid van een inactiverend BCRP-polymorfisme, kan resulteren in ophoping van potentieel toxische BCRP-substraten, zoals hormoonverstorende stoffen, heem en kynureninezuur. Dit kan een verstorende invloed hebben op een groot aantal cellulare processen die gereguleerd worden door BCRP. Daarom zou het transporteiwit een interessant aangrijpingspunt kunnen zijn in de ontwikkeling van nieuwe therapieën, zoals BCRP-inductie om ophoping van BCRP-substraten te voorkomen. Echter, er is meer onderzoek nodig om de haalbaarheid van het beïnvloeden van de BCRP-functie bij patiënten te bestuderen.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1H-NMR</td>
<td>1H-nuclear magnetic resonance</td>
</tr>
<tr>
<td>5αRed1</td>
<td>5α-reductase type 1</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>8-Bromoadenosine 3’,5’-cyclic monophosphate</td>
</tr>
<tr>
<td>AB</td>
<td>arachnoid barrier</td>
</tr>
<tr>
<td>ABC transporter</td>
<td>ATP binding cassette transporter</td>
</tr>
<tr>
<td>ABC</td>
<td>avidin-biotin complex</td>
</tr>
<tr>
<td>ABCB1/P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>ABCC/MRP</td>
<td>multidrug resistance-associated protein family</td>
</tr>
<tr>
<td>ABCC7/CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>ABCG2/BCRP</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BPA</td>
<td>bisphenol A</td>
</tr>
<tr>
<td>BPA-G</td>
<td>BPA-glucuronide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
</tr>
<tr>
<td>BTB</td>
<td>blood-testis barrier</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CMV</td>
<td>cross model validation</td>
</tr>
<tr>
<td>COW</td>
<td>correlation optimizing warping</td>
</tr>
<tr>
<td>CP</td>
<td>choroid plexus</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>Ct value</td>
<td>cycle threshold value</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450 enzyme</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DEHP</td>
<td>bis(2-ethylhexyl) phthalate</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco Eagle’s modified medium</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E₁S</td>
<td>estrone sulfate</td>
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<td>E₂17βG</td>
<td>estradiol glucuronide</td>
</tr>
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<td>EDC</td>
<td>endocrine disrupting chemical</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDX</td>
<td>energy-dispersive X-ray</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor-α</td>
</tr>
<tr>
<td>eYFP</td>
<td>enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FePP</td>
<td>heme</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>FTC</td>
<td>fumitremorgin C (BCRP inhibitor)</td>
</tr>
<tr>
<td>FTC</td>
<td>Friend leukemia virus B mouse strain</td>
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<tr>
<td>Gapdh</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GF120918</td>
<td>elacridar (mixed P-gp/BCRP inhibitor)</td>
</tr>
<tr>
<td>GLUT1</td>
<td>glucose transporter-1</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>H₂DCFDA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HE stain</td>
<td>hematoxylin and eosin stain</td>
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<tr>
<td>HEK293</td>
<td>human embryonic kidney cell line 293</td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
</tr>
<tr>
<td>HPG axis</td>
<td>hypothalamic–pituitary–gonadal axis</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>HSD</td>
<td>hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2 3-dioxygenase</td>
</tr>
<tr>
<td>ISF</td>
<td>brain interstitial fluid</td>
</tr>
<tr>
<td>KATII</td>
<td>kynurenine aminotransferase-II</td>
</tr>
<tr>
<td>Ki</td>
<td>equilibrium constant for inhibitory affinity</td>
</tr>
<tr>
<td>Kim-1</td>
<td>kidney injury molecule-1</td>
</tr>
<tr>
<td>Kiss1</td>
<td>kisspeptin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Km</td>
<td>Michaelis constant: the substrate concentration at which an enzyme-catalyzed reaction proceeds at 50% of its maximum velocity</td>
</tr>
<tr>
<td>KynA</td>
<td>kynurenic acid</td>
</tr>
<tr>
<td>LC</td>
<td>Leydig cell</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>HPLC combined with tandem mass spectrometry</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>Lhr</td>
<td>LH receptor</td>
</tr>
<tr>
<td>MA-10</td>
<td>murine Leydig cell line</td>
</tr>
<tr>
<td>MDCKII</td>
<td>Madine Darby canine kidney cell line II</td>
</tr>
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<td>MEHP</td>
<td>mono(2-ethylhexyl) phthalate</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<td>MTX</td>
<td>methotrexate</td>
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<td>N2a</td>
<td>malignant neuroblastoma cell line</td>
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<td>NAC</td>
<td>N-acetylcysteine</td>
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<td>NBD</td>
<td>nucleotide binding domain</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>Ngal</td>
<td>neutrophil gelatinase-associated lipocalin</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-d-aspartate-receptor</td>
</tr>
<tr>
<td>NMQ</td>
<td>N-methylquinidine</td>
</tr>
<tr>
<td>OAT</td>
<td>organic anion transporter</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PCs</td>
<td>principal components</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PFOA</td>
<td>perfluoroctanoic acid</td>
</tr>
<tr>
<td>PFOS</td>
<td>perfluorooctanesulfonic acid</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>QSAR</td>
<td>quantitative structure-activity-relationship</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCO</td>
<td>subcommissural organ</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
Chapter 9

SEM  standard error of the mean
SLC  solute carrier family
SnMP tin mesoporphyrin
SNP  single nucleotide polymorphism
StAR steroidogenic acute regulatory protein
TBBPA tetrabromobisphenol A
TMD  transmembrane domain
TRITC tetramethyl rhodamine iso-thiocyanate
TS buffer Tris-sucrose buffer
TSP-d4 sodium-3-(trimethylsilyl)propionate-2,2,3,3-d4
UPLC ultra performance liquid chromatography
URAT1 urate transporter 1
UV ultraviolet
Vmax maximum velocity of an enzymatic reaction
α7nAchR α7-nicotinic-acetylcholine-receptor

Note: of rodent genes and proteins, only the first character is capitalized
Curriculum vitae

List of publications


