2010-01-01

Effects Of The Loss Of Multidrug Resistance Associated Protein 1 On Steroid Homeostasis, Dendritic Cell Function And Compensatory Mechanisms

Jeffrey Charles Sivils
University of Texas at El Paso, jcsivils@yahoo.com

EFFECTS OF THE LOSS OF MULTIDRUG RESISTANCE ASSOCIATED PROTEIN 1 ON STEROID HOMEOSTASIS, DENDRITIC CELL FUNCTION AND COMPENSATORY MECHANISMS

JEFFREY CHARLES SIVILS

DEPARTMENT OF BIOLOGICAL SCIENCES

APPROVED

Kristine Garza , Ph.D., Chair

Lisa Bain, Ph.D.

J.R. Bristol, PhD

R.T. Miller, Ph.D.

Patricia D. Witherspoon, Ph.D.
Dean of the Graduate School

EFFECTS OF THE LOSS OF MULTI-DRUG RESISTANCE ASSOCIATED PROTEIN 1ON STEROID HOMEOSTASIS, DENDRITIC CELL FUNCTION AND COMPENSATORY MECHANISMS

by

JEFFREY CHARLES SIVILS,B.S.

DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

May 2010

Acknowledgements

I would like to thank my family and friends, especially my parents and Kelly, for the continued encouragement and support, which made this possible. I would also like to thank my committee members, Dr. Tina Garza, Dr. Lisa Bain, Dr. Jack Bristol, and Dr. Tim Miller, for all of their guidance and support. I would like to thank Dr. Lisa Bain for allowing me to start in her laboratory and for her guidance and seeing me to the end. I am also deeply grateful to Dr. Tina Garza for allowing me to join her laboratory and for her guidance and mentoring. I would not have finished without her continuing support and friendship. I would also like to thank Dr. Kyle Johnson for always having my back and Dr. Marc Cox who has been a wealth of information. I would also like to thank Dr. Bristol and Dr. Mayberry, and especially Dr. Larry Jones, they never gave up on me as an undergraduate, and I would not have made it here without them.

Abstract

The ATP-binding cassette (ABC) superfamily of membrane transporters use energy derived from ATP to eliminate a variety of exogenous and endogenous compounds from cells including anti cancer and anti viral drugs, metals steroids, bilirubin, cAMP, cGMP, leukotrienes , prostaglandins. The multi drug resistance associated protein family (MRP/ABBC) has been acknowledged as a major player involved in multi drug resistance (MDR), in which cancers stop responding a wide array of structurally and functionally unrelated chemotherapy drugs. Cancer is the second leading cause of death in adults in the United States. Although there have been great strides made the treatment of cancers over the last 50 years, there are still 1.5 million new cases and 0.5 million deaths a year in the US. The death rates for several cancers including cervical cancer and stomach cancer in both men and women are a third of what they were 40 years ago. However, for melanoma, esophageal, pancreatic and liver cancer death rates have actually increased. There are various ongoing lines of research attempting to develop new therapies and strategies to increases the efficacy of chemotherapy. One approach which has met with varying degrees of success has been the manipulation of drug transporters involved in MDR. Inhibiting the MRPs and there by reversing the MDR phenotype would allow the use of existing chemotherapy drugs and treatment regimes at lower doses , thereby decreasing the magnitude of the side effects experienced patient undergoing current treatment regimes. However the endogenous function of MRP1 has not been well characterized, therefore inhibiting MRP1 may have unforeseen consequences. We there for decided to look at an Mrp1 knock out mouse to determine any potential compensatory changes that might result from the loss of Mrp1. Work has previously been done examining changes in the liver of the Mrp1-/- mouse, we therefore looked at extra hepatic tissues. We discovered increased renal cytochrome P405 activity in the Mrp1 -/- mice. Phase I P450 activity was altered in the testes as well, with a reduction of Cyp2c11 activity and an induction of Cyp17 activity. In addition to alterations in phase I activity, Mrp1-/- mice had increased expression of the phase II conjugation enzyme sulfotransferase 1a1 in the small intestine, and a reduced expression in the lungs. Interestingly, we fund that Mrp2 and Mrp4 expression was reduced in the

lungs and Mrp2 was reduced in the kidneys of the Mrp1-/- mice. We also sought to determine any modifications that may have resulted from the loss of Mrp1 in the testes. As Mrp1 is highly expressed in the testes and is thought to be involved in maintaining steroid homeostasis by expelling excess estrogen from the testes, we examined both testicular and serum steroid levels. We discovered that the loss of Mrp1 leads to decreased steroid levels in both the testes and serum. There is a significant reduction of androstenedione, estradiol, testosterone, and dehydroepiandrosterone (DHEA) in the testes of Mrp1 knockout mice. In order to resolve the mechanisms involved in the reduction of serum steroid levels we examined testicular steroid biosynthesi pathways. We found no alterations in sulfotransferase or glucuronosyltransferase enzyme activity. So it appears that in order to circumvent the buildup of excess estrogen in the testes, steroid production is attenuated, resulting in lower androsteindione, testosterone, and estradiol in the testes. The decrease in circulating testosterone is in all likelihood responsible for the alterations seen in the kidney, ling and small intestines.

We also examined Mrp1-/- mice for alterations in drug disposition using tritiated vincristine for our test compound. The loss of Mrp1and the resulting alteration in Mrp expression leads to significant build up of vincristine in the kidney, small intestine, colon, and epididymis. This buildup of vincristine is almost certainly an attempt to eliminate the vincristine and results from the variation in transporter expression along the GI track and the kidney.

Mrp1 is also expressed in dendritic cells(DC) and considering Mrp1 and Mrp4 have been implicated in DC function and a required for proper DC trafficking we decided to determine if either of the transporters are required for efficient DC maturation and activation. By using the Mrp1-/- mouse model, and a specific Mrp4 inhibitor, we determined potential role Mrp1 and Mrp4 play DC function. The loss of either Mrp1 and/or Mrp4 hindered DC ability to produce IL12 when stimulated by LPS. If either of the transporters are lost during maturation, upon activation by LPS, the DCs have reduced induction of activation markers CD54, CD 80, CD86 and MHCII, as well as reduced IL12 production. As the ultimate

test of DC function is activation of T cells, after inhibition of transporter activity during maturation, we examined how effectively the DCs could activate T cells. Without functioning transporters, the ability DCs to effectively activate T cells is severely crippled.

Table of Contents

List of Tables

List of Figures

Chapter One: Introduction

1.1 Cancer

The last 50 years have seen great advances in the detection and treatment of malignant neoplasms and viral diseases. The development of chemotherapeutic treatments for cancer and anti-viral drugs for viral infections have led to the increased quality of life for many patients and complete relief of disease in others. However, cure rates for cancer are not 100%, and in patients with advanced metastatic neoplasms, the long term outlook is far less encouraging, with five year survival rates less than 5% in patients with advanced pancreatic cancer Other neoplasms have a somewhat better prognosis (Table 1) (Jamel et al, 2008). As many as 50% of lung cancers either do not respond or quit responding to chemotherapy drugs. Approximately 90% of primary breast cancers initially respond to treatment, however 30% of patients have refractory disease (Gonzalez-Angulo AM et al, 2007). In up to 90% of patients with metastatic diseases, treatment failure is due to drug resistance (Longley DB & Johnston PG, 2005).

Table 1 – Five year cancer survival rates.

Cancer	% Survival
Breast	88
Colon	64
Lung	15
Melanoma	91
Ovary	45
Prostate	98
Stomach	24
Pancreas	05
Bladder	80
Cervix	72

*Adapted from Cancer Statistics, 2008

Early diagnosis and improved treatment options have greatly increased survival, however mortality from cancer is still ultimately do to treatment failure Treatment failure has become an increasingly significant problem and has rendered many of the current chemotherapy regimens ineffective. There are two potential reasons resulting in a poor response to chemotherapy: 1) inability to get the drugs to cells at adequately high concentrations and 2) cancers cells becoming resistant to the chemotherapy drugs via epigenetic and genetic mechanisms leading to alterations in pharmacokinetics and increased drug efflux. In many cases, tumor cells simultaneously develop drug resistance to several groups of structurally and functionally diverse chemotherapy drugs. The drugs lose their cytotoxic activity, a phenomenon termed multidrug resistance (MDR). The two forms of MDR are intrinsic resistance, in which cells are resistant at the beginning of treatment, and acquired resistance, in the patients experience relapse. Failure of chemotherapy and antiviral therapy and the onset of MDR may be due to the increased expression of drug exporters and their ability to efflux therapeutic compounds.

In the later part of the last century, several cancer cell lines were shown to over-express certain proteins involved in the efflux of anticancer medication and were attributed to MDR. The P-glycoprotein (P-gp), a 170 KDa membrane glycoprotein, was the first ABC transporter discovered. P-gp conferred resistance to doxorubicin in cultured cells (Juliano and Ling, 1976, Riordan JR et al, 1985).

Later, two doxorubincin resistant cell lines, HL60/ADR and H69AR, were examined. It was determined that P-gp was not over-expressed in the two cell lines, although both demonstrated an MDR phenotype, but rather a possible new class of transporter (McGrath et al

1987, McGrath et al, 1988, Mirski et al, 1987). A 190kDa membrane protein was discovered and subsequently in 1992, Cole et al. cloned the new protein, the source of MDR in the lung cancer cell line and thus, multidrug resistance-associated protein 1 (MRP1) was discovered. MRP1 belongs to the superfamily of ATP-binding cassette (ABC) transporters, which contains 12 members; 9 multi-drug resistances associated related proteins (MRPs1-9), cystic fibrosis transmembrane conductance regulator (CFTR), and 2 sulfonylurea receptors (SUR1/2), with functions including ion transport, cell surface receptors, and extrusion of endogenous and exogenous compounds from cells. To date 7 rat and 8 mouse MRP orthologs have been characterized. There are 48 ABC transporters in humans, divided into 7 subfamilies: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG (reviewed in Haimeur et al 2004; Deeley et al 2006)

1.2 ATP-binding cassette transporters

The ABCC transporter family is divided into two groups depending on the number of hydrophobic membrane spanning domains the transporter contains. The short transporters, MRP4,5,8,and 9 each contain two membrane spanning domains, with 12 transmembrane helices, while MRP1-3,6 and 7 have three membrane spanning domains(MSD) containing 17 transmembrane helices (Figure 1). In addition to the MSD, all members of the ABCC family contain two nucleotide binding domains (NBDs) with both a Walker A and Walker B motif and the signature C motif, LSGGQ, involved in ATP binding and hydrolysis (Walker et al, 1982, Higgens et al, 1986,Ren et al, 2004). Energy derived from the hydrolysis of ATP is used to actively transport substrates across the cell membrane. Transport is thought to be carried out in

stages: 1) substrate binding which causes a conformational change in the protein and increases affinity of NBD1 for ATP, 2) ATP binds NBD1 and again cause a change in protein confirmation, 3) A second ATP binds NBD2 , an additional conformational change, lower affinity for the substrate, it is released; 4) the ADP fall of and the protein returns to its original confirmation (Payen et al, 2005, Yang et al, 2004, Hou et al, 2003) (See figure 2).

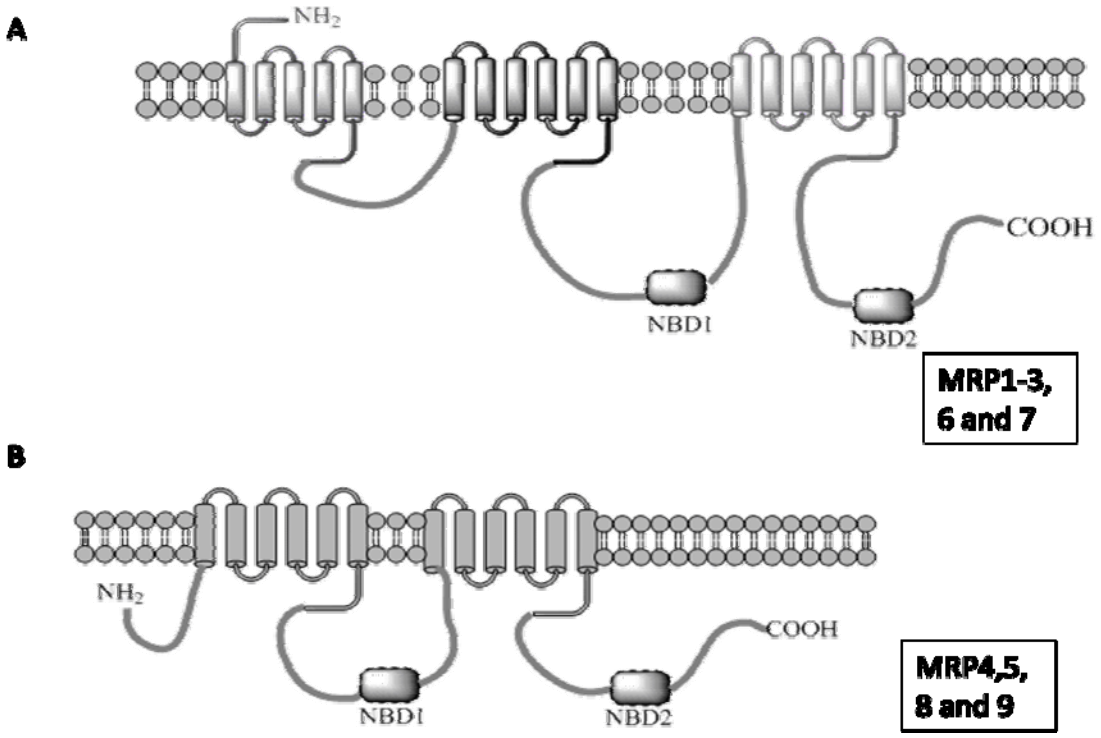


Figure 3 . Topology of MRP proteins.

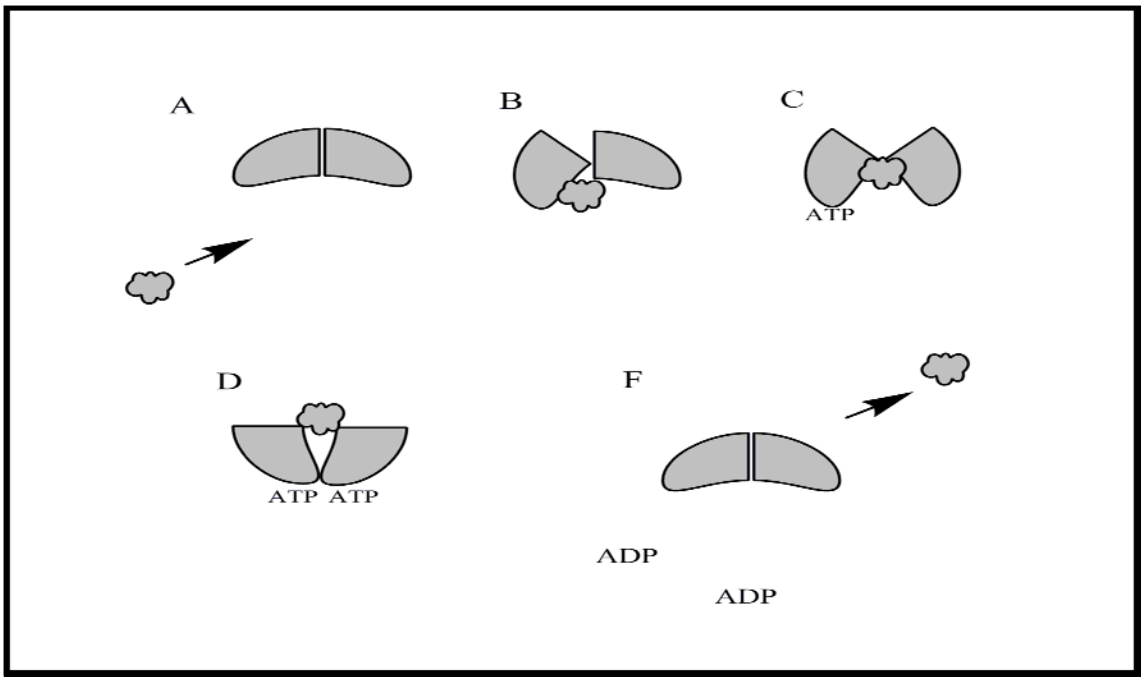


Figure 2. ATP mediated transport

A) No substrate bound to MRP. B) Substrate binds transporter and first conformational change C) Increased ATP affinity and ATP binds NBD1, conformational change. D) Second ATP binds NBD2, conformational change decreased substrate affinity, and transport. F) ADPs falls off and the transporter returns to resting confirmation (adapted from Borst P, et al, 2005).

MRP1/ABCC1 belongs to the ABCC subfamily of the ABC transporters. MRP1 can transport glucuronide, glutathione, sulfate conjugates, organic ions and nucleotide analogues. Physiological substrates include glutathione, GSSG, the proinflammatory agent leukotriene (C_4, D_4,E_4), S-glutathionyl prostaglandin, esterone 3-sulfate, dehydroepiandrosterone 3-sulphate (DHEAS),17β-glucuronosyl estradiol, bilirubin and bile acids (Gottesman et al, 2002, Leslie et al, 2001), chemotherapy drugs including camptothecin, colchicines, doxorubicin, daunorubicin, etoposie, methotrexate, taxol, and vincristine,(reviewed in Gottesman et al., 2002, Schinkel & Jonker, 2003, Haimeur et al, 2004, Deeley et al, 2005, Choudhuri & Klaassen, 2006, Ballator et

al, 2008, Toyoda, et al, 2008, Zhou et al, 2008, Dean, 2009), antibiotics and anti-virals such as ciprofloxacin, grepafloxacin, Saquinavir and ritonavir (Tami et al, 2000, Rodríguez-Ibáñez et al, 2003, Srinivas et al,1998) heavy metals such as arsenic and antimony (Chen et al, 1997), and toxins including aflatoxin B and the vinca alkaloids (Loe et al., 1996; Leslie et al, 2001) across cell membranes. MRP1 also transports a variety of anti HIV medications, including saquinavir and zidovudine, and this is one mechanism by which HIV treatments become ineffective (Srinivas et al, 1998, Jorajuria et al, 2004, Dallas et al, 2004, Eilers et al, 2008).

MRP1 is ubiquitously expressed at some level in most tissues (Cole et al 1996; Flens et al 1996). In mice, it is highly expressed in the lung, heart, kidney, testes and to a lesser extent, in the intestine and liver (Maher et al, 2005). In humans, there is high expression in the esophagus, intestine, lung, pancreas, heart, skin, peripheral blood monocytes, testes, blood-brain barrier, blood-choroid plexus barrier, and very low levels in the liver (Peng et al., 1999; Flens et al, 1996, Soontornmala1 et al, 2006). Within the cells, MRP1 is located in the cytoplasm or the basolateral membranes of polarized cells (Peng et al., 1999).

Although the true physiological role for MRP1 is not known, it appears to be essential for proper immune function, dendritic cell migration and maturation and is a T cell activation marker (Elliott et al, 2004, Lohoff et al 1998, van de Ven R et al, 2006, Randolph et al, 2001, Robbiani et al 2000). Another possible physiological role is in steroid homeostasis, as MRP1 expression is highest in tissues that respond to or are involved in synthesis of steroids, including the testes, ovaries and adrenal glands (Mayer et al, 2005, reviewed Klaassen, 2009).

Also included in the ABCC subfamily is MRP2/ABCC2. This protein , originally termed the canalicular multispecific organic anion transporter (cMOAT), was discovered in 1996 in Eisai hyperbilirubinemic rats (EHBR) (Elferink et al 1995, Paulusma et al, 1996, Taniguchi et al, 1996), although the phenotype of idiopathic jaundice had been known in humans since 1954 and in rats since 1985 (Dubin, 1954, Jenson et al, 1985). MRP2 transports similar substrates as MRP1, including glucuronide, sulfate, and glutathione organic ion conjugates (Jedlitschky et al, 1997, Evers et al, 1998,Cui et al, 1999, Kamisako et al, 1999) . MRP2 also transports mono- and bisglucuronosylbilirubin, bile salts as well as xenobiotic conjugates into bile for hepatobiliary excretion (Trauner et al, 2003). Other endogenous substrates include leukotriene C_4, 17β-glucuronosyl estradiol, and esterone 3-sulfate (Cui et al, 1999). One of MRP2's main functions is to excrete bilirubin into the bile. Humans with a mutation in the MRP2 gene, leading to a non functional transporter, have an inheritable disorder called Dubin-Johnson syndrome characterized by a mild form of hyperbilirubinemea similar to the EHBR rats (Kartenbeck et al, 1996, Ronald et al, 1995). Additionally, MRP2 removes a variety of chemotherapy agents, including anthracyclines, vinca alkaloids, cisplatin, etoposide, methotrexate, ,camptothecins, and doxorubicin (Hooijberg et al 1999; Kawabe et al, 1999; Cui et al, 1999; Maher et al, 2005). MRP2 is found mainly in the liver, kidney, and small intestine, but is also found in the colon, gallbladder, and placenta. Unlike MRP1, MRP2 is located on the apical, not the basolateral side of polarized cells where it extrudes compounds into luminal spaces (Paulusma at al, 1996).

MRP3/ABCC3, the third member, is found mainly in the liver, small intestine, colon, adreanal, and to a lesser extent, the pancreas, kidney, lung, brain, spleen, and prostate (Kool et al

1997; Belinsky et al, 1998; Scheffer et al, 2002). MRP3 is located on the basolateral side of the cells and has substrate specificity like that of MRP1 and MRP2, including glucuronide and sulfate conjugates, although it is a poor transporter of glutathione conjugates (Konig et al, 1999; Hirohashi et al 1999). Endogenous substrates include leukotriene C_4, 17β-glucuronosyl estradial, folate, bilirubin, and DHEAS (Lee et al, 2004, Akita et al, 2002). Unlike both MRP1 and 2, MRP3 transports taurocholate, glycocholate, and cholate in addition to mono- and bisglucuronosylbilirubin bile salts (Zang et al, 2003, Hirohashi et al, 2000, Akita et al, 2002) Anticancer drugs exported by MRP3 include methorexate, etoposide, and teniposide, however unlike MRP1 and MRP2, it is a poor transporter of the vinca alkaloids and anthracyclines (Kool et al, 1999; Zelcer et al, 2001). MRP3 also transports APAP glucuronide (Zamek-Gliszczynski et al, 2006). In the MRP2 deficient EHBR rat, there is an induction of hepatic MRP3 and higher blood levels of bilirubin presumably to compensate for the loss of MRP2 (Hirohashi et al, 1998).

\

MRP4/ABCC4, described in 1997 as the first of the short transporters, is ubiquitously expressed in most tissues, particularly in the prostate, testis, ovary, intestine, lymphocytes, pancreas and lung, and is located in either the apical or basolateral side of cells depending on the tissue (Lee et al, 1998, Lee et al, 2000). First discovered in a T cell leukemia resistant to 9-(2-phosphonyl methoxy) adenine (PMEA) and azidothymidine monophosphate (AZT), MRP4 can also confer resistance to antiviral, nucleoside and nucleotide analogues including the anti viral ganciclovir (Schuetz et al, 1999; Adachi et al ,2002; Chen et al, 2002; Reid et al, 2002). MRP4 is also an important transporter of cyclic AMP and GMP (Chen et al, 2001, Lee et al 2000). Many important antiviral and anticancer drugs are nucleoside analogues, making this and other nucleotide transporters of particular importance in humans. Additional physiological substrates

include 17β-glucuronosyl estradiol, DHEAS, prostaglandins, thromboxane folate, and bile salts (Chen et al, 2002, Zelcer et al, 2003, Rius et al, 2005).

MRP5/ABCC5 is expressed most in tissues, however at lower levels. Like MRP4, MRP5 transports nucleoside analogues as well as cyclic nucleotides (Reid et al, 2002 Jedlitschky et al, 2000). MRP5 also transports cAMP and cGMP, however has a 9-fold lower affinity for cAMP than MRP4, but a 5-fold higher one for cGMP (Jedlitschky et al, 2000). Drug substrates include PMEA, methotrexate, 5-fluorouracil, cadmium chloride, and potassium antimony (Wijinholds et al, 2000, Rius et al 2003, McAleer et al, 1999, Pratt et al, 2005).

MRP6/ABCC6, found mainly in the liver and kidney, has low levels in various other tissues, where it localizes to the basolateral membrane (Beck et al, 2005, Scheffer et al, 2008). MRP6 transports aromatic cyclic compounds (Gottesmen et al., 2002), but does not transport glutathione, glucuronide conjugates or cyclic nucleotides (Belinsky et al, 2002). It does however transport an endothelin receptor antagonist, BQ123, a cyclopentapeptide, and leukotriene C_4 (Madon et al, 2000) MRP6 does confer low level resistance to etoposide, doxorubicin, and cisplatin, but not to methotrexate or vinca alkaloids (Belinsky et al, 2002). MRP6 is of particular interest in humans, as inherited mutations of the MRP6 gene lead to a degenerative connective tissue disorder Pseudoxanthoma Elasticum (PXE), in which patients get calcification of the elastic fibers and irregular collagen fibers of the arteries, skin, and retina leading to cardiovascular difficulties, skin lessons, and blindness (Bergen et al, 2000, Mendelsohn et al,1978, Yap et al, 1992, Lebwohl et al, 1993) The exact mechanism and exactly how MRP6 is involved is unknown.

MRP7/ABCC10 is present in many tissues, but at generally very low levels. Its highest levels are in pancreas, liver, placenta, lung, kidney, brain, ovary, lymph nodes, spleen, white blood cells, and colon (Hooper et al, 2001; Koa et al, 2002; Allikmets R, et al, 1996). MRP7 to date has a much smaller range of physiological substrates as only two of the normal array of physiological substrates are transported by MRP7, 17β-glucuronosyl estradiol and leukotriene C_4 (Chen et al, 2003). The drug resistance profile, however is rather broad, and includes docetaxel, paclitaxel, vincristine, vinblastine, etoposide, SN-38, and the nucleoside cancer drugs cytarabine and gemcitabine (Hopper-Borge et al, 2004, Hopper-Borge et al, 2009). MRP7 also confers resistance to the antivirals 2,3-dideoxycytidne and PMEA (Hopper-Borge et al, 2009). There are a few caveats with MRP7: it is the only MRP able to transport the taxanes (Kruh et al, 2007), the structure of MRP7 is like that of MRP1, 2, and 6, however its amino acid sequence is more closely related to CFTR (Kruh et al, 2007), and HAL-E binds a peptide derived from a TMD from MRP7 and inhibits cell lysis by natural killer cells (Wooden et al, 2005)

MRP8/ABCC11 is widely expressed, although at low levels, with the highest expression found in liver, brain, and placenta (Cole et al, 1992, Gou et al, 2003). Like MRP4 and 5, MRP8 has only two MSDs (Bera et al, 2002). In MDCKII and in human HepG2 cells MRP8 localizes, like MRP2, to the apical side, however in the CNS, MRP8 is the only MRP found in the axonal processes of nerve cells (Bortfeld et al, 2005). MRP8's physiological substrates include several steroid conjugates, such as DHEAS, esterone–3 sulfate, 17β-glucuronosyl estradiol, leukotriene C_4, folate, the bile salts cholyglycine and cholyglycine, cAMP, and cGMP (Chen et al, 2005). MRP8 induces resistance to 5-fluorouracil, 5-fluoro-2'-deoxyuridine, 5-fluoro-5'-deoxuridne,

2',3'- dideoxycytidine and PMEA (Guo et al, 2003). MRP8 has a splice variant which lacks the second ATP binding domain (Yabuuchi et al, 2001). Additionally, there is no murine orthologue for MRP8 (Shimizu et al, 2003).

MRP9/ABCC12 has been far less characterized than the other transporters, with even the tissue distribution being debated. Transporter cellular localization and substrate profiles have yet to be determined. MRP9 is over-expressed in breast cancer cells and expressed in various other cancers including lung, prostate colon, prostate, ovarian, and pancreatic (Bera et al, 2002; Tammur et al, 2001, Yabuuchi et al, 2001). There have been 4 additional splice variants detected for MRP9 termed A, B, C, and D (Yabuuchi et al, 2001). Variants A, B and D lack the second ATP binding domain (Yabuuchi et al, 2001). MRP9 is located on chromosome 16q12.1 both(Tammur J, et al, . 2001). Due to its location on chromosome 16q12.1, it has been suggested that a mutation in MRP9 may be involved in inherited paroxysmal kinesigenic choreoathetosis disease (Yabuuchi et al, 2001)

Table 2. Names, tissue localization, and sequence homology with ABCC1

Gene	Name	Tissue distribution	Amino Acid Sequence Identity
ABCC1	MRP1	Ubiquitous (low in liver)	100%
ABCC2	MRP2	Liver, kidney, intestine, brain	50%
ABCC3	MRP3	Liver, kidney, adrenals, pancreas, gut, placenta, &prostate	58%
ABCC4	MRP4	Ubiquitous (↑ prostate ↓ liver)	41%
ABCC5	MRP5	Ubiquitous, low levels	38%
ABCC6	MRP6	Liver, kidney	46%
ABCC7	CFTR		30%
ABCC8	SUR1		36%
ABCC9	SUR 2A/2B		35/36%
ABCC10	MRP7	Ubiquitous low levels	35%
ABCC11	MRP8	Ubiquitous low levels	33%
ABCC12	MRP9	Ubiquitous low levels, testes	36%

Detoxification and elimination of lipophilic compounds from cells is accomplished in three phases. In general, phase I enzymes add a hydroxyl group to a compound, making it slightly more water soluble and add a conjugation site for phase II enzymes.

Phase II enzymes add organic anions such as glucuronide or sulfate, which greatly increases its water solubility and also target the compound for active transport out of the cell by one of the MRP transporters (Figure 4). For example, acetaminophen (APAP), a popular

extensively used over the counter analgesic and antipyretic is initially metabolized in the liver by several isoforms of phase I cytochrome P450's (Mitchell et al., 1973; Potter et al., 1973). Next, phase II enzymes either glucuronidate or sulfate APAP, which is then extruded from the cells by either MRP3 or MRP4 (Zamek-Gliszczynski et al, 2006)

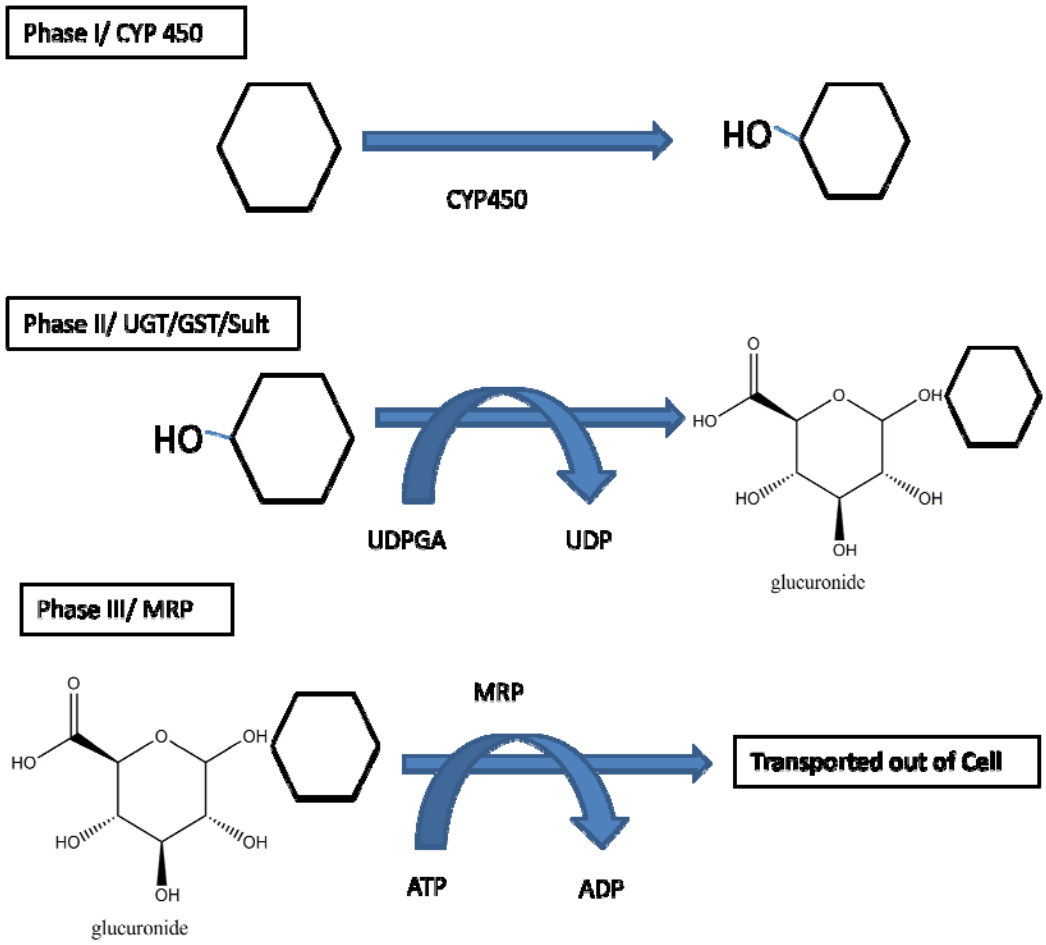


Figure 4 . Phases of xenobiotic metabolism and excretion

1.3 Phase 1 Enzymes

Phase I metabolism is exemplified by the cytochrome P450 (CYP) group of heme containing enzymes, which is one of the largest in terms of the total number of members of the superfamily, with 57 human isoforms, the sheer number of substrates, and their catalytic ability. CYP enzymes are involved in monooxygenation and hydroxylation of a variety of xenobiotic and endogenous substrates including, but not limited to, exogenous compounds, pharmaceuticals, fatty acids, lipids, and endogenous hormones. Biotransformation generally makes compounds more polar, but more importantly, it adds a good leaving group (OH) for further metabolism and conjugation by the phase II enzymes. Usually this leads to detoxification of the compound. However, in some cases, the compound becomes more toxic by means of bioactivation. Of the 15 P450's found to be involved in the metabolism of xenobiotics >75% of drugs are metabolized by members of 3 families; CYP 2D6, 2C9, and 3A4 (Guengerich, 2005, Yoshitomi et al, 2001).

1.4 Phase II Enzymes

Phase II xenobiotic metabolism generally involves bioconjugation with the tripeptide glutathione, glucuronic acid, or a sulfate group to either a functional group (OH, COOH, NH2, SH, CH, and halogens) on a compound or one which has been added during phase I metabolism. Phase II conjugation has two consequences: 1) it usually inactivates the compound; and 2) the compound becomes significantly more hydrophilic and can now more readily be removed from the cell. This is the main mean by which cells are protected from reactive oxygen species (ROS) and electrophiles. The compound can now be excreted via the kidneys and urine, or through the

liver and the feces. The three main enzymes involved in phase II metabolism are glucuronosyltransferases (UGT), sulfotransferases (SULT), and glutathione S-transferases (GST).

Glucuronosyltransferases mediate the addition of glucuronic acid to a phase I metabolite, thereby making it more water soluble and available for transport and excretion (Tukey and Strassburg, 2000). UGTs use the cofactor uridine diphospho-glucuronic acid (UDPGA) to supply the glucuronic acid which is conjugated to the compound.

The nomenclature for UGTs is similar to that of the P450 system. There are 19 human UGTs, arranged into 2 families UGT1 and UGT2, then divided into three subfamilies UGT1A, UGT2A and UGT2B of which there are 9, 3, and 7 genes respectively (Nakamura et al, 2008). The UGTs are found in numerous tissues, although mainly in the liver, kidney, bladder and the intestine (Strassberg et al, 1997, Strassberg et al, 1997, Radominska-Pandya et al, 1999, Tukey and Strassburg, 2000, Nakamura et al, 2008). One characteristic of glucuronidation not found with the other phase II products, is presence of β-glucuronidase produced by intestinal flora, which can cleave glucuronic acid, allowing for re-absorption and enterohepatic circulation. The compound is glucuronidated in the liver and excreted in the bile to the small intestine where gut bacteria cleave the glucuronic acid, and the compound is reabsorbed and transported to the liver and the processes is repeated.

Sulfotransferases (SULT) catalyzes the sulfonation of a variety of exogenous and endogenous compounds including monocyclic phenols, acetaminophen, paracetamol, DHEA,

estrone, 17β-estradiol, thyroid hormone (T_3), bile acids, androgens (Fujita et al, 1997, Fujita et al, 1999,Yamazoe et al, 1999, and as reviewed in Lindsay et al, 2008 and Gamage et al, 2005). There are two groups of SULTs. The membrane bound group are found in the Golgi and catalyzes endogenous peptides and lipids, while the second group resides in the cytosol and are the ones that catalyzes most exogenous as well as endogenous compounds. Sulfotransferases use 3'-phosphoadenosine-5' phosphosulfate (PAPS) as a cofactor that donates the sulfonate group in the reaction. This, like glucuronidation, this greatly increases the compound's water solubility and generally inactivates it as well. There are a several instances when SULTs bioactivate the compounds making them more cytotoxic, such as polycyclic aromatic hydrocarbons (PAH), some alcohols, and 2-nitropropane (Gamage et al, 2006, Banoglu , 2000)

The nomenclature devised for the SULTs is like that of the UGTs and P450s. There are 4 families with 45% amino acid similarity (SULT1, SULT2, SULT4, and SULT6) (Blanchard et al, 2004). The tissue distribution is somewhat more restricted than the UGTs, with the SULTs mainly found in liver, gi tract, kidney, lung, platelets, placenta, adreanal gland, skin and prostate (Lindsay et al, 2008).

The last of the phase II enzymes is the 45-55 KDa glutathione S –transferases (GST), a super family that catalyzes the conjugation of glutathione (GSH) to electrophilic phase I metabolites and reactive oxygen species (ROS). The importance of GSH can be discerned by the fact that all life expresses glutathione. GSTs bring the GSH active site and the electrophille together and also activate the sulfhydryl group, which allows a nucleophilic attack and conjugation (Armstrong, 1997). Again, the new metabolite is very water soluble and usually

inactivated. There are a few instances in which the GS-conjugate is more reactive than the unmodified compound, such as dichloromethane, dibromoethane, hexachlorobutadiene, or the reaction leads to depletion of cellular glutathione, such as those by quinines and isothiocyanates (Hayes et al, 2005) . There are many endogenous and xenobiotics substrates for GSTs, including benzo(a)pyrene 7,8-dihydrodiol-9,10 epoxide, DDT, atrozine, cisplatin ,acetaminophen, adriamycin, LTC_4, and prostaglandins (Hayes et al, 2005). In MRP1 mediated transport, GSH may be,conjugated to a substrate, required to initiate transport, or may be co transported the substrate (Moa et al, 2000, Loe et al, 1996)

There are 3 major GST families termed cytosolic, mitochondrial, and ER, which share >40% amino acid sequence identity. The cytosolic are the major xenobiotic metabolizing and make up ~95% of the cellular GST. The cytosolic GST are divided into 7 classes sharing ~25% amino acid identity, which are alpha, Mu, Pi, Sigma, Omega, Theta, and Zeta (Eaton et al, 1999, Hayes et al, 2005). The classes are further divided into subunits denoted by a number. Alpha and Mu have 5, Theta and Omega 2, and Pi, Sigma, Zeta each have only 1 subunit, exp. GSTA1-5(Eaton et al, 1999, Casarett & Doull's, 2007).

1.5 Compensatory changes in drug metabolizing enzymes

In the last 20 years there have been great strides made in the understanding of compensatory changes in the drug metabolism, resulting from the loss of various components within the pathway. Compensatory changes in phase I enzymes have been seen in several animal knock out models. The loss of Pgp (Mdr1a) in mice led to an induction of Cyp3A (Schuetz et al,

2000). In Mrp2-/- mice, hepatic expression of several Cyp450s are altered, with females having a 10-fold induction of Cyp2b13, a 2- fold induction of Cyp2b9, and males having a 2.2-fold increase in Cyp7a1, and a 2-fold reduction in Cyp4a14 (Chu et al, 2006). The loss of a functional nuclear receptor, leading to alterations in transcription, can also modify Cyp450 expression. In farnesoid X receptor (FXR) knockout mice, Cyp3a11 is induced, whereas Cyp7a1 and Cyp8b1 are down regulated (Marschall et al, 2006, Wagner et al 2003). In retinoid-related orphan receptor (RORα and RORγ) double knockout mice there is an induction of hepatic Cyp2b9, 2b10, 39a1, 4a14, 2b13, and a reduction in Cyp7b1, 2f2, and 8b1 expression (Kang et al, 2007). The loss of either the constitutive androstane receptor (CAR) and/or the pregnane X receptor (PXR) leads a reduction of Cyp7a in the liver (Uppal et al, 2005).

Compensatory changes are also seen in the expression of phase II enzymes. In HepG2 cells over expressing Cyp2E1, which may be induced in response to ethanol exposure, there is an increase in α and microsomal GST levels, presumably to protect the cells from ethanol induced oxidative stress (Mari and Cederbaum, 2001). In the CAR, PXR and CAR/PXR double knockout mice there is a reduction of GstA2 expression and an increase in Ugt1A1 (Uppal et al, 2005). In RORα null mice, Sult1E1 expression is induced (Wada et al, 2008). Mrp4 knockout mice have reduced levels of hepatic Sult2a (Mahfoud et al, 2004). In mice with a nonfunctional cystic fibrosis transmembrane receptor (CFTR)/ABCC7, there is increased Sult1E1 activity and mRNA expression (Falany et al, 2002, Falany et al, 2009).

Compensatory transporter induction has also been well documented. As mentioned previously, compensation was first shown in the Eisai hyperbilirubinemic rats (EHBR) and

Groningen Yellow/transporter- deficient rat (TR^-), in which Mrp3 and Pgp are induced in the liver, and Mrp4 and Pgp is induced in the kidneys, shifting the excretion of bilirubin from hepatic to renal (Elferink et al 1995, Jensen et al, 1985, Paulusma et al, 1996, Taniguchi et al, 1996 Chen et al, 2005). There is somewhat contradictory data in Mrp2-/- mice. Chu and colleges established induction of Mrp4 in the kidney, however they did not see an induction of Mrp3 in the liver, while Nezases and colleges saw a reduction of hepatic Mrp3 (Chu et al., 2006, Nezasa et al, 2006). In addition, Vlaming and colleges determined there was a 2-fold increases in liver Mrp3 and in kidney Mrp4, in Mrp2-/- mice , they did however use a different strain of mouse than the above two laboratories (Vlaming et al, 2006). In Fxr-/- mice, in response to bile acid loading, there is increased expression of hepatic and intestinal Mrp3 and Mrp4, and renal Mrp4 (Zollner et al, 2003, Wagner et al, 2003, Marschall et al, 2006). Administration of carbon tetrachloride (CCL_4) to mice leads to an induction of Mrp4 and a reduction in Oatp1a1 and 1a4 in the liver, presumably to prevent accumulation of toxic metabolites (Aleksunes et al 2006, Aleksunes et al, 2008). Compensatory induction of efflux transporters may be the result chemical exposure or in response to loss of a functional transporter or transporters.

Pascussi and colleagues, 2008 endeavored to provide a concise review of the complexities involved in the cross talk between nuclear receptors regulating xenobiotic metabolizing enzymes (Table 3). It also sheds light on possible mechanisms involved in compensation, particularly in the nuclear receptor knockout models.

Table 3. Transcriptional regulation of multi-drug resistance associated proteins. (Adapted Urquhart et al, 2007; Qadri et al, 2009: Okada et al, 2009; Scotto, 2003)

Gene	Transcription Factors
Mrp1	PXR, Sp1, p53, Ap1?
Mrp2	PXR, CAR, FXR, RXR, HNF1, HNF4, Nrf2, Sp1? Ap1?
Mrp3	PXR, LXR?,CAR?, Nrf2, Sp1
Mrp4	PXR,FXR, Nrf2

In contrast to nuclear receptor induction of the transporter expression, during LPS induced inflammatory cholestasis and the resulting released cytokines, there is a decrease in both hepatic Mrp2 and Mrp3 expression (Hartmann et al, 2002). As mentioned above transporter regulation is a complex network of mechanisms and much still remains to be discerned.

1.6 MRP1 transport of steroid hormones and steroid metabolism

Although MRP1 is expressed in most tissues, high expression is seen in tissues involved in steroid hormone metabolism, such as the testes, ovaries, prostate, and adrenal glands (Bart et al, 2004, Maher et al, 2005, Nishimura et al, 2005). Also given MRP1's ability to transport several steroid conjugates, such as esterone 3-sulfate, with high affinity, as well as 17β-glucuronosyl estradiol, and DHEAS, MRP1 may play a role in steroid homeostasis (Qian et al, 2001, Jedlitscky et al, 1996, Zelcer et al, 2003). In addition to MRP1, MRP4 is widely distributed, but at a low levels in most tissues. Expression is highest in the kidney, however expression is also high in the prostate and testes (sertoli and leydig cells) compared to the remaining tissues (Ho et

al, 2008, Augustine et al, 2005). Like MRP1, MRP4 transports DHEAS with high affinity and 17β-glucuronosyl estradiol (Chen et al, 2001, Zelcer et al, 2003).

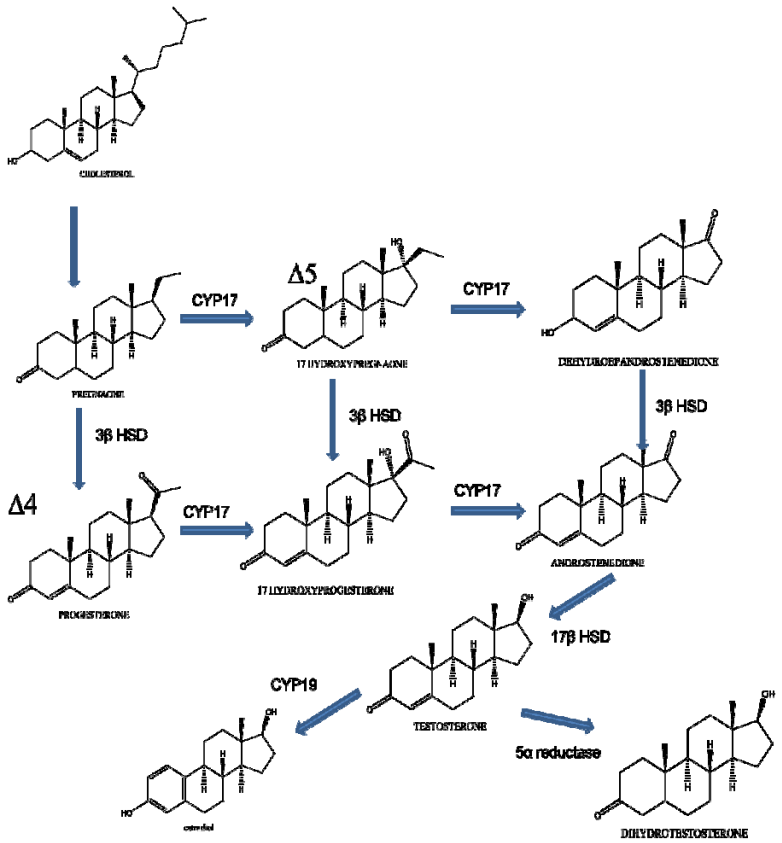


Figure5. Testicular Steroid Synthesis.

Steroid synthesis in the testes begins with side chain cleavage of cholesterol to form pregnenolone. Synthesis can then follow one of two paths: the Δ4 pathway forms progesterone, while the Δ5 pathway forms 17hydroxypregnalone (Fig.5). Δ5 products can shift to the □4 pathway via 3β-hydroxysteroid dehydrogenase. The ultimate product in both pathways in the testes is estradiol. However, excessive build up of estrogens in the testes can damage developing spermatozoa (Flen et al, 1996, Wijnholds et al, 1998., Reviewed Leslie et al, 2001). With high expression of MRP1 in the testes, and expression of Sult1E1 also in the testes, and high affinity

for esterone 3-sulfate, MRP1 may play a role in protecting developing spermatozoa from the feminizing effects of elevated testicular estrogen.

With respect to Mrp4s tissue distribution and substrate profile, it may contribute to DHEA/DHEAS homeostasis. Given DHEAS is converted to DHT in the prostate, and Mrp4 transports DHEAS with high affinity and has high expression in the prostate, Mrp4 may be involved in the regulation DHT, by adjusting the amount of available DHEA (Zelecer et al, 2003).

1.7 Dendritic Cells

Dendritic cells (DCs), sentinels of the immune system, are potent antigen processing and presenting cells (APCs). DCs are bone marrow derived mononuclear cells which, are MHC class II positive and are the only cells with the ability to stimulate naïve T cells (Heath & Carbone, 2009). DCs are found throughout the body in all tissues, especially where there is an interface between the body and the environment. There are four major types of DCs: langerhans, interstitial myleod derived (MDCs), monocyte-derived, and plasmacytoid-derived (PDCs). DCs possess a heterogeneous haemopoietic lieneage, different types have been shown to display a differential morphology, phenotype and function (Kamath A.T., et al, 2000, Kamath AT, et al 2002, Heath WR & Carbone FR, 2009). All subsets share the ability to stimulate naïve T cells and induce proliferation.

It has been suggested that the so-called myeloid derived DCs have a stimulatory function and lymphoid-derived have tolerogenic function, however, this idea has recently come under

scrutiny (McLellan AD,et al, 2000, Navak N et al, 2008) Tolerance for self antigens is an important process,so for the body will not to mount an immune response against itself. Tolerance involves loss of proliferation of T cells after stimulation with a self antigen and the creation of regulatory T cells, which suppress the immune response.

DC precursors travel via the bloodstream to peripheral tissues where they differentiate into the immature DCs and can begin taking up antigens, processing and presenting them by the major histocompatibility complex (MHC)(Rodriguez et al, 1995, Sallusto et al 1994 Sallusto et al, 1995).

DCs play a critical role in the adaptive immune system. They are the key antigen presenting cells (APC) of peptides to T and B cells. DCs process and present both endogenous and exogenous antigens. DCs are the only APC capable of stimulation naïve T cells.Immature DCs processes both intracellular antigens produced from intercellular bacteria, parasites and viruses inside of the DC and extracellular antigens including bacteria, viruses, proteins, and cellular debris which are internalized via pahgocytosis, pinocytosis, or endocytosis (Rodriguez et al, 1995, Sallusto et al 1994 Sallusto et al, 1995). Larger protein antigens are processed, broken down into peptides, and loaded onto MHC I or II molecules and transported to the cell surface for presentation to antigen specific T cell (Mellman et al, 1998). Intracellular proteins are degraded, loaded onto MHCI in the E.R. and the MHCI-peptide complex is transported to the cell surface via the golgi for presentation to CD8 cytotoxic T cells (CTC) (Mellman et al, 1998). Extracellular proteins are degraded in endosome-lysosomes to peptides, which are then loaded on to MCH II. The MCHII is then transported to the cell surface for presentation to CD4 Helper T cells (Th) (Sallusto et al 1994 Sallusto et al, 1995).

DCs in the peripheral tissue express high levels of intercellular MHCII and CD1a, are proficient antigen phagocytes, can rapidly respond to stimuli, and express little adhesion and costimulatry molecules (CD40, CD54, CD58, CD80 and CD86).

Maturation is occurring as antigens are being processed for presentation on one of the MCH molecules. During maturation, DCs go from being poor stimulatory cells to those with decreased phagocytic ability. They gain motility, and begin migration from peripheral tissues to lymphoid tissues, begin to express makers such as CD83, and, have an increased ability to stimulate T cells (Banchereau et al, 1998). . Maturation beings when the DC is stimulated by host inflammatory molecules such as CD40L, TNF-α, IL-1, IL-6, and IFN-α. Maturation can also be stimulated by foreign products including LPS from gram – bacterial cell walls. These foreign antigens are recognized by toll like receptors (TLR). TLR recognize different types of molecules, TRL2 responds to gram positive and TLR4 to gram negative cell wall, TLR5 respond to flagellin, and TLR9 to CpG motifs (Guermonprez et al, 2002) Upon recognition of an antigen by a TLR, DC maturation is initiated by a intricate signaling cascade.

As DCs mature, there is an increase in the expression of adhesion and costimulatory molecules involved in DC –T cell interactions. Molecules that are up-regulated include CD40, CD54, CD58, CD80, CD86 as well as MCH I and II. There is also increased excretion, of IL12, IL10 or IFN in response to bacterial or viral infection (Guermonprez et al, 2002).

Upon maturation there are also altered changes in chemokines and chemokine receptors. Expression of CCR1 CCR2, CCR5, and CCR6 DCs allow immature DCs to move to the site of

infection. After DCs have acquired antigen and begin maturing, here is a down-regulation ofCCr1, CCR2 CCR5, and CCR6, and an up regulation of CC7 (Sallusto and Lanzavecchia, 2000). This allows DCs to migrate from peripheral tissues to lymph nodes or the spleen in response to chemokines CCL19 and CCL 21, and leukotrienes (reviewed in Lanzavecchia et al, 20010; Randolph et al, 2005) . Mature DCs also secrete TARC, MDC, IP-10, RANTES, MIP-1α, and MIP1β to recruit T cells and monocytes (Sallusto and Lanzavecchia, 2000).

Once matured DCs move to lymph nodes, they interact with, and present antigen to T cells. Interaction with T cell receptor (TCR) and CD40L lead to survival of the DC (Caux et al, 1994). Once in the lymph node, DCs can interact with hundreds of T cells. The DC presenting antigens will eventually form a DC-T cell interaction, priming the T cells. This is a three step process; first there is a quick interaction with many T cells, next a long interaction inducing activation markers and cytokine secretion, lastly DC-T cells separate, the T cells migrate and proliferate and leave the lymph nodes (Kleijmeer et al, 1995). In order for T cells to proliferate and become cytokine –secreting cells, called effector cells, there must be sufficient priming by the DC (Lanzavecchia and Sallusto, 2001). Effective priming needs a mature DC, sufficiently long DC-T cell interaction, and high affinity of the TCR for the MHC. Proper T cell priming by the DC is necessary for long term T cell survival, as well as differentiation into effector and memory cells (Kleijmeer et al, 1995). If there is effective priming, T helper cells can differentiate in two directions as either Th1 cells that produce IFNγ and aid cytotoxic T cells, or Th2 cells that secrete IL-4, IL-5, and IL-3 in support of humeral immunity (Rissoan et al, 1999). The path the Th cells take is dependent on the cytokines the DC is secreting. If the DC is releasing IL-2,IL-18, and IL-27, the cells become Th1; if the DC is secreting CCL17 or CCL22 ,or there is no IL-12,

the cells move to become Th2 (Rissoan et al, 1999). The determining factor as to which cytokine a DC will secrete is dependent on which antigen the DC is interacting with In order for cytotoxic T cell (CD8/CTL) response there must be a DC-CD4 Th interaction between CD40-CD40L.

In conclusion, DC's are the most potent APC, and the only cells able to activate naïve T cells. DC antigen aquistion and presentation is required for proper T cell stimulation leading to activation of and long term survival of CD4 and CD 8 T cells (as reviewed by Kindt et al, 2007, Adams et al, 2005, Janeway et al, 2001).

Chapter two: The Loss of Multidrug Resistance Associated Protein one Leads to Alterations in Phase I, Phase II, and Phase III Metabolizing Enzymes Expression and Drug Disposition

Abstract:

The multidrug resistance-associated protein-1 (MRP1/ABCC1) is a 190kDa membrane-bound glycoprotein that mediates cellular efflux of a variety of xenobiotics, typically as glucuronide, sulfate, or glutathione conjugates. We examined alterations in phase I, II, and III detoxification protein activity or gene expression in the kidney, lungs, and small intestine between FVB mice and mice that lacked the multidrug resistance associated protein 1 (FVB/mrp1-/-) mice. In the kidney, 16ά-OH (Cyp2b9, 2c11), 7ά/15β-OH (Cyp 1a1/2 & 2a1),and 16β-OH (Cyp2b1), hydroxylation was significantly upregulated 1.53-fold, 1.48-fold and 1.18-fold respectively. Significant changes in phase II metabolizing enzyme expression included a 2.3-fold increase in sulfotransferase 1a1 mRNA levels in the small intestine and 1.2-fold reduction of Sult1a1 mRNA levels in the lung. No changes were detected in sulfotransferase 1a2 or glucuronosyltransferase expression in the FVB/mrp1-/- mice. Mrp4 expression was reduced in the lungs, andMrp2 expression was decreased in the kidney of Mrp1 knock out mice. To determine the impact of the compensatory changes FVB/Mrp1 -/- mice were dosed with [^{3}H] vincristine and its localization into the organs was examined. FVB/Mrp1-/- mice have increased accumulation of vincristine in the kidney, small intestine, colon, and epididymis. All of the enzymes differentially expressed in the Mrp1-/- mice have some degree gender predominance. Thus changes in the expression are presumably due to alterations in circulating serum steroid levels seen in the FVB/Mrp1-/- mice.

However, the changes do not appear to alter drug disposition in the lungs or small intestine, but may contribute to increased retention of vincristine in the kidney.

2.1 Introduction

The efflux of various endobiotics and xenobiotics is due in part to members of the ABCC subfamily of ATP-binding cassette (ABC) transporters, which contains 13 genes, with functions ranging from ion transport, cell surface receptors, and toxin (Kruh et al 2001) (reviewed in Haimeur et al, 2004 Wang et al 2008 ,Toyoda et al, 2008,Stanly et al, 2009). One member, the multidrug resistance associated protein 1 (MRP1) transports glucuronide, glutathione, sulfate conjugates, and nucleotide analogues, leukotriene C_4, chemotherapy drugs like doxorubicin, colchicines, (Jedlitschky et al. , 1996) (Gottesman et al., 2002) (Leslie et al, 2001A), heavy metals such as arsenic and antimony (Leslie et al, 2001B), and toxins including aflatoxin B , the vinca alkaloids, 4-(methyllnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) a tobacco metabolite and methoxychlor (Loe et al., 1997;Leslie et al, 2001B;Tribull et al, 2003) across cell membranes. MRP1 is expressed ubiquitously in most tissues on the basolateral side of polarized cells; however it is at very low expression levels in the liver (Peng et al. 1999).

Other members of the ABCC subfamily include MRP2 which is found mainly in the liver, kidney, and intestine and unlike MRP1, it is located on the apical side, rather than the basolateral side of polarized cells (Paulsma et al, 1996). MRP3 is found mainly in the liver, pancreas, kidney and intestine (Cherrington et al., 2002; Belinsky et al., 1998; Kool et al., 1997), and like MRP1, is located on the basolateral side of the cells. All three transporters have

overlapping substrate specificities. MRP4 is ubiquitously expressed in tissues, particularly in the prostate, testis, ovary, intestine, and lung, and is on the apical side of cells (Lee et al., 1998) (Kruh et al., 2001). It is an important transporter of not only organic acids but also nucleoside analogues such as PMEA, an anti-HIV nucleotide analogue, and cyclic nucleosides such as cyclic AMP (Schuetz et al., 1999) (Kruh et al., 2001). MRP5 is expressed in most tissues and like MRP4 transports nucleoside analogues as well as cyclic nucleotides including cAMP and cGMP (Jedlitschky et al., 2000).

The expression of MRP transporters is also inducible by a variety of drugs and toxins (Schrenk et al., 2001). For example, both MRP1 and MRP3 are inducible via nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin or ibuprofen in human colon cancer cells (Tatebe et al., 2002). It has also been shown that transporters like MRP1 can be upregulated in response to endotoxin-induced cholestasis and during other types of hepatobiliary organic ion transport impairment (Vos et al., 1998; Tatebe et al., 2002).

There are naturally occurring models demonstrating compensatory mechanisms to balance the loss of an ABCC transporter. Eisai hyperbilirubinemic rats and Groningen Yellow transport deficient rat (TR^{--}),which have an inherited nonfunctional Mrp2, appear to compensate by inducing hepatic Mrp3 and renal Mrp4 (Ogawa et al., 2000; Akita et al., 2001, Jansen et al, 1985, Paulusma et al, 1996). The increase in Mrp3 expression diverts the elimination of bilirubin from the liver via Mrp2,; to the kidney via Mrp3. Rats with a nonfunctional Mrp2 also have increased expression of Mrp4 in the kidney (Chen et al., 2005). There is conflicting data in mice lacking Mrp2 (Mrp2-/-). One group did not show induction of Mrp3 in the liver, however there

was an increase in hepatic and renal Mrp4 (Chu et al., 2006), A second group demonstrated a modest ~60% induction of Mrp3 in the same knock out mouse strain (C57BL/6) (Nezasa et al, 2006). A third group using a different stain of Mrp2-/- mice demonstrated a 2 fold increases in hepatic Mrp3 and renal Mrp4, similar to the changes seen in the rat models (Vlaming et al, 2006). Mice lacking Mrp1 or Mrp3 show no obvious changes in phenotype, save a decrease in leukotriene-induced inflammatory response in Mrp1 -/- mice (Wijnholds et al., 1997; Zelcer et al, 2006).

Prior to transport by the MRP family, endogenous and exogenous compounds typically need to go through several detoxification steps to both increase their water solubility and alter their structure such that they become substrates for the transporters. In the first step, or phase I metabolism, the compound undergoes monooxygenation and hydroxylation, which is often mediated by the cytochrome P450 (CYP) enzymes. These enzymes are involved in the metabolism of a variety of xenobiotic and endogenous substrates such as carcinogens, drugs, and steroids (reviewed in Danielson 2002). The second step, or phase II metabolism, is mediated by enzymes such as glutathione S-transferases (GST), glucuronosyltransferases (UGT), and sulfotransferases. These enzymes attach either a glycoprotein, sugar or sulfate respectively, to phase I metabolites. This vastly increases the compounds water solubility and in most cases leads to inactivation of the compound. Phase II enzymes may also be induced by various exogenous and endogenous substrates (Reviewed Xu et al, 2005).

As with induction of different transporters to compensate for the loss of another transporter, compensatory induction phase I and phase II enzymes due to loss of an ABC

transporter has also been demonstrated. For example, there is induction of Cyp3a in mice lacking P-glycoprotein (Schuetz et al., 2000). Induction of glutathione has also been shown in mice lacking ABCC transporters, as there were increased levels of glutathione in the lung, heart, kidney, muscle, colon, and testes in Mrp1-/- mice as compared to the wild-type mice (Lorico et al., 1997). There is a 3.5 and 5.5 fold increase in Ugt1a expression in Mrp2 deficient TR- rats (Johnson et al, 2006).

Previous work in our laboratory has shown FVB/mrp1-/- mice have increased expression of hepatic Mrp2 and Mrp5 mRNA, as well as a decrease in hepatic activity of sulfotransferases and an increase glucuronosyltransferases activity (Bain et al 2003). Thus, we decided to examine extrahepatic tissues, including the kidneys, lungs, and small intestine to determine if there were also changes in the expression of phase I, II and III enzymes in response to the loss of Mrp1.

2.2 Materials and Methods:

2.2.1 Collection of tissues from FVB and FVB/mrp1 -/- mice

Eight FVB and FVB/mrp -/- mice were purchased from Taconic Farms (Germantown, NY) at five weeks of age, kept at 25^{o} C and 50% humidity, and provided water and food *ad libitum*. At nine weeks of age, the mice were euthanized with a CO_2 overdose. The lung, intestine, and kidneys were divided into 2 sections: One placed into TRI–Reagent (Sigma Chemical Company, St. Louis, MO) and stored at –80^{o} C for extraction of total RNA and the other frozen at –80^{o}C for cytosol and microsome preparation.

2.2.2 Testosterone hydroxylation assay

Phase I activity was measured by the testosterone hydroxylation assay (Baldwin and LeBlanc, 1992). The P450's regio- and steriospecifically hydroxylate testosterone, so one can use this assay to measure multiple P450's at the same time. Individual tissues were homogenized in cytosol buffer (10mM HEPES, pH 7.4, 1mM EDTA, 10% glycerol, 2µg/mL each of aprotinin, leupeptin, and pepstatin) Microsomes were separated and collected by differential centrifugation, first at 10,000xg/ 10 min, transferring the supernatant, and again at 100,000xg/ 60 min. The supernatant containing the cytosol was stored at –80^{o}C. The pellet containing the microsomes was centrifuged again at 100,000xg/ 60 min and then suspended in microsome buffer (0.1M potassium phosphate, 0.1mM EDTA, 20% glycerol at pH7.4) and stored at –80^{o}C. The Bradford assay was done to determine protein concentrations (Bradford, 1976).

Microsomal protein (100-200□g) was incubated in 0.1M phosphate buffer (pH7.4) with 40nmol [^{14}C] testosterone (53.6mCi/mmol; New England Nuclear, Boston, MA) (Baldwin and LeBlanc, 1992). Subsequently 1mM NADPH was added and incubated for 10 min at 37^{o}C in a shaking water bath. To terminate the reaction, 2mL ethyl acetate was added, the samples vortexed, and then centrifuged at 1,000xg /5min. The aqueous layer was transferred to a new tube and 2mL of ethyl acetate is added, vortexed, and the samples again were spun at 1000xg/ 5min. The 2 ethyl acetate extracts containing the hydroxytestosterone products were combined. The samples were evaporated under nitrogen, resuspended in ethyl acetate, and spotted on a thin layer chromatography (TLC) plates. The plates were subsequently developed in 80% methylene chloride/20% acetone and dried. Next they were developed in 70% chloroform/17.5% ethyl acetate/12.5% ethanol, and then exposed to autoradiography film. Using the film as a template,

the metabolites were cut from the TLC plate and quantitated by liquid scintillation. Activity was measured by pmol of metabolite produced /min/mg microsomal protein. Student's t-test was used to determine statistical significance in phase I activity between the wild type and Mrp1 (-/-) mice.

2.2.3 QPCR

Tissue samples were homogenized in Tri Reagent to recover total RNA, treated with RNase free DNase. and their concentration determined by spectrophotometry. cDNA was prepared from 2µg RNA using MMLV buffer, 400µM dNTPs, 100ng of random hexamers, 10ng of Rnasin, and 1U MMLV-RT, and incubated at 37^{o}C for 1 hour. The cDNA was stored at –20^{o}C until used for PCR. A standard was prepared for each gene to be analyzed using PCR. Briefly, 400□M gene-specific primers (Table 1) were incubated with 100ng cDNA, 400µM dNTPs, and unit Taq polymerase,. The gene is amplified in a thermocyler, using a gene-specific annealing step (Table 1), for 40 cycles. The PCR product was purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). The concentration of the purified sample of DNA for use in the standard curve was determined by spectrophotometry.

QPCR was run for each of the transporters and18S RNA as a housekeeper, using Sybr Green. Serial dilutions were made with the DNA standard and run in triplicate using real time PCR to determine the number of RNA molecules for each gene examined. This was normalized to 18S concentrations, and Student's t-test was used to determine significant expression differences between the wild type and the Mrp1 -/- mice.

2.2.4 Alterations in vincristine disposition

FVB and FVB/mrp1-/- mice (n=3) were injected IP with 1mg/kg of [^{3}H] vincristine sulfate (5.3 Ci/mmol) (American Radiolabeled Chemicals, St. Louis, MO). Twelve hours post injection mice were euthanized by CO_2 overdose, and the kidney, small intestine, lung epididymis liver, gallbladder, heart, lung, stomach, spleen brain colon and testes were removed, weighed, and a portion homogenized in 2% BSA in PBS. [^{3}H] vincristine disposition was determined for each organ by scintillation counting. Data is expressed as average ng vincristine/g tissue + standard deviation, statistical differences (*) as determined by student's t-test ($p<0.05$)

2.2.5 Statistical Analysis:

Was performed using Prism GraphPad software (La Jolla, CA). Statistical differences of ($p \leq 0.05$) were considered significant.

2.3 Results

2.3.1 Phase I activity is changed in a compensatory manner between FVB and FVB/Mrp1 -/- mice:

Cytochrome P450s regio- and stereo-specifically hydroxylate testosterone, so this assay was used to determine changes in phase I enzyme activity between FVB and FVB/Mrp1-/- mice, . While there were no changes in phase I activity in the lung,.16ά-OH (Cyp2b9, 2c11), 7ά/15β-OH (Cyp 1a1/2 & 2a1), 16β-OH (Cyp2b1), and metabolite A formation was increased 1.53 fold, 1.48-

fold,1.18-fold and 1.94-fold, respectively, in the kidney of mice lacking mrp1 (Table 2). We hypothesize that these changes in phase I activity are in response to the loss of Mrp1 and may lead to changes in phase II and phase III interaction. Protein was extracted from the small intestine and quantified, we unable to resolve any usable data.

2.3.2 Phase II enzyme expression is changed in a compensatory manner between FVB and FVB/Mrp1 –/- mice:

Ugt and Sult mRNA levels were examined in the kidneys, lung, and the small intestine by real-time PCR. There were no significant changes in the expression of any of the Ugts (Figure 1). Additionally, Ugt1a1 levels were below the detection limit in the small intestine. Sult 1a1 was induced 2.34 fold in the small intestine , and reduced 1.22 fold in the lung of mice lacking Mrp1 (Figure 2A), while no changes were detected in Sult 1a2 (Figure 2B).

2.3.3 Phase III transporter mRNA expression in FVB and FVB mrp1-/- mice

Considering the large overlap in substrates among the ABCC transporters, we expected there to be an induction of several of the remaining Mrps in the Mrp1-/- mice. Since Mrp1 is expressed at relatively low levels in the liver, extrahepatic tissues such as the kidney, lung, and small intestine were examined for compensatory changes. mRNA levels of Mrp2, Mrp3, Mrp4, and Mrp5 were determined by real time PCR. In contrast to the liver, the only significant changes detected were a 1.8 fold a decrease of Mrp2 in the kidney and a 1.3 fold reduction of Mrp4 in the lung. The low expression of Mrp2 in the small intestine is was unexpected as Mrp2 is highly expressed in the mouse small intestine (Maher et al, 2005). This was surprising as we had expected either a compensatory increase resulting from the loss of Mrp1 or no changes in baseline expression.

2.3.4 Changes in drug disposition and elimination in Mrp1-/- mice

As Mrp1 is expressed in most tissues and there is considerable overlap in transporter expression and substrate specificity, the loss of Mrp1 appears to have limited effect in terms of compensation by the remaining transporters at basal levels. After examining the activity or expression levels of the phase I, phase II and phase III drug metabolizing enzymes, we wanted to examine the effect the loss of Mrp1 might have on drug disposition. Both wildtype FVB and FVB Mrp1-/- were challenged with [^{3}H] vincristine, a known high affinity substrate for Mrp1 (Cole et al, 1994). There was an increase in vincristine levels by 3.7, 3.2, and 4.9 fold in the kidney, small intestine, and epididymis, respectively in the knockout mice as compared to the wildtype (Table 3). There were no significant changes found in liver, gallbladder, heart, lung, stomach, spleen, brain, or testes. The lack of compensatory transporter induction appears to severely alter the elimination profile of vincristine in the Mrp1-/- mice.

2.4 Discussion

This study indicated that loss of Mrp1 leads to alterations extra hepatic phase I, phase II and phase III metabolizing enzymes and drug disposition. FVB/mrp1-/- mice have increased cytochrome P450 activity in the kidney, a decreased in sulfotransferase expression in the lung, and diminished Mrp mRNA levels in both the kidney and lung. Typically, studies involving compensatory responses after the loss of a transporter focus on examining the liver (Casarett & Doulls,2008), since this organ is the site of highest detoxification activity and readily eliminates compounds into the bile. However, the baseline expression of Mrp1 in the liver is exceedingly low (Roelofsen et al, 1997). Tissues expressing high levels of Mrp1 include the lung, kidney,

heart, adrenal, and testes (Reviewed in Klaassen and Aleksunes, 2010), while the small intestine has low levels (Peng et al, 1999, Zimmerman et al. 2005). The lung is the only organ that singularly functions as an excretory and intake organ, save the skin. Therefore it is exposed to both environmental and endogenous compounds, both native and biotransformed. The kidney and small intestine have high metabolic activity, and function in both toxicant uptake and elimination. Thus, we investigated compensatory changes in Mrp1 knockout mice in these three tissues.

Mice lacking Mrp1 had increases in 16α-OH, 7α/15β-OH, 16β-OH,testosterone hydroxylation activationin their kidneys, which correspond to Cyp2c7, 2a1, and 3a4. Cyp 2c7 and Cyp2a1 are female predominate, and Mrp1-/- mice have been shown to have lower levels of circulating testosterone and higher levels of circulating estradiol (Sivils et al, 2010). Testosterone has been shown to have an inhibitory effect on Cyp2a1 expression, although the effect on Cyp2c7 expression is inconclusive (Waxmam et al, 1985, Bandiera et al, 1992, Ortiz de Montallano, 2005). Cyp 3a4 activity has also be shown to be modulated by steroids, as androgens induced triazolam 4-hydroxylation, but reduced TZM 1-hydroxylation by Cyp3a4 (Nakamura et al, 2002, Cheung et al, 2006, Yu et al, 2005, reviewed Walbold et al, 2003). Since Cyp 1a1, 2c7, and 3a4 all metabolize steroids including estrogens, the increased activity in the kidney of Mrp1 knockout mice may be due to increased serum estrogen (Sivils et al, 2010) and an attempt to metabolize and eliminate the estrogen via the kidney.

Phase I enzyme activity was not altered in the livers of Mrp1 knockout mice (Bain and Feldman, 2003). This is likely due to the constitutively high P450 and low Mrp1 expression in

the liver (Casarett and Doulls, 2008, Cole et al, 1998) In contrast, the liver has high Mrp2 expression and several Cyp450s are differentially expressed (Chu et al, 2006). There was a 2.2-fold induction of Cyp7a1 and a 2.5-fold reduction in males, and a 10-, and 2.0-fold reduction in Cyp4a14, Cyp 2b13 in females Cyp2b9 , in the livers of Mrp2 -/- mice.(Chu et al, 20006)which was hypothesized to be due to downstream changes in phase ll and phase lll metabolism (Chu et al, 2006). The loss of Mrp2 leads to an increase in serum bilirubin and bile salt levels, and an elevated rate of excretion of bile salts in the bile; leading to a decreased accumulation bile salt levels in the liver. ,The decreased bile acid levels up regulate Cyp7a1expression via a negative feedback mechanism(Hunt et al, 2000, Moffit et al, 2006, Eloranta et al, 2005). Both Cyp2b9 and 2b13 are expressed in the liver, however there substrates, are as of yet undetermined (Hashita et al, 2008). Interestingly, the one P450 that showed a 2.65-fold reduction in the liver of Mpr1-/- mice was the male specific Cyp2c13, although this was not significant due to high variability (Bain and Feldman, 2003). This enzyme hydroxylates testosterone at the 6β and15α position and progesterone at the 6β and 16α position (Kitareewan and Walz, 1994), and its reduction in mice lacking Mrp1 was probably due to the decreased levels of serum testosterone (Sivils et al, 2010)

Phase II compensation due to the loss of an ABCC transporter has been shown to date in two studies. Our laboratory has previously shown that there is an increase in hepatic glucuronosyltransferase activity and decreased sulfotransferase activity in Mrp1 knockout mice (Bain and Feldman 2003). Additionally, Mrp4 knockout mice have increased hepatic Sult2a levels (Mahfoud et al, 2004). In the present study Sult1a1 expression was reduced in the lung which may be related to alterations in serum steroid levels. Sult1a1 is the only Sult with higher expression in the male compared to female mice, which occurs only in the lung (Alnouti, 2006).

Thus, the reduction in Sult1a1 expression may be due to the increased serum estrogen levels (Sivils et al, 2010). There were no changes Sult 1a2 levels were below the detection limit in the small intestine; however Sult 1a2 is expressed at levels far below Sult 1a1 in all tissues thus far examined (Dooley et al, 2000, Enokizano et al, 2007).

There are several well characterized examples of compensative induction of one or more MRPs in the response to the loss of an MRP transporter. Surprisingly, mice lacking Mrp1 have reductions in the extra hepatic expression of other Mrp transporters, including reduced Mrp4 expression in the lung and reduced Mrp2 expression in the kidneys. This is in contrast to previously published studies dhowing hepatic induction of Mrp2 and Mrp5 in the livers of Mrp1 knockout mice and induction of Mrp3 and Mrp4 in the livers and kidneys of mice and rats lacking Mrp2 (Bain and Feldman, 2003, Cho et al, 2006,Chen et al, 2005). The lack of any significant compensatory induction of extrahepatic Mrps may indicate that outside of the liver baseline expression levels of the remaining transporters is significant to handle the increased export burden.

Dosing both FVB and FVB/Mrp1-/- mice with a well characterized non-endogenous Mrp1 substrate, which overlaps with as many transporters as possible ,may overload the baseline transport capacity of the remaining transporters, and lead to a detectable compensatory transporter induction.

To determine whether the changes in phase I, II, and III enzyme expression inj the Mrp1 -/- mice lead to changes in drug disposition, we dosed FVB and Mrp1-/- mice with [^{3}H] labeled vincristine. Increased accumulation of vincristine occurred in the small intestine, kidney, colon

and epididymis. Aside from the epididmis, vincristine accumulated in the excretory tissues of mice lacking Mrp1. Although Mrp2 is expressed in the small intestine, highest expression is in the duodenum and as you move threw to the ileum expression decreases, and finally, Mrp2 is not expressed in the colon (Klaassen and Aleksunes, 2010). The high levels found in the intestine and colon may be due to re absorption and enterohepatic circulation (Maher et al 2005, Zimmermann et al, 2005). The vincristine begins to accumulate in cells of the gastrointestinal (GI) track as it moves through during excretion. As we looked at the small intestine as a single organ we were only able to determine total vincristine accumulation. The change in vincristine disposition is most likely due to the loss of Mrp1coupled to decreased Mrp2 expression in the intestine., which in turn leads to the accumulation in excretory tissues as the FVB/Mrp1-/- mouse attempts to expel the vincristine in the feces (Loe et al, 1996). Increased accumulation in the kidney is in all probability an attempt to eliminate the excess vincristine via the urine. As the vincristine moves through the nephron, it is reabsorbed. Without a functional Mrp1 and with the decreased expression of Mrp2 our study found in the kidneys, the vincristine cannot be extruded into the blood via Mrp1 nor can be effectively excreted into the urine by Mrp2 (Ines et al, 1994).

The blood-testes barrier serves to protect developing germ cells for toxic insult by endogenous and exogenouns compounds (Reviewed in Leslie et al, 2004). Mrp1 expression is high in the murine testes and is considered to be a critical element of the blood-testes barrier (Bart et al, 2004). With the loss of Mrp1 in the testes, the blood testes barrier would be compromised, and the vincristine could no longer be pumped out of the testes into the blood stream, and would rather accumulate in the epididymis to be ultimately eliminated (Tribull et al, 2003). The increased retention of vincristine in the tissues of Mrp1-/- mice may be promising in

terms of clinical treatment of MDR tumor cells over expressing Mrp1 and Mrp2. A specific Mrp1/Mrp2 inhibitor may be able to increase the efficacy of cytotoxic drugs that are substrates for both Mrp1 and Mrp2, by increasing the intercellular concentration and thereby the antineoplastic activity.

In conclusion, changes in phase I, phase II and phase III enzymes levels and activity may very well be due less to compensatory changes; rather it may be a result of alterations in serum steroid levels. All of the enzymes showed at least some degree of sexually dimorphic expression levels. Therefore, considering the potential clinical implications determining alterations in drug metabolism and disposition resulting from the loss of or manipulation of Mrp1 activity deserves further investigation.

Table 1: Mouse primers used for PCR and Real Time PCR.

Primer	Forward 5'to 3'	Reverse 5' to 3'	T_m
18S RNA	CATGACCACAGAGTCCATGC	GTCATACCAGGAAATGAGC	63
MRP2	CTGCCTCTTCAGAATCTTAG	CCATCAGTTTCCTTATGGGTCC	58
MRP3	GCTGGCAAGTCTTCCATGAC	CGTGTGCAGGTGGGACAGC	63
MRP4	CATTGCACACAGATTGAACACC	GTCGAGGGCTGTCCATTGG	63
MRP5	GGAATGTATTGCCCAGCTACC	GCAGCAAACATGGCATAGAATC	63
UGT 1a1	CATACAATCATTCGCACATCC	GTGTTGAGACCTGGAGTGG	64
UGT1a2	CTGCTCTGACCTTCGGCATCAGC	CTGGCAGCTGTGCTTTCTTCG	64
UGT2a5	GAAATGCAGCTCTTGAGAACAGC	CAGCGGGATTGTCTGGGTAGC	64
SULT1a1	GTGCATTGGCCATACTAGTC	GTCAGGAAGAGGCTGATTCTTGC	64
SULT1a2	CACTCGCAGCCATCCCTGG	CTCGACCTTAGGACTTCTGC	63

Table 2: Cytochrome P450 activity determined by testosterone hydroxylation. The activity of multiple P450 isoforms and their ability to hydroxylate testosterone on different regions of the molecule was determined (n=6).

	Kidney			Lung	
Product	**FVB**	**MRP1-/1**		**FVB**	**MRP1-/1**
Metabolite A	**2.31± 1.9**	**4.50± 0.3***		**-**	**-**
15ά-OH	**22.46± 10.9**	**30.53± 3.1**		**0.67± 0.4**	**0.25± 0.3**
16ά-OH	**7.92± 3.6**	**12.14± 1.3***		**1.44± 0.5**	**1.19± 0.4**
7ά/15β-OH	**1.68± 0.6**	**2.50± 0.5***		**0.79± 0.2**	**0.74± 0.4**
6ά-OH	**2.64±1.1**	**3.45± 0.3**		**-**	**-**
6β-OH	**9.14± 4.6**	**12.67± 1.6**		**4.08± 1.2**	**3.49± 0.8**
16β-OH	**6.74± 2.3**	**7.97± 1.6***		**1.83± 0.3**	**1.84± 0.8**
2ά-OH	**12.31± 4.17**	**13.61± 3.0**		**22.79± 7.1**	**24.04± 12.0**

Significant $p < 0.05$ (*).

Table 3. Organ disposition of [^{3}H] vincristine in FVB wildtype and FVB/mrp1-/- mice. FVB and Mrp1-/- were injected with 1mg/kg [^{3}H] vincristine sulfate (5.3Ci/mmol), euthanized at 12 hours, organs removed and weighed. Portions were homogenized in 2% BSA/PBS and assayed for radioactivity by scintillation counting. Data is presented as average ng of vincristine/ g of tissue $\pm$ S.D. Statistical differences were determined by Student's t-test (*, $p \leq 0.05$)

Tissue	FVB (ng vinc/g)	Mrp1-/- (ng vinc/g)	Fold Change
Liver	2.8 $\pm$ 1.8	2.9 $\pm$ 2.3	1.0
Gall Bladder	5412.5 $\pm$ 1134.4	7155.1 $\pm$ 4754.0	1.3
Kidney	11.3 $\pm$ 8.0	36.3 $\pm$ 11.0	3.2*
Colon	543.0 $\pm$ 227.7	1302.7 $\pm$ 269.5	2.4*
Small Intestine	127.5 $\pm$ 52.1	468.3 $\pm$ 35.2	3.7*
Epididymis	111.2 $\pm$ 40.9	541.3 $\pm$ 111.1	4.9*
Heart	35.2 $\pm$ 24.0	43.8 $\pm$ 14.8	1.2
Lung	38.0 $\pm$ 21.5	64.5 $\pm$ 12.2	1.7
Stomach	1491.9 $\pm$ 2431.3	640.4 $\pm$ 475.7	0.4
Spleen	275.6 $\pm$ 79.9	251.4 $\pm$ 65.5	0.9
Testes	56.7 $\pm$ 32.9	100.8 $\pm$ 53.7	1.8
Brain	1.6 $\pm$ 0.7	2.1 $\pm$ 1.4	1.3
Feces	829 $\pm$ 315.1	470.7 $\pm$ 324.2	0.6

Figure legend:

Figure 1. Levels of mRNA in the kidneys, lung, and small intestine of FVB and FVB/Mrp1-/- mice mRNA levels of Ugt1a1 (A), Ugt1a2 (B), and Ugt2B(C) were determined by real-time PCR using primers specific for each Ugt. Data is the mean of 5-6 animals, each run in triplicate. Data is expressed as number of molecules of RNA/100 ng cDNA $\pm$ S.D. and data normalized to 18S. Statistical differences (*) were determined by students t-test ($p<0.05$). ND=Not detected.

Figure 2. mRNA levels of Sult1a1 (A) and Sult1a2 (B) in the kidneys, lung, and small intestine ofFVB and FVB/mrp1-/- mice. RNA levels were determined by real-time PCR using primers specific for each SULT. Data is the mean of 5-6 animals, each run in triplicate. Data is expressed as number of molecules of RNA / 100 ng cDNA +/- S.D. and normalized to 18S. Statistical differences (*) were determined by students t-test ($p<0.05$). ND=Not detected.

Figure 3. MRP mRNA levels in FVB and FVB/mrp1-/- mice, in the kidneys, lung, and small intestine. RNA levels were determined by real-time PCR using primers specific for each Mrp2 (a), Mrp3 (b), Mrp4 (c), and Mrp5 (d). The only significant changes were decreased expression of Mrp2 in the kidney. Data is the mean of 5-6 animals, each run in triplicate. Data is expressed as number of molecules of RNA/100 ng cDNA, +/- S.D. and normalized to 18S. Statistical differences (*) were determined by students t-test ($p<0.05$). ND=Not detected.

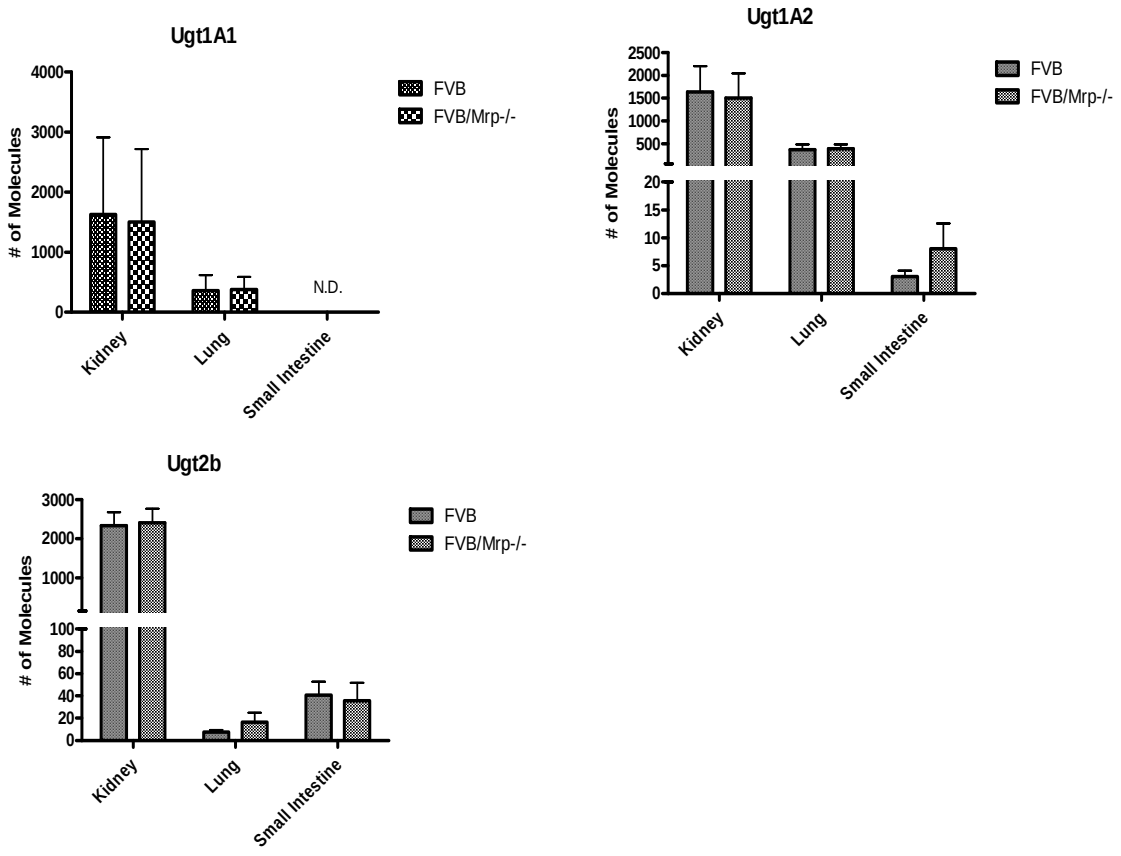

Ugt1A1
# of Molecules
4000
3000
2000
1000
0
N.D.
Kidney
Lung
Small Intestine
FVB
FVB/Mrp-/-
Ugt1A2
# of Molecules
2500
2000
1500
1000
500
20
15
10
5
0
Kidney
Lung
Small Intestine
FVB
FVB/Mrp-/-
Ugt2b
# of Molecules
3000
2000
1000
100
80
60
40
20
0
Kidney
Lung
Small Intestine
FVB
FVB/Mrp-/-

Figure 1.

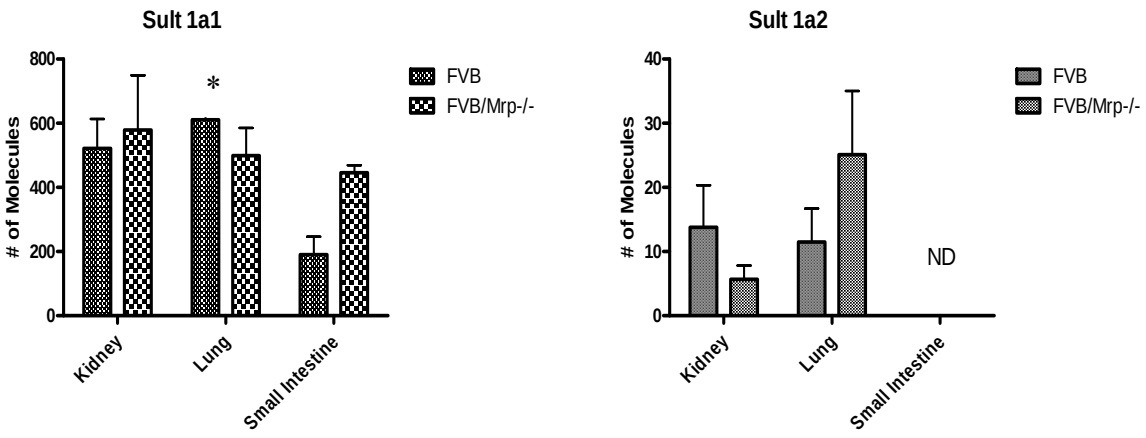

Sult 1a1
800
600
400
200
0
# of Molecules
*
FVB
FVB/Mrp-/-
Kidney
Lung
Small Intestine
Sult 1a2
40
30
20
10
0
# of Molecules
ND
FVB
FVB/Mrp-/-
Kidney
Lung
Small Intestine

Figure 2.

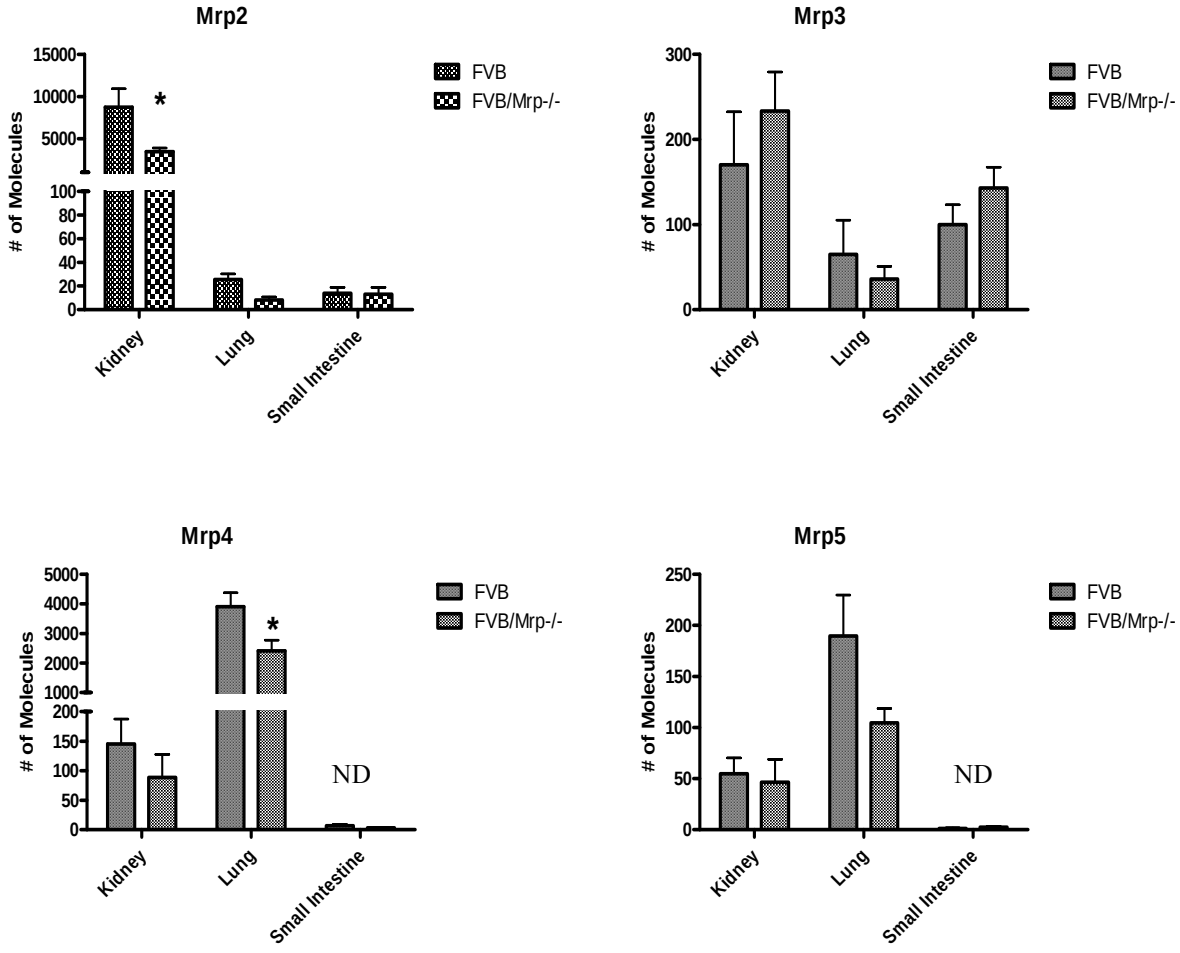

Mrp2
# of Molecules
15000
10000
5000
100
80
60
40
20
0
*
FVB
FVB/Mrp-/-
Kidney
Lung
Small Intestine
Mrp3
# of Molecules
300
200
100
0
FVB
FVB/Mrp-/-
Kidney
Lung
Small Intestine
Mrp4
# of Molecules
5000
4000
3000
2000
1000
200
150
100
50
0
*
ND
FVB
FVB/Mrp-/-
Kidney
Lung
Small Intestine
Mrp5
# of Molecules
250
200
150
100
50
0
ND
FVB
FVB/Mrp-/-
Kidney
Lung
Small Intestine

Figure 3

Chapter 3: Mice lacking Mrp1 have reduced testicular steroid hormone levels and alterations in steroid biosynthetic enzymes

Published: *General and Comparative Endocrinology.* May 15, 2010;167(1):51-59

JEFFREY C. SIVILS[1], IVEN GONZALEZ[1,3] AND LISA J. BAIN[2]*

[1]Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas, USA, and

[2]Department of Biological Sciences, Clemson University, Clemson, South Carolina, USA

[3]Current address: University of Texas Health Science Center at Houston Dental Branch, Houston, Texas, USA

*Address correspondence to: Lisa Bain, Clemson University, 132 Long Hall, Clemson, SC 29634. Phone: 1-864-656-5050; FAX: 1-864- 656-0435; email: lbain@clemson.edu

Abstract

The multidrug resistance-associated protein 1 (MRP1/ABCC1) is a member of the ABC active transporter family that can transport several steroid hormone conjugates, including 17β-estradiol glucuronide, dehydroepiandrosterone sulfate (DHEAS), and estrone 3-sulfate. The present study investigated the role that MRP1 plays in maintaining proper hormone levels in the serum and testes. Serum and testicular steroid hormone levels were examined in both wild-type mice and Mrp1 null mice. Serum testosterone levels were reduced 5-fold in mice lacking Mrp1, while testicular androstenedione, testosterone, estradiol, and dehydroepiandrosterone (DHEA) were significantly reduced by 1.7- to 4.5-fold in Mrp1 knockout mice. Investigating the mechanisms responsible for the reduction in steroid hormones in *Mrp1-/-* mice revealed no differences in the expression or activity of enzymes that inactivate steroids, the sulfotransferases or glucuronosyltransferases. However, steroid biosynthetic enzyme levels in the testes were altered. Cyp17 protein levels were increased by 1.6-fold, while Cyp17 activity using progesterone as a substrate was also increased by 1.4-2.0-fold in mice lacking Mrp1. Additionally, the ratio of 17β-hydroxysteroid dehydrogenase to 3β- hydroxysteroid dehydrogenase, and steroidogenic factor 1 to 3β-hydroxysteroid dehydrogenase were significantly increased in the testes of Mrp1-/- mice. These results indicate that *Mrp1-/-* mice have lowered steroid hormones levels, and suggests that upregulation of steroid biosynthetic enzymes may be an attempt to maintain proper steroid hormone homeostasis.

Key Words: Multidrug resistance-associated protein 1, ATP-binding cassette, testosterone, androstenedione, testes, Cyp17, 17β -hydroxysteroid dehydrogenase

3.1. Introduction

Members of the multidrug resistance-associated protein (MRP or ABCC) subfamily of transporters are responsible for the elimination of numerous endogenous ligands, drugs, and toxicants (Deeley, Westlake et al. 2006; Bakos and Homolya 2007). One member of the family, the multidrug resistance-associated protein 1 (MRP1 or ABCC1), extrudes phase II metabolites of steroid hormones such as 17β-estradiol glucuronide, dehydroepiandrosterone sulfate (DHEAS), and estrone 3-sulfate (Loe, Almquist et al. 1996; Qian, Song et al. 2001; Zelcer, Reid et al. 2003; Leslie, Deeley et al. 2005), along with other endogenous and exogenous substrates. MRP1 and other members of the ABCC family help maintain the blood-brain barrier, blood-cerebrospinal fluid barrier, and the blood-testes barrier, by removing xenobiotics and endogenous compounds from the nervous system or testes and transporting them back into the bloodstream (Wijnholds, Scheffer et al. 1998; Dallas, Zhu et al. 2003; Nies, Jedlitschky et al. 2004). In human tissues, MRP1 is expressed in the testes, adrenals, prostate, skin, esophagus, small intestine, large intestine, lung, heart, amnion epithelium, and the pancreas (Flens, Zaman et al. 1996; Zelcer, Reid et al. 2003; Aye, Paxton et al. 2007). In mice, Mrp1 is expressed in testes in both Sertoli and Leydig cells, as well as in the colon, heart, small intestine, kidney, and lungs (Stride, Valdimarsson et al. 1996; Peng, Cluzeaud et al. 1999). Organs that synthesize and respond to steroid hormones, such as the adrenals, testes and ovaries appear to have the highest levels of expression (Maher, Slitt et al. 2005).

In addition to cellular and organ localization, evidence from knockout mice also supports the idea that one function of MRP1 is to protect cells from damage. For example, etoposide

treatment damages the mucosal layer of the tongue in mice lacking Mrp1 (Wijnholds, Scheffer et al. 1998), while vincristine treatment of Mrp1 knockout mice results in toxicity to the bone marrow and reduces survival by 4-fold (Johnson, Finch et al. 2001; van Tellingen, Buckle et al. 2003). In addition, the presence of Mrp1 reduces tissue accumulation of the antibiotic grepafloxacin (Sasabe, Kato et al. 2004; Li, Kato et al. 2005). Triple knockout mice that lack Mdr1a, Mdr1b, and Mrp1, exposed to cigarette smoke for 6 months, had reduced numbers of inflammatory cells and IL-8 levels in the lungs of mice compared to control mice, which suggests an impaired inflammatory response (van der Deen, Timens et al. 2007). In the testes, Mrp1 is thought to play a role in maintaining the blood-testes barrier, preventing accumulation of toxicants as well as preventing the build-up of estrogen-like compounds (Wijnholds, Scheffer et al. 1998; Tribull, Bruner et al. 2003).

Although it has long been known that MRP1 can transport a variety of steroid hormones using *in vitro* models, its role in modulation of steroid hormone homeostasis in an animal model has not been previously described. In the testes, hormones are derived from pregnenolone after conversion through two different potential pathways. Th Δ^5 steroidogenic pathway involves the conversion of pregnenolone to DHEA and androstenediol, prior to the formation of testosterone. This is the dominant pathway in humans (Fluck, Miller et al. 2003). The Δ^4 pathway begins with the conversion of pregnenolone into progesterone, with androstenedione ultimately being converted into testosterone. This is the predominant pathway in rodents (Fevold, Lorence et al. 1989; Mathieu, Auchus et al. 2002), although 3β-hydroxysteroid dehydrogenase can convert hormones from the Δ^5 pathway into substrates involved in theΔ^4 pathway.

Testosterone and estradiol are both synthesized in the testes. Although both are needed for proper testicular functioning and for spermatogenesis, estradiol concentrations must be maintained at low levels to prevent testicular feminization and protect developing spermatozoa. To maintain proper estrogen levels, sulfotransferase 1E1 (sult1e1 or EST) catalyzes the formation of estrone 3-sulfate, which inactivates the hormone (reviewed in (Strott 1996; Song and Melner 2000). Because the resulting product is hydrophilic, investigators have hypothesized that estrone 3-sulfate is transported out via Mrp1 (Qian, Song et al. 2001). Therefore, this study examined the differences in steroid hormones levels and steroid hormone metabolizing enzymes in Mrp1 knockout mice to determine whether Mrp1 played a role in regulating circulating and testicular steroid hormone levels.

3.2. Materials and methods

3.2.1 FVB and FVB/mrp1-/- mice

Three sets of male FVB (control or wild-type mice) and Mrp1 knockout mice (FVB/*Mrp1-/-*) were obtained from Taconic Farms (Germantown, NY) at 4-5 weeks of age. They were individually housed until 10 weeks of age at 25$\pm$2^{o}C and 50% humidity and fed TestDiet 5001 rodent chow (Richmond, IN) before euthanization via a CO_2 overdose. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at El Paso. In the first set of mice, testes, livers, and blood were removed from six animals from each strain. One testis was snap-frozen in liquid nitrogen to prepare cytosol and microsomes, while the other was placed in Tri-Reagent (Sigma Chemical, St. Louis, MO) to obtain RNA. All samples were stored at -80^{o}C. Blood was collected, serum prepared by centrifugation, and stored at –20^{o}C for steroid hormone analyses. In the second and third set of

experiments, six additional male FVB and Mrp1-/- mice were obtained, housed, and euthanized as above. The testes were removed and weighed. Blood was collected, serum prepared by centrifugation, and stored at –20^{o}C for steroid hormone analyses. One testis was snap-frozen in liquid nitrogen to prepare cytosol and microsomes. The other testis was homogenized in 0.1M potassium phosphate buffer, pH 7.4, and testicular steroid hormones were extracted twice by the addition of 2mL of diethyl ether. The two extracts were combined, evaporated under nitrogen and resuspended in 1mL phosphate buffer (Jeyaraj, Grossman et al. 2005). The aqueous portions of the testes extracts were used to examine dehydroepiandrosterone sulfate (DHEAS). Extracts were stored at –20^{o}C.

3.2.2 Steroid hormone levels in serum and testes

Testosterone, progesterone, estradiol, and DHEAS concentrations were determined by EIA kits (Calbiotech, Spring Valley, CA for DHEAS; Cayman Chemical, Ann Arbor, MI for testosterone, progesterone, and estradiol), while androstenedione and DHEA concentrations were determined by RIA (Diagnostic Systems Laboratories, Webster, TX). To determine testosterone concentrations, 10µL of a 1:20 dilution of the testes extracts or 10µL of serum was used. For progesterone concentrations, 25µL of the testes extract and serum were used. For estradiol concentrations, 25µL serum and 100µL of the testicular extracts was used. For androstenedione, 5µL of both serum and testes extract were used. For DHEA, 50µL of serum and 100µL of the testicular extracts were used, while for DHEAS, 1µL of serum or 100µL of the aqueous portion of the testes sample was used. All samples were run in duplicate or triplicate. Results are expressed as amount of hormone per mL serum or amount of hormone per gram of tissue.

3.2.3 Changes in RNA abundance by QPCR

Total RNA from the testes and liver of each mouse was isolated using TRI-Reagent (Sigma, St. Louis, MO) and then treated with RNase-free DNase. To prepare cDNA, total RNA (2μg) was incubated with 50ng random hexamers, RNAsin, 10mM dNTP mix, and 200U Moloney murine leukemia virus (MMLV) reverse transcriptase at 37^{o}C for 1 hour. Quantitative PCR was performed in Bio-Rad's I-Cycler (Hercules, CA) using 0.2mM dNTPs, 0.25X Sybr green, 1U Taq polymerase (SABiosciences, Frederick, MD), along individual sets of primers for uridineglucuronosyltransferase 2b (Ugt2b), sulfotransferase 1e1 (Sult1e1), 3β-hydroxysteroid dehydrogenase 1 (3β-HSD1), 17β-HSD3, androgen receptor (AR), lutenizing hormone receptor (LHR), Cyp17, Cyp11A, steriodogenic acute regulatory protein (StAR), Cyp19, estrogen receptor α (Esr1), estrogen receptor β (Esr2), and steroidogenic factor-1 (SF-1) (Table 1). 18S rRNA was used as the housekeeper for Ugt2b and Sult1e1 to facilitate comparisons between the liver and testes, while GAPDH were used as housekeeper for all other genes. All PCR products had a denaturing step of 95°C for 15 seconds, an annealing /extension step at 61^{o}C (51^{o}C for GAPDH) for 1 minute for a total of 40 cycles. The cycle threshold values obtained from the real-time PCR were converted into starting number of molecules per 100ng cDNA using known concentrations of the specific gene product, which was normalized to the housekeeping gene. The standards were prepared by RT-PCR and sequenced to confirm their identity.

3.2.4 Estrogen sulfotransferase activity

One testes and a portion of the liver from each mouse was individually homogenized in buffer (250mM sucrose, 1mM EDTA, 10mM Tris, pH 7.4, containing 2µg/mL each aprotinin, leupeptin, and pepstatin) with a Dounce homogenizer. Microsomes were prepared by centrifuging the homogenate at 10,000xg for 10 minutes. The supernatant was removed and centrifuged at 100,000xg for 60 minutes. The cytosol was removed and stored at -80^{o}C. The microsomal pellet was resuspended and recentrifuged. The final pellet containing the microsomes was resuspended in homogenization buffer containing 10% glycerol and stored at –80^{o}C. Protein concentrations were determined according to Bradford (Bradford 1976) using bovine serum albumin as the standard. Estrogen sulfotransferase (EST) activity was determined by preincubating 100µg of either liver or testes cytosolic protein in 50 mM Tris-HCl, 7mM $MgCl_2$ buffer, pH 7.4, containing 100nM [^{3}H] estradiol (100Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) at 37^{o}C for 3 minutes. The reactions were started by the addition of 20µM PAPS and incubated for 30 minutes at 37^{o}C. The reactions were terminated by the addition of 250 mM Tris-HCl (pH 8.7) to alkalinize the solution and chloroform to separate the estradiol from its sulfated product (Miki, Nakata et al. 2002). EST formation was determined by removing 100µl of the aqueous phase and counting on a liquid scintillation counter.

3.2.5 Immunoblotting

Testicular microsomal proteins (2-20µg) were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose (Harlow and Lane 1988). The nitrocellulose membrane was blocked in 5% nonfat milk and then incubated with the primary antibody in TBST (Ugt2b:1:400 dilution; Cyp17: 1:200 dilution; both from Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing, the

blot was incubated with the appropriate secondary antibody (1:200-1:5000 dilution; Santa Cruz) in TBST. The blot was developed and the intensity of the bands quantified using the ImmunStar AP kit (BioRad) or the Luminol HRP kit (Santa Cruz) on a ChemiDoc XRS molecular imager (BioRad). Blots were stripped in 100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl, pH 6.7 at 50^{o}C. After rinsing in TBST, blots were reprobed using an actin (1:500 dilution; Sigma) or GAPDH antibody (1:1000 dilution; Imgenex, San Diego, CA) as a loading control, and were quantified as above.

3.2.6 Progesterone metabolism by CYP17

CYP17 hydroxylase assays were performed to assess hormone production using progesterone as a precursor. Testicular microsomal protein (50µg) was preincubated in 100mM potassium phosphate buffer (pH 7.4) containing 1µM([^{3}H]progesterone (120Ci/mmol, American Radiolabeled Chemicals) in a shaking water bath at 37^{o}C for 3 minutes. 1mM NADPH was added to initiate the reactions and the tubes were incubated for 20 minutes. The reaction was terminated and progesterone metabolites were extracted twice by the addition of 2mL ethyl acetate. The extracts were evaporated under nitrogen, dissolved in 35µL ethyl acetate, and spotted onto TLC plates. The TLC plates were resolved using a 3:1 mixture of chloroform and ethyl acetate. The identities of metabolites were determined by co-migration of authentic standards (Steraloids, Newport, RI). The TLC plates were exposed to autoradiography film, and individual metabolites were cut from the plates and quantitated by scintillation counting to determine the specific activity for each metabolite.

3.2.7 Statistical Analysis

Samples from each group were averaged and significant differences were determined by Student's t-test or Mann-Whitney ($p \leq 0.05$) using Graphpad Prism Software (San Diego, CA). Linear regression analyses were also performed using Graphpad Prism.

3.3 Results

3.3.1 Reductions in steroid hormone concentrations in Mrp1 knockout mice

Because MRP1 is known to transport several steroid hormones *in vitro*, their concentrations in the serum of wild-type and Mrp1 knockout mice were examined. Serum testosterone concentrations were reduced by 5-fold in the FVB/Mrp1-/- mice, although there were no differences in serum progesterone, androstenedione, DHEA, DHEAS, or estradiol levels (Figure 1). We hypothesized that since testosterone concentrations were reduced in the serum, its levels in the testes would be increased. Surprisingly, testosterone levels were actually reduced by 2.4-fold in the testes of mice lacking Mrp1, indicating low testosterone production (Figure 2). Progesterone levels were similar between both strains of mice. However, the other progesterone metabolites examined, androstenedione and estradiol, were significantly reduced by 4.5-fold and 1.7-fold, respectively, in Mrp1 knockout mice. Likewise, testicular DHEA concentrations were lowered by 2.1-fold (Figure 2).

Next, we determined whether testicular hormone concentrations correlated with one another using linear regression. Progesterone levels tended not to correlate with the other hormones, but the levels of DHEA, androstenedione, testosterone, and estradiol did highly correlate with one another (Table 2). Indeed, when comparing the ratio of two hormones in the testes of an individual mouse, there is always a higher level of progesterone in the Mrp1 knockout mice versus the control mice, relative to the other downstream hormones, including androstenedione and testosterone, as well as DHEA (Table 3). In contrast, there is a trend towards a reduction in testicular androstenedione levels relative to all other downstream hormones. The changes in steroid hormone concentrations in the *Mrp1-/-* mice, coupled with a higher relative ratio of progesterone versus androstenedione suggests that either (1) there is increased conjugation, inactivation, and/or elimination of androstenedione, testosterone, and estradiol in mice lacking Mrp1, or (2) an enzyme in the steroid hormone biosynthetic pathway is altered in mice lacking Mrp1, likely immediately preceding the formation of androstenedione.

3.3.2 Alterations in steroid conjugation

To test these hypotheses, the expression and activity of sulfotransferase 1e1 (SULT1E1) and uridine diphosphoglucuronoysltransferase 2b (UGT2B) were examined in the testes to determine whether increased conjugation was responsible for the reductions in testicular steroid hormone concentrations. There were no differences in SULT1E1 RNA expression or enzyme activity or in Ugt2b RNA and protein levels in the testes of wild-type and *Mrp1-/-* mice (Figure 3). There are also no changes in SULT1E1 activity and in UGT2b RNA expression in the liver of the two strains of mice (data not shown). Thus, an increase in androgen inactivation due to

increased conjugation does not appear to cause the reductions in testicular androstenedione, testosterone, or estradiol levels.

3.3.3. Changes in steroid biosynthetic enzymes

To test the second hypothesis, the levels of steroidogenic enzyme mRNA expression in the testes along with the transcription factor SF-1, as well as the androgen, estrogen, and lutenizing hormone receptors, were examined by QPCR to determine whether there were differences between control and Mrp1 knockout mice. There were no changes in the expression of StAR, Cyp11a, Cyp19, LHR, ERα, or ERβ mRNA levels (data not shown). AR levels were increased in the mice lacking Mrp1, but this was not quite statistically significant ($p=0.06$; data not shown). We also examined changes in other enzymes in the steroid biosynthetic pathway, including 3β-HSD1, which catalyzes the conversion of pregnenolone into progesterone, 17β-HSD3, which converts androstenedione into testosterone, and SF-1, which is a transcription factor involved in regulation of the enzymes in this pathway. Levels of 3β-HSD1 in the testes were unchanged between wild-type and *Mrp1-/-* mice (Table 4). 17β-HSD3 expression appeared to be upregulated in the mice lacking Mrp1, but this was not quite statistically significant ($p=0.068$; Table 4). However, the ratio of 17β-HSD3/3β-HSD1 expression and SF-1/3β-HSD1 in each individual mouse was significantly increased (Table 4). This indicates that mice lacking Mrp1 have upregulated steroidogenic gene expression to compensate for the lowered production of hormones.

Ultimately, it is the conversion of progesterone to androstenedione along the Δ^4 pathway that appears to be most impacted, because progesterone levels in the testes were similar between FVB and *Mrp1-/-* mice while androstenedione levels were reduced in *Mrp1-/-* mice. Therefore, we examined Cyp17 protein and activity. Cyp17 catalyzes the formation of 17-OH progesterone from progesterone, and then the formation of androstenedione from 17-OH progesterone. Transcript levels of Cyp17 were increased by 1.6-fold but this was not quite statistically different (data not shown). There was a significant 1.6-fold increase in Cyp17 protein in the testes of Mrp1 knockout mice (Figure 4A and B). The increase in protein expression was corroborated by with an increase in Cyp17 activity, with the production of 17-OH progesterone increased by 1.4-fold and the production of androstenedione increased by 2- fold (Figure 4C). Taken together, these data indicate that mice lacking the Mrp1 transporter have upregulated steroid biosynthetic enzymes in an attempt to mitigate the reduction in serum and testicular hormones.

3.4. Discussion

Maintaining steroid hormone homeostasis is essential for ensuring sexual differentiation, development, and reproduction. Estrogens are required for proper development and maintenance of the male reproductive tract, as well as for male fertility ((Hess 2003; Akingbemi 2005), but too high of estrogen levels have shown to be detrimental (Akingbemi 2005; Delbès, Levacher et al. 2006; Toppari 2008). For example, administration of exogenous estradiol to adult male rats resulted in significant decreases in serum and testicular testosterone levels. Depending on the estradiol concentration administered, serum testosterone levels were reduced between 4.4- to 18-

fold while testicular testosterone levels were reduced 7.8- to 11.6-fold (D'Souza, Gill-Sharma et al. 2005). Although the true endogenous function of MRP1 is not known, it has been hypothesized that MRP1 acts to maintain steroid hormone homeostasis because it can actively transport the estrogen metabolites 17β-estradiol glucuronide and estrone 3-sulfate, as well as DHEAS (Loe, Almquist et al. 1996; Zelcer, Reid et al. 2003; Chen, Guo et al. 2005). Thus, because Mrp1 expression is quite high in the testes (Flens, Zaman et al. 1996; Stride, Valdimarsson et al. 1996; Peng, Cluzeaud et al. 1999; Maher, Slitt et al. 2005), one role that Mrp1 likely plays is to help protect developing spermatozoa from excessively high steroid hormone levels.

The present study indicates that mice lacking the Mrp1 transporter have reduced concentrations of androstenedione, testostereone, estradiol, and DHEA in their testes, which to our knowledge, is the first time tissue reductions in steroid hormone levels has been shown in mice lacking one of the MRP family members. Furthermore, linear regression demonstrated that all of the testicular hormone concentrations correlated with one another except for progesterone. For example, mice lacking Mrp1 have 2.8- and 3.3-fold more testicular progesterone than testosterone and androstenedione, respectively. There is a similar trend with a higher ratio of progesterone to estradiol in the knockout mice. In contrast, testicular androstenedione levels were lower relative to all other downstream hormones. Although these were not statistically significant, the hormone ratios in the Mrp1 knockout mice were consistently half the values of the control mice (0.46-0.55 fold reduction), with all *p*-values being 0.075-0.099. These data suggest that the enzyme that converts progesterone into androstenedione, Cyp17, might be altered in these mice.

Indeed, Cyp17 protein levels and activity were significantly increased between 1.4- to 2-fold in the testes of Mrp1 knockout mice. Transcript levels of Cyp17 were also increased by 1.6-fold but this was not quite statistically different (data not shown). It appears that Cyp17 is therefore upregulated in *Mrp1-/-* mouse testes to compensate for the low levels of androstenedione and testosterone. Although there are several transcription factors that regulate Cyp17 expression, the predominant one appears to be SF-1 (Nr5a1) (Mellon, Compagnone et al. 1998; Zhang, Compagnone et al. 2001; Busygina, Vasiliev et al. 2005; Ozbay, Leon et al. 2006; Patel, Beshay et al. 2009; Shi, Schonemann et al. 2009). SF-1 mRNA levels were increased by 1.7-fold in the testes of mice lacking Mrp1, although this was not statistically different. Earlier studies had demonstrated that Cyp17 is regulated by LH secretion via the cAMP signaling pathway (Anakwe and Payne 1987; Sewer and Waterman 2002). cAMP can increase Cyp17 mRNA levels, and this effect can be reversed with testosterone exposure (Payne and Sha 1991). These studies suggest that reduced testosterone levels would cause an increase in Cyp17 expression, consistent with our findings.

The compensatory upregulation in Cyp17 is corroborated by a 1-6-fold increase in the transcript levels of 17β-Hsd3, which is the enzyme that converts androtenedione into testosterone. mRNA levels of the third enzyme in the pathway, 3β-Hsd1, which converts pregenolone into progesterone, was not altered between the two strains. However, the ratio of 17β-Hsd3 to 3βHsd1 in the testes was significantly increased in mice lacking Mrp1, again suggesting an upregulation of steroidgenic enzymes after the formation of progesterone. A recent study determined that testicular 17β-Hsd activity in mice was 0.0153 μmol NAD/minute/ mg protein

and 3β-Hsd activity was 0.0246μmol NADH/minute/ mg protein (Harini et al., 2009).This ratio of 0.62 in their study is in line with our findings in FVB mice, in which the ratio of 17β-Hsd to 3β-Hsd transcript levels was 0.7. In the *Mrp1-/-* mice, this ratio is much higher, again indicating an upregulation of 17β-Hsd3. Like Cyp17, 17β-Hsd3 also requires LH and androgen stimulation to maintain proper expression (Baker, Sha et al. 1997; Tsai-Morris, Khanum et al. 1999). However, cAMP stimulation is not needed for the expression of 3β-HSD (reviewed in Payne and Youngblood, 1995). Indeed, the promoter region of 3β-HSD does not have typical steroid regulatory elements, and so it is assumed that its regulation is mediated by indirect interactions with other transcription factors, such as Stat proteins and NF-κB (Reichardt, Kaestner et al. 1998; Simard, Ricketts et al. 2005). Because of the indirect interactions, many investigators consider 3β-HSD to be constitutively expressed enzyme. This is in agreement with the present study, in which 3β-HSD1 levels were unchanged between the two strains of mice.

Additional potential mechanisms responsible for the reductions in testicular steroid hormones might be due to changes in cholesterol uptake. However, we did not see changes in StAR gene expression or in serum cholesterol levels (data not shown), and other studies have not reported pathological differences in the testes between untreated wild-type and Mrp1 knockout mice that would indicate altered cholesterol uptake or storage (Wijnholds, Scheffer et al. 1998; Tribull, Bruner et al. 2003). Mice lacking estrogen sulfotransferase have increased lipid deposition in Leydig cells, but the accumulation of cholesterol esters is found only in older animals (>18 months). Two- to three-month old *Sult1e-/-* mice did not display this phenotype (Tong, Christenson et al. 2004). The mice in our study were 10 weeks old, and this may have impeded our ability to detect additional differences such as lipid deposition between the two strains.

Another mechanism causing changes in steroid hormones might be due to alterations in LH levels. Serum LH was assayed, but due to the limited amount of serum, we were never able to obtain values above the detection limits of the assay. However, LH receptor gene expression was unchanged between the two strains of mice (data not shown). Additionally, reduced steroid hormone concentrations do not always translate into increased LH levels. For example, administration of exogenous estradiol to adult male rats resulted in significant decreases in serum and testicular testosterone levels. At the lowest concentration of estradiol, both serum FSH and LH were reduced, but returned to control levels with higher estradiol concentrations (D'Souza, Gill-Sharma et al. 2005). Indeed, constant stimulation of gonadal cells by LH may alter negative feedback loops either in the HPG axis or short loops within the gonadal tissue itself (Taniguchi, Couse et al. 2007).

It has been long known that steroid hormone concentrations in the gonads are regulated, in part, through negative feedback loops. However, after chronic stimulation or exposure to a stressor, an organism may over- or under-compensate for that given perturbation, which has been part of the difficulty in modeling dose-responses to stressors in the reproductive system (Andersen, Thomas et al. 2005). The results of the present study indicate that mice have the ability to compensate for the loss of Mrp1 in their testes by upregulating Cyp17 and altering the ratio of 17βHsd to 3βHsd. The compensatory responces in the testes sen in our study are corroborated by recent studies in a model fish species. Fathead minnows were exposed to the Cyp19/aromatase inhibitor fadrozole, which reduced estradiol levels in the serum and ovaries, but increased Cyp19, Cyp11a, StAR, and FSH receptor mRNA levels (Villeneuve, Mueller et al. 2009). Exposure to prochloraz, which inhibits both Cyp19 and Cyp17, along with acting as an androgen

receptor antagonist, reduced testosterone and estradiol levels in the serum of male fish, but increased testicular expression of AR, Cyp17, and Cyp11a mRNA (Ankley, Bencic et al. 2009). In both of these studies, the investigators noticed an overcompensation once the stressor was removed, indicating that the increase in transcript levels serve to offset the reductions in steroid hormone levels. This is similar to the findings of the current study, in which androstenedione and testosterone concentrations were low, yet transcript, protein, and activity of Cyp17 were increased.

In conclusion, mice lacking Mrp1 have reduced concentrations of testicular steroids, including androstenedione, testostereone, estradiol, and DHEA, but have increased steroid biosynthetic enzymes. These results suggest that upregulation of steroid biosynthetic enzymes may be an attempt to maintain proper steroid hormone homeostasis due to the loss of the Mrp1 transporter.

Acknowledgments

The authors thank W.S. Baldwin and G.A. LeBlanc for critically reviewing this work. This study was supported by NIH grants ES012417, ES012417-01S1, and Clemson University Faculty Development funds.

References

Akingbemi, B.T., 2005. Estrogen regulation of testicular function. Reprod .Biol. Endocrinol. 3, 51.

Anakwe, O.O., Payne, A.H., 1987. Noncoordinate regulation of *de novo* synthesis of cytochrome P450 cholesterol side-chain cleavage and cytochrome $P450_{17a}$-hdryoxylase $C_{17\text{-}20}$ lyase in mouse Leydig cell cultures: relation to steroid production. Mol. Endocrinol .1, 595-603.

Andersen, M.E., Thomas, R.S., Gaido, K.W., Conolly, R.B., 2005. Dose-response modeling in reproductive toxicology in the systems biology era. Reprod.Toxicol. 19, 327-337.

Ankley, G.T., Bencic, D.C., Cavallin, J.E., Jensen, K.M., Kahl, M.D., Makynen, E.A., Martinovic, D., Mueller, N.D., Wehmas, L.C., Villeneuve, D.L., 2009. Dynamic nature of alterations in the endocrine system of fathead minnows exposed to the fungicide prochloraz. Toxicol. Sci. 112, 344-353.

Aye, I.L., Paxton, J.W., Evseenko, D.A., Keelan, J.A., 2007. Expression, localisation and activity of ATP binding cassette (ABC) family of drug transporters in human amnion membranes. Placenta 28, 868-877.

Baker, P.J., Sha, J.H., O'Shaughnessy, P.J., 1997. Localisation and regulation of 17β-hydroxysteroid dehydrogenase type 3 mRNA during development in the mouse testis Mol. Cell. Endocrin. 133, 127-133.

Bakos, E., Homolya, L., 2007. Portrait of multifaceted transporter, the multidrug resistance-associated protein 1 (MRP1/ABCC1). Pflugers Arch. 453, 621-641.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. Anal. Biochem. 72, 248-254.

Busygina, T.V., Vasiliev, G.V., Klimova, N.V., Ignatieva, E.V., Osadchuk, A.V., 2005. Binding sites for transcription factor SF-1 in promoter regions of genes encoding mouse steroidogenesis enzymes 3betaHSDI and P450c17. Biochemistry (Mosc) 70, 1152-1156.

Chen, Z.S., Guo, Y., Belinsky, M.G., Kotova, E., Kruh, G.D., 2005. Transport of bile acids, sulfated steroids, estradiol 17-b-D-glucuronide, and leukotriene C_4 by human multidrug resistance protein 8 (ABCC11). Mol. Pharmacol. 67, 545-557.

D'Souza, R., Gill-Sharma, M.K., Pathak, S., Kedia, N., Kumar, R., Balasinor, N., 2005. Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats. Mol. Cell. Endocrinol. 28, 41-48.

Dallas, S., Zhu, X., Baruchel, S., Schlichter, L., Bendayan, R., 2003. Functional expression of the multidrug resistance protein 1 in microglia. J. Pharmacol. Exp. Ther. 307, 282-290.

Deeley, R.G., Westlake, C., Cole, S.P., 2006. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol. Rev. 86, 849-899.

Delbès, G., Levacher, C., Habert, R., 2006. Estrogen effects on fetal and neonatal testicular development. Reproduction 132, 527-538.

Fevold, H.R., Lorence, M.C., McCarthy, J.L., Trant, J.M., Kagimoto, M., Waterman, M.R., Mason, J.I., 1989. Rat P450-17a from testis: characterization of a full-length cDNA encoding a unique steroid hydroxylase capable of catalyzing both D^4 and D^5-steroid-17, 20-lyase reactions. Mol. Endocrinol. 3, 968-975.

Flens, M.J., Zaman, G.J.R., van der Valk, P., Izquierdo, M.A., Schroeijers, A.B., Scheffer, G.L., van der Groep, P., de Haas, M., Meijer, C.J.L.M., Scheper, R.J., 1996. Tissue distribution of the multidrug resistance protein. Amer. J. Pathol. 148, 1237-1247.

Fluck, C.E., Miller, W.L., Auchus, R.J., 2003. The 17,20-lyase activity of cytochrome P450c17 from human fetal testis favors the D^5 steroidogenic pathway. J. Clin. Endocrinol. Metab. 88, 3762-3766.

Harini, C., Sainath, S.B., Reddy, P.S., 2009. Recovery of suppressed male reproduction in mice exposed to progesterone during embryonic development by testosterone. Reproduction 137, 439-448.

Harlow, E., Lane, D., 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hess, R.A., 2003. Estrogen in the adult male reproductive tract: a review. Reprod. Biol. Endocrinol. 9, 52.

Jeyaraj, D.A., Grossman, G., Petrusz, P., 2005. Altered bioavailability of testosterone in androgen-binding protein-transgenic mice. Steroids 70, 704-714.

Johnson, D.R., Finch, R.A., Lin, Z.P., Zeiss, C.J., Sartorelli, A.C., 2001. The pharmacological phenotype of combined multidrug-resistance mdr1a/1b- and mrp1-deficient mice. Cancer Res. 61, 1469-1476.

Leslie, E.M., Deeley, R.G., Cole, S.P., 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 204, 216-237.

Li, Q., Kato, Y., Sai, Y., Imai, T., Tsuji, A., 2005. Multidrug resistance-associated protein 1 functions as an efflux pump of xenobiotics in the skin. Pharm. Res. 22, 842-846.

Loe, D.W., Almquist, K.C., Cole, S.P.C., Deeley, R.G., 1996. ATP-dependent 17b-estradiol 17-(b-D-glucuronide) transport by multidrug resistance protein (MRP). J. Biol. Chem. 271, 9683-9689.

Maher, J.M., Slitt, A.L., Cherrington, N., Cheng, X., Klaassen, C.D., 2005. Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice. Drug Metab. Dispos. 33, 947-955.

Mathieu, A.P., Auchus, R.J., LeHoux, J.G., 2002. Comparison of the hamster and human adrenal P450c17 (17a-hydroxylase/17,20-lyase) using site directed mutagenesis and molecular modeling. J. Steroid Biochem. Mol. Biol. 80, 99-107.

Mellon, S.H., Compagnone, N.A., Zhang, P., 1998. Orphan receptors, proto-oncogenes and other nuclear factors regulate P450C17 gene transcription. Endocr. Res. 24, 505-513.

Miki, Y., Nakata, T., Suzuki, T., Darnel, A.D., Moriya, T., Kaneko, C., Hidaka, K., Shiotsu, Y., Kusaka, H., Sasano, H., 2002. Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues. J. Clin. Endocrinol. Metab. 87, 5760-5768.

Nies, A.T., Jedlitschky, G., König, J., Herold-Mende, C., Steiner, H.H., Schmitt, H.P., Keppler, D., 2004. Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. Neuroscience 129, 349-360.

Ozbay, R., Leon, A., Patel, P., Sewer, M.B., 2006. Cyclic adenosine 5'-monophosphate-dependent sphingosine-1-phosphate biosynthesis induces human CYP17 gene transcription by

activating cleavage of sterol regulatory element binding protein 1. Endocrinology 147, 1427–1437.

Patel, S.S., Beshay, V.E., Escobar, J.C., Suzuki, T., Carr, B.R., 2009. Molecular mechanism for repression of 17{alpha}-hydroxylase expression and androstenedione production in granulosa cells. J. Clin. Endocrinol. Metab. Epub.

Payne, A.H., Sha, L.L., 1991. Multiple mechanisms for regulation of 3ß-hydroxysteroid dehydrogenase/5-4 isomerase, 17-hydroxylase/C17–20 lyase cytochrome P450, and cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid levels in primary cultures of mouse Leydig cells. Endocrinology 129, 1429–1435.

Payne, A.H., Youngblood, G.L., 1995. Regulation of expression of steroidogenic enzymes in Leydig cells. Biol. Reprod. 52, 217-225.

Peng, K.-C., Cluzeaud, F., Bens, M., Van Huyen, J.P., Wioland, M.A., Lacave, R., Vandewalle, A., 1999. Tissue and cell distribution of the multidrug resistance-associated protein (MRP) in mouse intestine and kidney. J. Histochem. Cytochem. 47, 757-768.

Qian, Y.-M., Song, W.-C., Cui, H., Cole, S.P., Deeley, R.G., 2001. Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1. J. Biol. Chem. 276, 6404-6411.

Reichardt, H.M., Kaestner, K.H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., Schutz, G., 1998. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93, 531–541.

Sasabe, H., Kato, Y., Suzuki, T., Itose, M., Miyamoto, G., Sugiyama, Y., 2004. Differential involvement of multidrug resistance-associated protein 1 and P-glycoprotein in tissue distribution and excretion of grepafloxacin in mice. J. Pharmacol. Exp. Ther. 310, 648-655.

Sewer, M.B., Waterman, M.R., 2002. Adrenocorticotropin/cyclic adenosine 3',5'-monophosphate-mediated transcription of the human CYP17 gene in the adrenal cortex is dependent on phosphatase activity. Endocrinology 143, 1769–1777.

Shi, Y., Schonemann, M.D., Mellon, S.H., 2009. Regulation of P450c17 expression in the early embryo depends on GATA factors. Endocrinology 150, 946-956.

Simard, J., Ricketts, M.-L., Gingras, S., Soucy, P., Feltus, F.A., Melner, M.H., 2005. Molecular biology of the 3ß-hydroxysteroid dehydrogenase/5-4 isomerase gene family Endocr. Rev. 26, 525-582.

Song, W.C., Melner, M.H., 2000. Steroid transformation enzymes as critical regulators of steroid action in vivo. Endocrinology 141, 1587-1589.

Stride, B.D., Valdimarsson, G., Gerlach, J.H., Wilson, G.M., Cole, S.P.C., Deeley, R.G., 1996. Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter. Mol. Pharmacol. 49, 962-971.

Strott, C.A., 1996. Steroid sulfotransferases. Endocr. Rev. 17, 670-697.

Taniguchi, F., Couse, J.F., Rodriguez, K.F., Emmen, J.M., Poirier, D., Korach, K.S., 2007. Estrogen receptor-alpha mediates an intraovarian negative feedback loop on thecal cell steroidogenesis via modulation of Cyp17a1 (cytochrome P450, steroid 17alpha-hydroxylase/17,20 lyase) expression. FASEB J. 21, 586-595.

Tong, M.H., Christenson, L.K., Song, W.C., 2004. Aberrant cholesterol transport and impaired steroidogenesis in Leydig cells lacking estrogen sulfotransferase. Endocrinology 145, 2487-2497.

Toppari, J., 2008. Environmental endocrine disrupters. Sex. Dev. 2, 260-267.

Tribull, T.E., Bruner, R.H., Bain, L.J., 2003. The multidrug resistance-associated protein 1 (MRP1/ABCC1) transports methoxychlor and protects the seminiferous epithelium from injury. Toxicol. Lett. 142, 61-70.

Tsai-Morris, C.H., Khanum, A., Tang, P.-Z., Dufau, M.L., 1999. The rat 17ß-hydroxysteroid dehydrogenase type III: molecular cloning and gonadotropin regulation. Endocrinology 40, 3534-3542.

van der Deen, M., Timens, W., Timmer-Bosscha, H., van der Strate, B.W., Scheper, R.J., Postma, D.S., de Vries, E.G., Kerstjens, H.A., 2007. Reduced inflammatory response in cigarette smoke exposed Mrp1/Mdr1a/1b deficient mice. Respir. Res. 8, 49.

van Tellingen, O., Buckle, T., Jonker, J.W., van der Valk, M.A., Beijnen, J.H., 2003. P-glycoprotein and Mrp1 collectively protect the bone marrow from vincristine-induced toxicity in vivo. Br. J. Cancer 89, 1776-1782.

Villeneuve, D.L., Mueller, N.D., Martinovic, D., Makynen, E.A., Kahl, M.D., Jensen, K.M., Durhan, E.J., Cavallin, J.E., Bencic, D., Ankley, G.T., 2009. Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor. Environ. Health Perspect. 117, 624-631.

Wijnholds, J., Scheffer, G.L., van der Valk, M., van der Valk, P., Beijnen, J.H., Scheper, R.J., Borst, P., 1998. Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. J. Exp. Med. 188, 797-808.

Zelcer, N., Reid, G., Wielinga, P., Kuil, A., van der Heijden, I., Schuetz, J.D., Borst, P., 2003. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). Biochem. J. 371, 361-367.

Zhang, P., Compagnone, N.A., Fiore, C., Vigne, J.L., Culp, P., Musci, T.J., Mellon, S.H., 2001. Developmental gonadal expression of the transcription factor SET and its target gene, P450c17 (17-hydroxylase/c17,20 lyase). DNA Cell. Biol. 20, 613–624.

Figure legend:

Figure 1. Alterations in steroid hormone levels in the serum. All serum samples were run in duplicate or triplicate. Values are the average $\pm$ standard deviation of 4-6 mice per group. Statistical differences (*) were determined using Student's t-test ($p \leq 0.05$).

Figure 2. Alterations in steroid hormone concentrations in the testes. All samples testicular samples were run in duplicate or triplicate. Values are the average $\pm$ standard deviation for 5-6 mice per group. Statistical differences (*) were determined using Student's t-test ($p \leq 0.05$).

Figure 3. Sulfotransferase and glucuronosyltransferase expression and activity do not differ in the testes of FVB and FVB/*Mrp1-/-* mice. Sult1e1 and Ugt2b RNA expression was determined by QPCR (A). The data is expressed in number of molecules/100ng cDNA $\pm$ standard deviation. All samples were normalized to 18S rRNA, with each sample run in triplicate (n=5=6). SULT1E1 activity was measured by EST metabolite formation and is

expressed as pmol/mg protein (B). All samples were run in triplicate (n=6) and the data is expressed as the average of two separate assays. UGT2B protein levels were determined by immunoblotting (C) and quantified by densitometry, using GAPDH as a loading control. Data is expressed as GAPDH-corrected raw intensity values (n=4) and are the average of two separate blots (D).

Figure 4. The ratio of 17samples were normalized to 18S rRNA, with ea*Mrp1-/-* mice. The ratio of 17β-HSD3 to 3β-HSD1 mRNA expression (A) and SF-1 to 3βHSD1 (B) for each individual mouse was calculated. Statistical differences (*) were determined using Student's t-test. ($p \leq 0.05$).

Figure 5. Increased testicular Cyp17 protein expression and activity in *Mrp1-/-* mice. Protein levels of Cyp17 were determined by immunoblotting (A) and quantified by densitometry (B), using actin as a loading control. Data is expressed as actin-corrected raw density values (n=4) and are representative of three separate blots. Cyp17 activity was determined by scintillation counting (C), and the data is presented as the average $\pm$ standard deviation (n=6) of 2 different assays. Statistical differences (*) were determined using Student's t-test ($p \leq 0.05$).

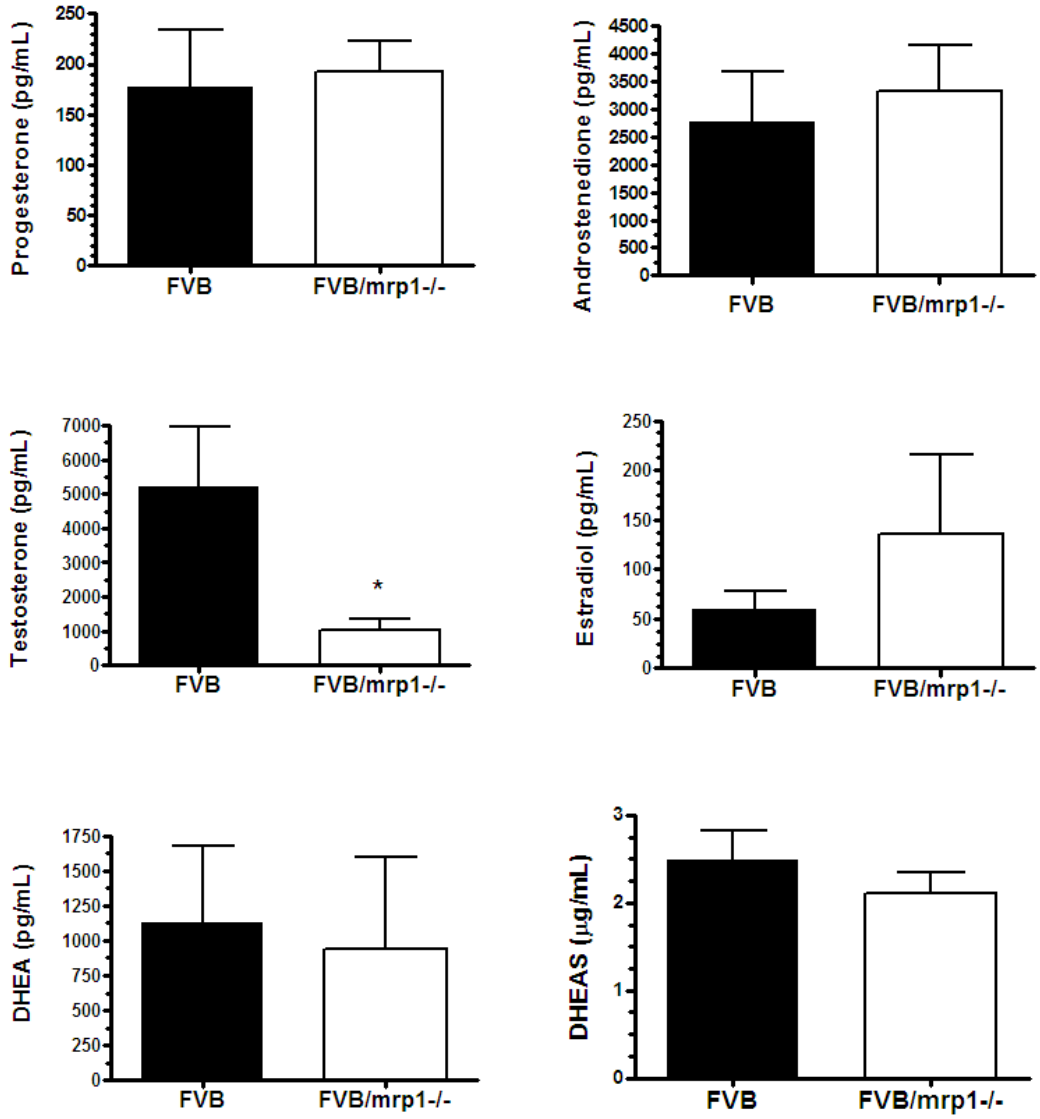

Progesterone (pg/mL)
250
200
150
100
50
0
FVB
FVB/mrp1-/-
Androstenedione (pg/mL)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
FVB
FVB/mrp1-/-
Testosterone (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
*
FVB
FVB/mrp1-/-
Estradiol (pg/mL)
250
200
150
100
50
0
FVB
FVB/mrp1-/-
DHEA (pg/mL)
1750
1500
1250
1000
750
500
250
0
FVB
FVB/mrp1-/-
DHEAS (µg/mL)
3
2
1
0
FVB
FVB/mrp1-/-

Figure 1

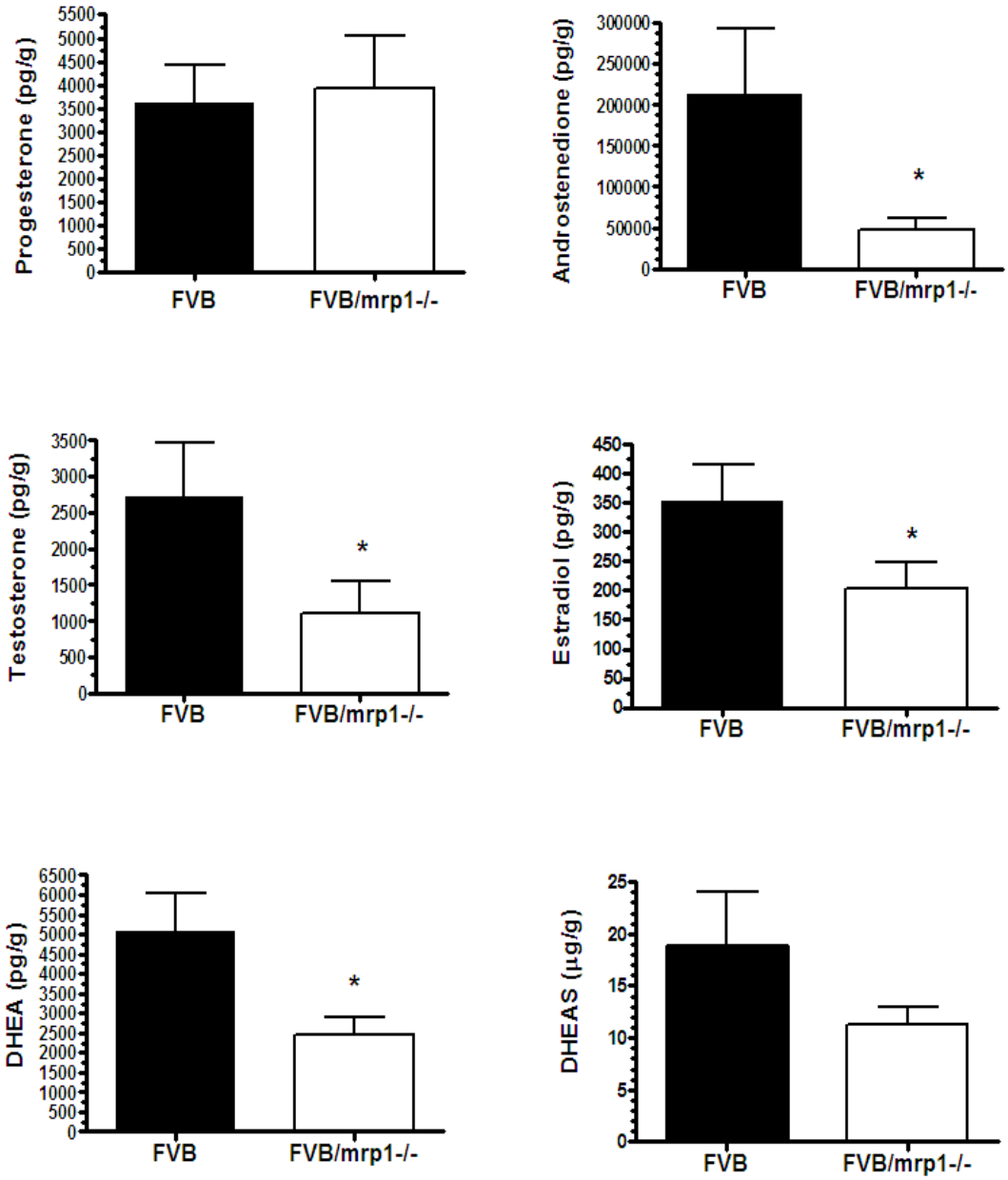

Progesterone (pg/g)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
FVB
FVB/mrp1-/-
Androstenedione (pg/g)
300000
250000
200000
150000
100000
50000
0
*
FVB
FVB/mrp1-/-
Testosterone (pg/g)
3500
3000
2500
2000
1500
1000
500
0
*
FVB
FVB/mrp1-/-
Estradiol (pg/g)
450
400
350
300
250
200
150
100
50
0
*
FVB
FVB/mrp1-/-
DHEA (pg/g)
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
FVB
FVB/mrp1-/-
DHEAS (µg/g)
25
20
15
10
5
0
FVB
FVB/mrp1-/-

Figure 2

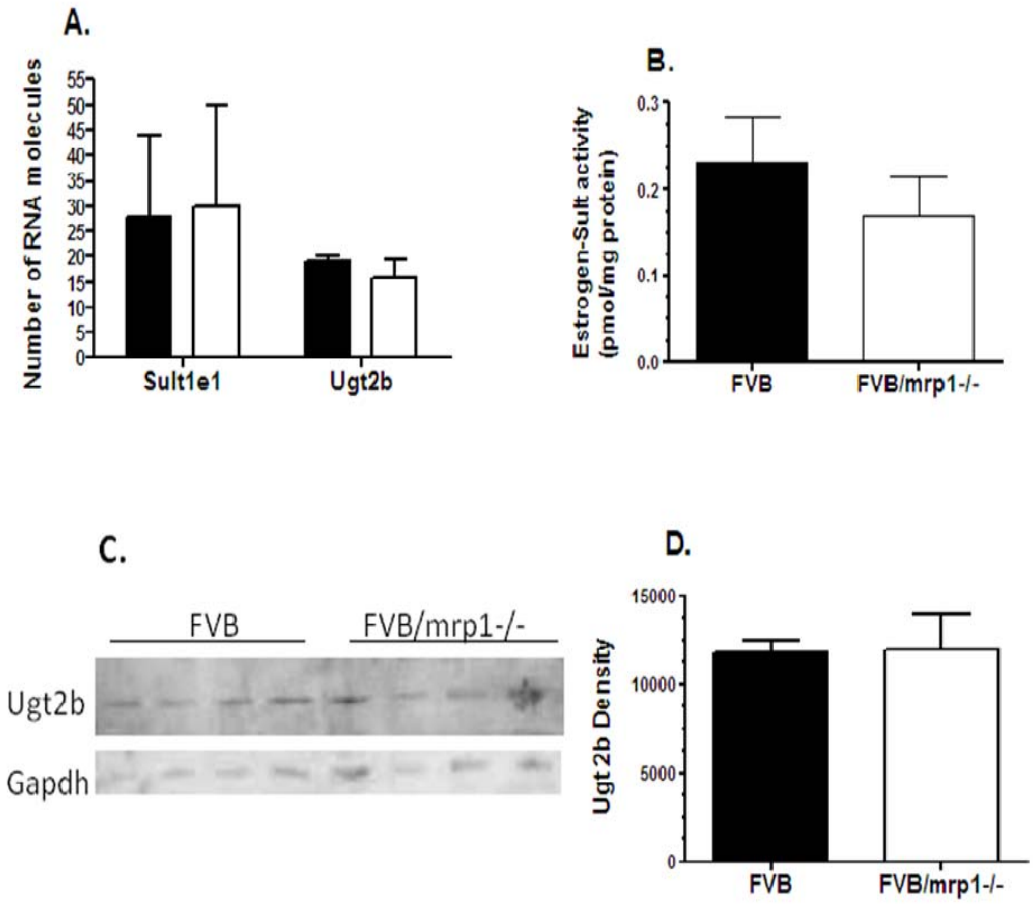

A.
Number of RNA molecules
55
50
45
40
35
30
25
20
15
10
5
0
Sult1e1
Ugt2b
B.
Estrogen-Sult activity (pmol/mg protein)
0.3
0.2
0.1
0.0
FVB
FVB/mrp1-/-
C.
FVB
FVB/mrp1-/-
Ugt2b
Gapdh
D.
Ugt 2b Density
15000
10000
5000
0
FVB
FVB/mrp1-/-

Figure 3.

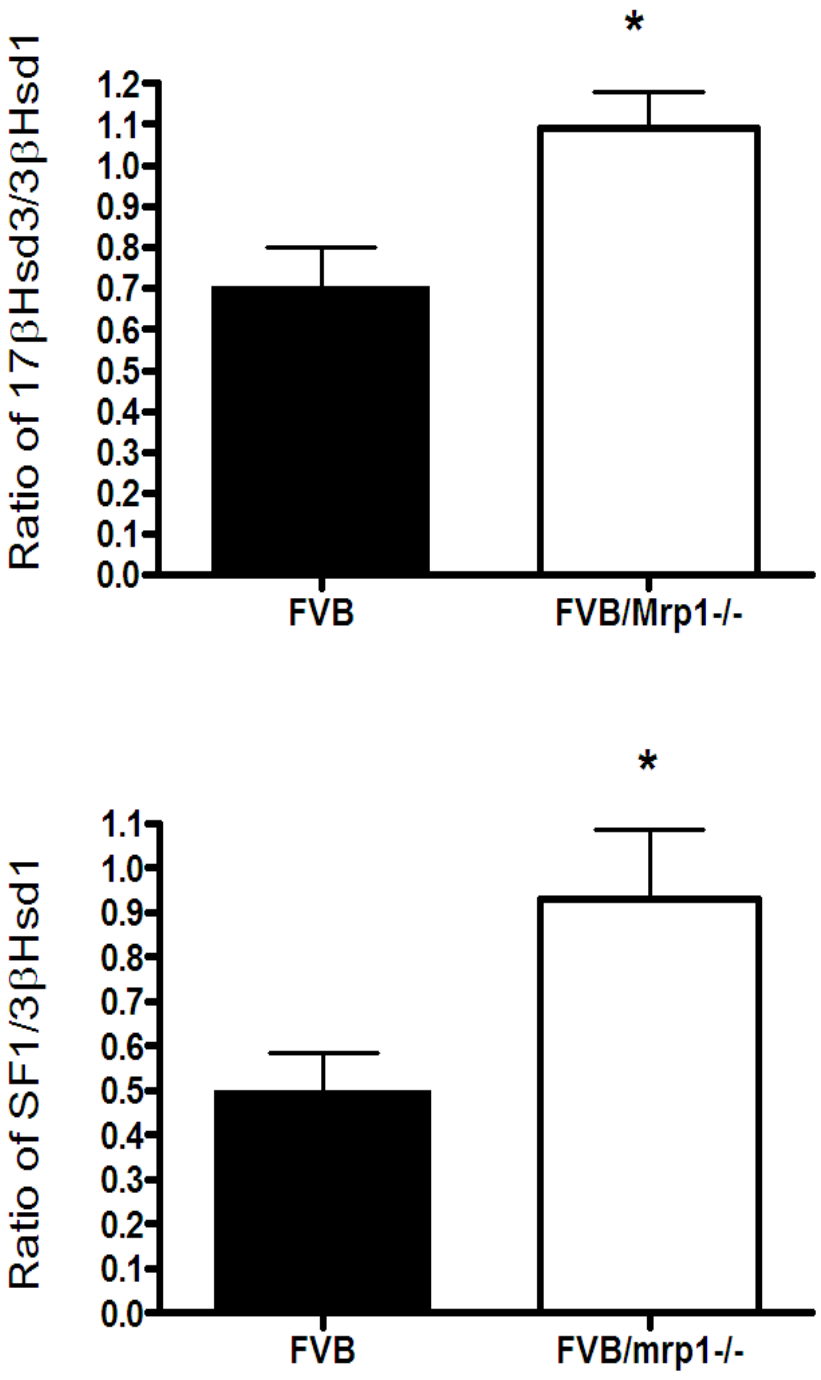

Ratio of 17βHsd3/3βHsd1
*
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FVB
FVB/Mrp1-/-
Ratio of SF1/3βHsd1
*
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FVB
FVB/mrp1-/-

Figure 4

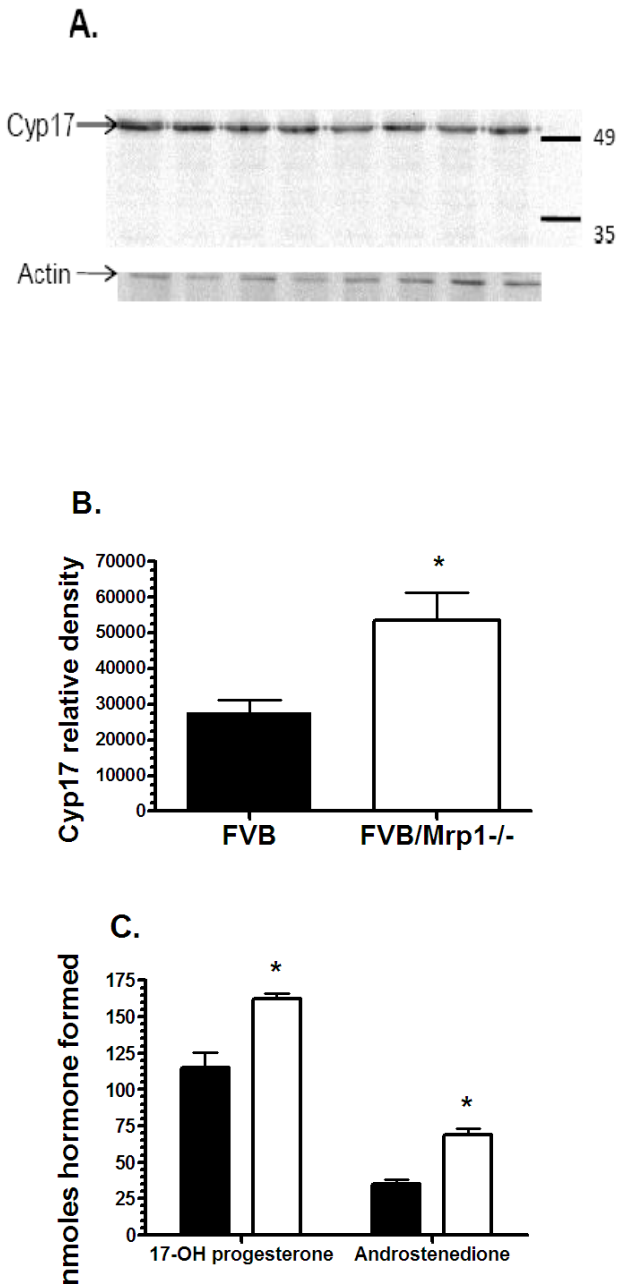

A.
Cyp17
49
35
Actin
B.
Cyp17 relative density
70000
60000
50000
40000
30000
20000
10000
0
*
FVB
FVB/Mrp1-/-
C.
nmoles hormone formed
175
150
125
100
75
50
25
0
*
*
17-OH progesterone
Androstenedione

Figure 5

Table 1. Primer sets for QPCR

Name	Accession #	Forward	Reverse
Ugt2b	NP_690024	5'-agttgagacaatgggccaag-3'	5'- gttgggtgaggaaactccaa-3'
Sult1e1	BC034891	5'- tgatgccagaggaaatgatg-3'	5'-tgggaagtggttcttccagt-3'
3βHSD1	BC052659	5'- tagcaagtacagaggcacaagcca-3'	5'- tgtgagtgggttagtgactggcaa-3'
17βHSD3	U66827	5'- taacaagatgaccaagaccgccga-3'	5'- gatttcatgagcaaggcagccaca-3'
AR	X53779	5'-tcaagggaggttacgccaaaggat-3'	5'-acagagccagcggaaagttgtagt-3'
LHR	M81310	5'- cgcagtgttcacgaaggcatttca-3'	5'- tccttctgtaaagttcagcccggt-3'
Esr1	NM_007956	5'- gaaggccgaaatgaaatgggtgct-3'	5'- tcaaggacaaggcagggctattct-3'
Esr2	U81451	5'- agctggctgggctggtatttatct-3'	5'- tgcccacttctctctcacacactt-3'
SF-1	NM_139051	5'- agtctgacttggaaggattggcct-3'	5'- aggtcgatttgatgaccacaccgt-3'
Cyp17	NM_007809	5'- accgtctttcaatgaccggactca-3'	5'- ttatcgtgatgcagtgcccagaga-3'
Cyp11a	NM_019779	5'- caggccaacattaccgagat-3'	5'- cgcagcatctcctgtacctt-3'
StAR	NM_011485	5'- gatgtgggcaaggtgtttc-3'	5'- gcggtccacaagttcttcat-3'
Cyp19	NM_007810	5'- ccaggtgaagacactgcaaa-3'	5'- atttccacaaggtgcctgtc-3'
18S rRNA	NR_003278	5'-ttgacggaagggcaccaccag-3'	5'-cgattccgtgggtggtggtgc-3'
GAPDH	BC096042	5'- gccttccgtgttcctacc-3'	5'- gcctgcttcaccaccttc-3'

Table 2. Correlations between testicular concentrations of steroid hormones. Hormone concentrations were determined by EIA or RIA in the testes of FVB and *Mrp1-/-* mice. Correlations were determined by linear regression.

	Progesterone	Androstenedione	DHEA	Testosterone	Estradiol
Progesterone	------	**N.S.**	**N.S.**	***r*=0.66** ***p*=0.027**	**N.S.**
Androstenedione		-----	***r*=0.87** ***p*=0.001**	***r*=0.83** ***p*=0.030**	***r*=0.71** ***p*=0.021**
DHEA			-----	***r*=0.87** ***p*=0.001**	***r*=0.83** ***p*=0.003**
Testosterone				-----	***r*=0.79** ***p*=0.004**
Estradiol					-----

N.S. is not significant

Table 3. Ratios of testicular hormones. The ratios of testicular hormones in each individual mouse was averaged and compared between FVB and *Mrp1-/-* mice. Statistical differences were determined by Student's t-test ($p<0.05$).

	FVB ratio	*Mrp1-/-* ratio	Fold difference
Progesterone/androstenedione	**0.03±0.03**	**0.10±0.05**	**3.3***
Progesterone /testosterone	**1.5±0.5**	**4.2±2.0**	**2.8***
Progesterone /estradiol	**10.0±2.2**	**24.4±16.4**	**2.4**
Progesterone /DHEA	**0.8±0.3**	**1.7±0.8**	**2.3***
Androstenedione/testosterone	**79.3±35.1**	**44.2±15.7**	**0.6**
Androstenedione/estradiol	**591.5±356.9**	**282.3±97.8**	**0.5**
Androstenedione/DHEA	**39.1±23.1**	**18.0±5.8**	**0.5**
Testosterone/estradiol	**7.2±2.3**	**5.9±3.7**	**0.8**
Testosterone/DHEA	**0.5±0.2**	**0.4±0.2**	**0.8**
Estradiol/DHEA	**0.07+0.02**	**0.07+0.03**	**0.9**

***statistically significant using Student's t-test ($p\leq0.05$)**

Table 4. mRNA concentrations of steroidogenic enzymes. The number of mRNA molecules for each enzyme was determined by QPCR and normalized to GAPDH expression. Fold difference was determined by divide the transcript levels in the Mrp1-/- mice by the transcript levels in the FVB mice.

	FVB mRNA	*Mrp1-/-* mRNA	Fold difference
3β-hydroxysteroid dehydrogenase 1	**121.8±50.4**	**122.0±47.8**	**1.0**
17β-hydroxysteroid dehydrogenase 3	**80.0±23.6**	**130.8±56.2**	**1.6**
SF-1	**48.4+21.0**	**83.2+44.3**	**1.7**

***statistically significant using Student's t-test ($p \leq 0.05$)**
N=6-7 mice; values are the average of two experiments

Chapter 4: Both Mrp1 and Mrp4 are Required for Efficient Denritic Cell Maturation and Activation

Jeffrey Sivils and Kristine Garza

Abstract:

The ATP-binding cassette (ABC) superfamily of membrane transporters uses energy derived from ATP to eliminate a variety of exogenous and endogenous compounds from cells including steroids, bilirubin, cAMP, cGMP, leukotriene $C_{4,}$ D_4 and E_4, prostaglandin E_1, E_2, $F_{a\alpha}$. It has been established that Mrp1 and Mrp4 are required for proper dendritic cell (DC) trafficking from peripheral tissues to lymph nodes and that Mrp1 may play a role in DC maturation. However, the inhibitor used to determine Mrp1 function in DC maturation was not specific to Mrp1 and can inhibit most of the Mrp transporters to some degree. As such, we have verified that murine DC express Mrp 1 - 5 and evaluated the role of Mrp1 and Mrp4 in DC migration; specifically, we assessed the function of these two transporters in DC maturation and activation. Bone marrow-derived DC (BM-DC) from Mrp1 knockout mice were treated with a specific Mrp4 inhibitor, Sildenafil, to distinguish the possible role of Mrp1 and 4 on DC maturation and activation. DC maturation was assessed following an 8 day bone marrow culture induced to become DC and DC activation was induced with lipopolysaccharide (LPS). Maturation and activation wasmeasured as a function of cell surface marker expression, interleukin (IL)-12 production, and ability to activate T cells. Sildenafil-treated wild-type (WT) and Mrp1- knock-out (KO) DC showed decreased up-regulation of activation markers, IL-12 production and ability to activate T cells upon treatment with LPS. Inhibition of BM-DC activation was more pronounced with the combined loss of Mrp1 and 4. Our data demonstrates that the absence of Mrp4, and/or Mrp1, reduces LPS-induced BM-DC maturation suggesting that multiple Mrp's may participate in DC maturation

4.1 Introduction.

The multidrug-resistance associated proteins (MRP) initially were thought to be exclusively involved in the removal of exogenous and endogenous substrates, including chemotherapy drugs, from cells. The ability of MRPs to pump out a large array of structurally and functionally dissimilar compounds leads to a multidrug resistance (MDR) phenotype in which tumor cells fail to respond to a prescribed chemotherapy régime. The last 13 years has revealed numerous additional endogenous substrates and functions performed by various MRPs. One particular MRP1 high affinity substrate, leutriene C_4 (LTC_4), is essential in generating an effective immune response (Jedlitscky et al, 1994). Additionally LTB4, LTD4, LTE_4 , prostaglandin A2 are Mrp1 substrates and LTC_4 , LTB_4, prostaglandins E_1, E_2, $F_{2\alpha}$, cAMP, cGMP are substrates MRP4 (Leier et al, 1994; Van Aubel et al, 2002;Reid et al, 200), all of which have been shown to play a role in effective immunity.

Dendritic cells (DC) are the bridge between the innate and adaptive immune systems, and are the only antigen presenting cells (APC) able to stimulate naïve T cells. DC reside in the periphery to surveil the interphase between the inside of the body and the environment. DC are continuously trafficking through peripheral tissue and migrating to draining lymph nodes, carrying and presenting material collected along the way. Howeer, the increased numbers of DC reach the lymph nodes upon inflammatory stimuli. Once DC have encountered and processed foreign antigen within an inflammatory enviroment, they migrate to draining lymph nodes where they present peptide to and activate naïve Tcells for the induction of an exquisitely specific immune response. Mrp1 has been shown to berequired for murine DC to efficiently migrate to draining lymph nodes (Robbiani et al, 2000). Upon extrusion from cells via Mrp1, LTC_4 is rapidly

converted to LTD_4 and further to LTE_4. Leutrienes then aid in chemotaxis, as DC home in on the chemokine C-C motif ligand (CCL19) for migration to draining lymph nodes (Robbiani et al, 2000). Blocking LTC_4 extrusion from DC significantly inhibits their trafficking to lymph nodes (Robbiani et al, 2000). Mrp1 appears to be required for the mobilization of tissue DC to draining lymph nodes. Taking into consideration the number of high affinity immune modulating substrates of Mrp1 and the role Mrp1 plays in DC chemotaxis, van de Ven and colleagues examined the potential role of MRP1 in the maturation of human monocyte-derived DC and the human acute myloid leukemia cell line MUT-3. The MRP inhibitor, MK571, was added to maturing interstitial DC on days 0, 3, and 6 (Van de Ven et al, 2006). They saw marked phenotypic changes relative to control DC, including lower expression of cluster of differentiation 40 (CD40), cluster of differentiation 86 (CD86), and major histocaptability complex (MHC), as well as a decreased ability to stimulate T cells (Van de Ven et al, 2006). It appeared that MRP1 was required for optimal DC maturation; however, MK571 is a global MRP inhibitor. Thus, the changes seen in DC maturation cannot be solely attributed to alterations in MRP1 function.

Recently, MRP4 has been show to transport LTB_4 and LTC_4; Van de Ven and colleagues therefore examined the role of MRP4 in human DC migration (Van de Ven et al, 2008). Using Sildenafil (20 and 60 μM), a specific MRP4 inhibitor, and MRP4 short hairpin RNA (shRNA), they determined that MRP4 is also involved in DC migration (Van de Ven et al, 2008). The chemical inhibition of MRP4 significantly decreased the percentage of cells that migrated toward the chemokine C-C motif ligands CCL19 and CCL2 by ~ 80% and ~ 60%, respectively (Van de Vel et al, 2008). The shMRP4 knock down also decreased DC migration to CCL19 by ~80% even in the presence of the MRP4 substrates PGE_2, LTB_4, LTD_4 and 8Br-cGMP (Van de Ven et

al, 2008). In addition to migration studies, Van de Ven and colleges assessed immune function in Mrp4 and Mrp4/5 knock-out mice. They evaluated bone-marrow (BM) derivedDC (BM-DC) phenotype between wild type, $Mrp4^{-/-}$ and $Mrp4/5^{-/-}$ mice by flow cytometry. They also examined what impact the loss of either Mrp4 or Mrp4/5 had on DC ability to stimulate polyclonal splenocyte cell proliferation and antibody isotype production (Van de Ven et al, 2008). There were no significant alterations in DC migration, phenotype, proliferation, or antibody isotype production in the absence of MRP4 (Van de Ven et al, 2008). This suggested that either MRP4 is not required for DC function or that other MRP's are compensating for the loss of MRP4.

As Van de Ven and colleagues looked at the involvement of either Mrp1 or Mrp4 and considering the substrate overlap between the two transporters, we decided to establish whether Mrp1 and Mrp4 might work in concert to insure effective DC maturation and activation. Our data suggest that both Mrp1 and Mrp4 are required for efficient DC maturation and activation, and that with the loss of one, the other may act in a compensatory manner to at least allow partial DC function.

4.2 Material and Methods:

4.2.1 Animals.

FVB and FVBMRrp -/- mice were purchased from Taconic Farms (Germantown, NY) and C57BL/6 mice were bred in house. The animals were housed at 25^{o} C and 50% humidity, with a 12-12 day-night cycle, and were provided water and standard pellet chow *ad libitum*. At ten weeks of age, the mice were euthanized with isoflurane and cervical dislocation at which time

bone marrow was isolated for generation of dendritic cells. All experiments were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) and the University of Texas at El Paso guidelines.

4.2.2 Chemicals and Cytokines.

Sildenafil was obtained threw commercial sources and MK571 was purchased from Calbiochem (La Jolla, CA). Interluekin-4 and granulocye colony stimulating facter (GMC-FS) were purchased from Paprotech Inc., Rocky Hill, NJ. Lipoploysacharide , Concanavalin A, and paraformaldehyde were purchased from Sigma Aldrich (St. Louis, MO).

4.2.3 Bone marrow-derived immature dendritic cells (BM-DC). Bone marrow was flushed from long bones of the indicated mice and red blood cells were lysed using an ammonium chloride buffer (0.15M NH4Cl and 0.17 M Tris Base) (Sigma). Collected cells were induced to differentiate into DCs by culturing in RPMI (Invitrogen) media containing 10% Fetal Bovine Serum (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), 10 ng/ml Granulocyte-Macrophage-ColonyStimulatingFactor(Peprotech), and10 ng/ml Interleukin-4 (Peprotech). Cells were incubated for eight days @ 37^{o}C, 5% CO_2, refreshing the media every other day. BM-DCs were matured and used either as a single population or were enriched by magnetic activated cell sorting.

4.2.4 Magnetic Activated Cell Sorting (**MACS**). MACS was used to enrich bone marrow-derived DCs . Briefly, single cell suspensions of BM cultures were incubated in 50% mouse serum (Rockland) in IMAG buffer (2% FBS in 1X phosphate buffered saline) (Hyclone).

Antibody-conjugated magnetic beads (Miltenyi) against the dendritic cell marker CD11c, were added at a ratio of 90 μL for every 10^6 cells as determined by trypan blue exclusion. Following incubation on ice, the cells were placed onto a positive selection magnetic column (Miltenyi) and washed 3 times with IMAG buffer. Upon removal from the magnet, the enriched cell suspension was released from the column using pressure. Cell purity, as determined by flow cytometry, was consistently >95%.

4.2.5 Polymerase chain reaction (PCR) –

PCR was performed on and transporter expression determined by extracting total RNA from BM-DC collected from FVB and FVB/ MRP1-/- mice and prepairing cDNA. Primers were designed for Mrp 1-5, and PCR reactions were performed at the appropriate annealing temperature for 40 cycles. PCR products were resolved on a 2% agarose gel and visualized on a transilluminator.

4.2.6 Enzyme-linked immunosorbant assay (ELISA).

Cellular production of IL-12 was measured by ELISA. Microtiter ELISA plates were coated with anti-IL-12 capture antibody (100μl/well) over night at 4^oC. Plates were blocked at room temperature with 3% bovine serum albumin in PBS. Cell culture supernatants were added to the plates (100μl/well) together with detection antibody (0.01μg/ml) for 2 hours. Plates were washed and strepavidin-HRP was added (0.05*μg/ml)* for an incubation period of 30 min. Plates were developed with 3, 3'5, 5'-tetramethylbenzidine (TMB Genscript) and read at 450nm of spectrometer. Concentrations were calculated against murine recombinant cytokines (BD Pharmingen).

4.2.7 Flow Cytometry.

To assess the number of cells expressing and/or the intensity of activation markers, single cell suspensions were blocked with 50% normal mouse serum (NMS) in FACS buffer (1X PBS, 10% FBS, and 0.5mM EDTA) for 15 minutes on ice. The cells were then stained with antibodies for CD11c, MHC II, CD54, CD80 ,and CD86 conjugated to either fluorescein isothiocyanate (FITC), phycoerytherin (PE), and/or Biotin-streptavidin PECy5 (Pharmingen). Cells were then fixed in 1% paraformaldehyde. Flow cytometery was performed on a Beckman Coulter FC500 instrument. Samples were gated for live cells based on forward and side scatter parameters and 10,000 events per sample were collected and analyzed using CXP software (Beckman Coulter).

4.2.8 Mixed Lymphocyte Reactions.

BM-DCs from FVB and FVB/Mrp-/- mice were used as stimulators of T cells enriched from lymph nodes of C57/B mice. BMD-DCs were isolated as described above and matured either in media alone, with MK571 (25μM), or Sildenafil (20μM), irradiated in an XRad160 gamma-irradiator (Precision Instruments; 1200 rads), then co-cultured with responder T cells at 1:4 and 1:8 ratio. T cell activation was measured 96 hours later as a function of proliferation. To assess proliferation, cells were pulsed with [^{3}H]-thymidine (PerkinElmer, 0.5 μCi) during the final 18 hrs of culture (overnight pulse). Cells were harvested and proliferation was determined by scintillation counting. Unenriched lymph node cells stimulated with Concanavalin A (Con A) were used as a positive control and T cells in culture medium alone were used as a negative control.

4.2.9 T cell isolation and purification

Lymph nodes from C57B1/6 mice were collected, and homogenized to a single cell suspension. T cells were enriched by MACS, using anti-CD4 for positive selection. Cell concentration was determined trypan exclusion and counting on a hemocytometer

4.2.10 DC rescue assay-

To determine the maturation and activation of DCs grown in the presence of MRP inhibitor (MK571, 25 μM), two sets of control cells and two sets of cells with the inhibitor were grown as above. Every two days one set of control cells and one set of treated cells were collected, centrifuged, and the media was removed. Control cell media was replaced (Figure 1.). The media for the treatment cells was replaced with the control media, cytokines refreshed and inhibitor added to 25 μM. On day 6 cells were treated with 10 μM LPS for 24 hr and flow cytometry was preformed as described above.

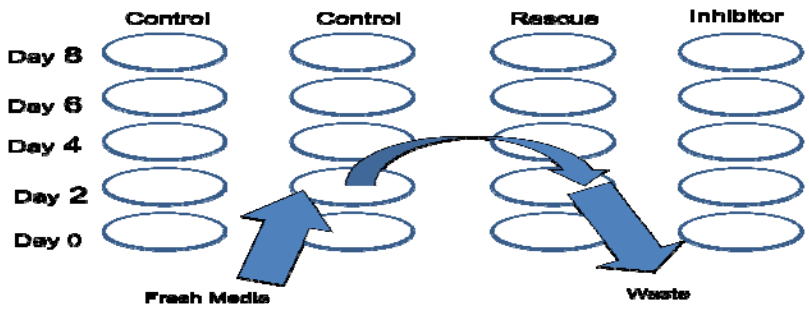


Figure 4.1 Assay set up to determine the ability of control cell supernatants to rescue DC marturation. Lane 1 is the control lane, lane 2 is the control lane used for rescue media added to the media with MK571, lane 3. Lane 3 is the MK571 rescue lane. Lane 4 is the MK571 inhibited lane.

4.3 Results

4.3.1 Mrp expression in murine dendritic cells.

Both Mrp1 and Mrp4 expression has been previously described. To test our hypothesis that Mrp's are required for DC maturation and activation, we first verified transporter expression on activated BM-DC. Total messenger RNA (mRNA) was isolated from BM-DCs generated from FVB and FVB/Mrp1$^{-/-}$ mice .l The BM-DC were harvested after maturation (at the end of the 8 day BM culture) and following activation with the bacterial endotoxin, LPS. PCR was performed to determine expression of Mrp1, Mrp2, Mrp3, Mrp4, and Mrp5. As shown in Figure 2, expression was detected for all of the Mrps, with the exception of Mrp2..

4.3.2 Inhibition of Mrp1 and Mrp4 reduces IL-12 production upon BM-DC activation with LPS but does not alter activation marker expression levels.

Both Mrp1 and Mrp4 have been implicated in DC activation, however the data is still inconclusive. To establish whether Mrp1 and/or Mrp4 are required for DC activation (response to microbial stimuli), BM-DC were assessed for IL-12 production and up-regulation of activation markers upon treatment with LPS. BM-DC, generated from FVB and FVB/Mrp1$^{-/-}$ mice, were activated with LPS (10 μg/mL) and incubated overnight. Cells were incubated in media alone (negative control), in in the presence of Mrp general inhibitor, MK571 (25μM), or in the presence of the Mrp4-specific inhibitor, Sildenafil (20 μM). The general inhibitor (MK571) prevented the induction of IL-12 production by BM-DC and the inhibition was not enhanced upon further loss of Mrp1 (Figure 3A). The lack of either Mrp1 or Mrp4 resulted in a significant

reduction in the production of IL-12 by BM-DC (Figure 3A). The combined loss of Mrp1 and Mrp4 (FVB/Mrp1$^{-/-}$ + Sildenafil) however, did not act cooperatively to inhibit the ability of BM-DC to produce IL-12 in response to LPS.

For DC to effectively interact with T cells and promote an effective immune response, several activation markers must be up-regulated on the DC surface including the adhesion molecule CD54 and the co-stimulatory molecule CD86. BM-DC from both FVB and FVB/Mrp1$^{-/-}$ were treated with MK571 or Sildenafil, and activated with LPS. The cells were stained for the activation markers CD54 and CD 86, and examined by flow cytometery. There were no significant alterations in marker expression between the treated and the untreated control DC, in either the wildtype or the Mrp1 knock-out BM-DC (Figure 3B).

4.3.3 Inhibition of Mrps during maturation impedes IL-12 production and up-regulation of markers upon activation with LPS.

To test the second part of our hypothesis, that Mrp1 and/or Mrp4 are needed during maturation, and that the loss of Mrp1, Mrp4 or both during maturation will alter DC function, transporter function was inhibited during maturation of BM-DC from both FVB and FVB/Mrp1$^{-/-}$ cells. The inhibitors were added on day 0 and refreshed every 2 days along with the maturation-inducing cytokines; on day 8, the BM-DC cultures were administered 10 ng/ml of LPS and assessed for IL-12 production and activation marker expression 24 hrs later. There was a 5.4 fold decrease in the production of IL-12 in the FVB/Mrp1$^{-/-}$ mice as compared to wildtype in response to LPS (Figure 4A). Incubation with MK571 led to a significant 5.4 fold decrease in the wildtype compared to LPS wildtype control and 1.9 fold decrease in knock-out as compared to the FVB/Mrp1$^{-/-}$ control (Figure 4A). Sildenafil caused a significant 3.3 fold reduction in

wildtype compared to LPS wildtype control, and a 2.0 fold reduction in the FVB/Mrp1$^{-/-}$ as compared to FVB/Mrp1-/- LPS control (Figure 4A). Additionally in comparing the treated FVB/Mrp1$^{-/-}$ against the wildtype LPS control there was a 10.0 and 10.8 fold decrease in IL-12 production by MK571 and Sildenafil, respectively (Figure 4A). Considering the high variability in IL-12 production between wild type and FVB/Mrp1$^{-/-}$ mice, IL-12 levels were expressed as a percent the perspective LPS controls. The FVB/Mrp1$^{-/-}$ BM-DC treated with MK571 produce 46.19% less IL-12, and BM-DC treated with Sildenafil produced 39.93% less IL-12 compared to the LPS control cells. Wildtype BM-DC treated with MK571 produced 81.62% less IL-12 and Sildenafil-treated cells produced 69.11% less IL-12 than control cells (Figure 4B). It would appear that losing both Mrp1 and Mrp4 has a greater effect on maturation as measured by IL-12 production than just the loss of Mrp1 or Mrp4 alone.

To further assess the potential role of Mrp1 and/or Mrp4 on DC maturation, the expression levels of cell surface DC activation markers were assessed. BM-cells from both FVB and FV/Mrp1$^{-/-}$ were collected and plated as described, except this time only one inhibitor was used (Sildenafil 20μM) and was again added throughout the maturation period. As before, following a 24 hr stimulation with LPS on day 8, the BM-DC were stained for CD11c (DC-specific marker), MHC class II, CD54, CD80 and CD86. The flow was gated specifically for DC using CD11c, and the percentage of cells expressing each marker was ascertained. Foreach population, 80% of the cells were expressing CD11c (Figure 5); however, the percentage of FVB and FVB/Mrp1$^{-/-}$ CD11c^{+} cells expressing the activation markers was significantly altered (summarized in Table1). In adition, the FVB-derived DC treated with the inhibitor also had a significantly lower percentage of cells expressing MHC class II, CD54, CD80 and CD86 (Table

1). A change in the percentage of Mrp-4 inhibitor-treated FVB/Mrp1$^{-/-}$ cells expressing DC activation markers was only decreased with respect to CD54 expression (Figure 5).

Also evaluated was mean intensity or relative number of individual cell surface markers on the cell. The only significant difference between wildtype DC and knock-out DC was in the Sildenafil-treated knockout BM-DC. CD54 on the knockout DC was expressed 5.48 fold lower than on the wildtype cells when Mrp4 was also blocked (Figure 6). Together, the IL-12 data and the activation marker data suggest that both Mrp1 and Mrp4 are critical for DC maturation. The lack of either transporter significantly inhibits IL-12 production and significantly alters the percentage of DC that upregulate activation markers suggesting that both transporters and necessary to generate DC that can properly respond to microbial stimulation.

4.3.4 Optimal T cell activation appears to require both Mrp1 and Mrp4 as determined by MLR

In order to bridge the innate branch of the immune system to the acquired, DC need to present antigen to T cells, this in turn triggers the adaptive response. To determine the role of Mrp1 and/or Mrp4 play in the ability of DC to activate naïve T cells a Mixed Lymphocyte Reaction (MLR) was performed with BM-DC acquired from both FVB and FVB/Mrp1$^{-/-}$ mice. The DC were matured either in the presence of MK571 (25μM), Sildenafil (20μM), or media alone; on day eight, the cells were irradiated and co-incubated with T cells isolated from C57Bl/6 mice. Seventy two hours later, the co-cultures were pulsed for 24 hours with [^{3}H]-thymidine.

As shown in Figure 7, DC harvested from FVB/Mrp1$^{-/-}$ mice were not as proficient at activating allogeneic T cells as those from wild type FVB mice (Figure 7A). The addition of MK571 reduced the ability of wild type and FVB/Mrp1$^{-/-}$ BM-DC to activate T cells 2.0 and 1.4 fold,

respectively. The FVB/Mrp1$^{-/-}$ mice, both untreated and treated with MK571, showed a similar ability to stimulate T cells as the wildtype treated with MK571 (Figure 7A). Both wild type and knock-out DC treated with Sildenafil were 2.9 and 3.5 fold less efficient in stimulating allogeneic T cells, respectively, and less efficient than the cells treated with MK571 (Figure 7A). Curiously, even when cells were treated with the T cell mitogen ConA as a positive control, Sildenafil inhibited BM-DC effectiveness of activating allogeneic T cells. The significant differences in the ability of BM-DC to activate allogenic T cells in more readily seen when comparing proliferative responses of T cells (at a DC to T cell ratio of 1:4) relative to the positive control of Con A or wild type (Figure 7B).

4.3.5 Rescue of DC maturation in chemically inhibited BM-DC.

As there are several immune modulating substrates for both Mrp1 and Mrp4 we have hypothesized that DC expel an autocrine factor via the Mrps while undergoing maturation, which is required to complete maturation. To determine if Mr1 and/or Mrp4 is pumping out a molecule or molecules having autocine action on the DC, required for maturation and/or activation, a rescue experiment was performed. It was hypothesized the unknown factors released into the media through the transporters by control DC during maturation could be harvested and used to rescue maturation of BM-DC maturing in the presence of the inhibitor. Two sets of BM-DC were matured in media alone and two sets in the presence of MK571 (25 μM). One media and one inhibited set BM-DC were allowed to mature as previously described. For the remaining set of inhibitor-treated BM-DC, the media was removed every two days and was replaced with the media from the remaining control BM-DC plus with fresh media and inhibitor (to maintain chemical inhibition of the Mrps). After 6 days of maturation and an additional 24 hours of

activation with LPS, BM-DC were examined for activation markers expressed by flow cytometry. As shown in Figure 8, the mean intensity of DC cell surface activation markers, CD54, CD80, and CD86, on the cells expressing CD11c statistically increased on the rescued cells. This preliminary data suggests that the supernatants from normal maturing BM-DC can recover DC maturation when Mrps are inhibited.

4.4 Discussion

Several groups have looked at the potential roles of MRP1 or MRP4 play in DC function. Inhibition of MRP1or MRP4 function blocked DC migration to lymph nodes, (Robbiani, Finch et al. 2000; Honig, Fu et al. 2003; van de Ven, Scheffer et al. 2008). Initially the loss of MRP1 was thought to obstruct DC differentiation however this was determined with using MK571 to block MRP1, which has since been determined to inhibit most of the MRPs to some extent (van de Ven, de Jong et al. 2006). Later, the same group looked specifically at Mrp4 in mice; it was determined that murine Mrp4 was not required for DC migration or for mounting an immune response; this did not exclude Mrp1 from the previous study (van de Ven, de Jong et al. 2006; van de Ven, de Groot et al. 2009). Considering the functional and substrate overlap seen in the more traditional roles played by the MRPs in the elimination of endogenous and exogenous compounds, and that neither Mrp1 nor Mrp4 knock-out mice are severely immune-compromised, we decided to look at the potential role of both Mrp1 and Mrp4 in DC function (Wijnholds et al, 1997, van de Ven et al, 2009). Using an Mrp1 knock-out mouse, the global Mrp inhibitor MK571 and the Mrp4-specific inhibitor, Sildenafil, we concluded that both Mrp1 and Mrp4 are required for DC maturation and ultimately effective DC induction of adaptive immunity.

To confirm the expression of Mrp1 and Mrp4, mRNA was extracted from activated FVB mouse BM-DC, reversed transcribed and PCR was performed. All but Mrp2 was present; this concurs with determined human DC MRP expression (Skazik, Heise et al. 2008)(Fig. 1).

To look at the possible role of Mrp1 and Mrp4 on DC activation, DC were cultured as described in Materials and Methods. On day 8, DC were stimulated with LPS. BM-DC activation was measured as a function of IL-12 production and expression of cell surface activations markers. We next wanted to determine if inhibiting the transporters either together or one at a time would impair DC ability to initiate an immune response. The global MRP inhibitor, MK571 has been shown to effectively block Mrp1 mediated efflux of known Mrp1 substrates (van de Ven et al, 2006). Sildenafil has also been shown to effectively inhibit Mrp4 mediated efflux of Mrp4 substrates (van de Ven et al, 2008). The production of IL-12 in the wild type and the Mrp1 -/- cells was significantly lowered by the loss of either Mrp1 and/or Mrp4. However blocking either Mrp1 or Mrp4 with the Mrp inhibitors at the time of activation did not affect DC ability to up-regulate the activation markers CD54 or CD86 when exposed to LPS. Conversely, the decrease in IL-12 production may hamper the responding T cells and attenuate the immune response. Even considering BM-DC IL12 production was decreased by inhibiting Mrp function during activation, we concluded that any significant effect on DC function occur prior to their activation, hence we looked at DC maturation.

BM-DC were cultured as above, except the inhibitors were added on day zero and refreshed every two days. Previously inhibition by MK571 had been shown to reduce the production of IL12 in activated DC by greater than 50%, and MK571 blocked interstitial langerhans DC differentiation and maturation, assessed by a decreased expression of the cell surface markers CD14, CD1a, CD1c, langerin,CD40, CD54, CD86, CD86, and HLA-DR

(human MHC class II) (van de Ven, de Jong et al. 2006). DC were exposed to LPS and either MK571 or Sildenafil, and incubated over night. Cells were again assayed for IL-12 production and expression of the DC differentiation marker CD11c and the activation markers MHCII, CD54, CD80 and CD86. Control DC treated with MK571 were severely impaired in their ability to produce IL-12; this was comparable to the data in the literature. Inhibition of Mrp4 also impeded the production of IL-12 from controls cells, although not as severely. Interestingly activated $Mrp1^{-/-}$ cells produce IL-12 levels similar to the control cells exposed to MK571. MK571 and Siledafil reduced the production of IL-12 further in $Mrp1^{-/-}$ cells compared to wild type cells. Global transporter inhibition in the wild type cells reduced IL-12 production to a greater extent than by just blocking Mrp4 alone. Conversely, in the $Mrp1^{-/-}$ cells blocking all of the transporters with MK571 reduced IL-12 production to the same extent as did inhibiting just Mrp4. This would appear to suggest that both Mrp1 and Mrp4 are required for optimum IL-12 production in activated DC. Taking into consideration that MK571 had a similar effect on control and $Mrp1^{-/-}$ cells, when the cells were examined to determine Mrp4's role, they were treated with the Mrp4 inhibitor. In all of the populations; control, treated and untreated, and $Mrp1^{-/-}$ treated and untreated ~80% of cells expressed CD11c, at equivalent intensity. The loss of Mrp1 or Mrp4 did not prevent BM-cells from maturing into the DC lineage. However, when treated with MK571 immature interstitial langerhans cells showed decreased expression of the activation markers, however only CD1a, CD1c and langerin markers were significantly reduced (van de Ven, de Jong et al. 2006). Inhibiting either Mrp1 or Mrp4 may not prevent BM cells from maturing to DC; however it may impede their ability to become fully activated upon stimulation. This proved to be the case as inhibiting Mrp4 during maturation lead to a dramatic shift down in the percentages of cells expressing the activation markers MCH II, CD54, CD80,

and CD86 in wild type cells cells exposed to Sildenafil compared to untreated wildtype cells activated with LPS. Additionally there was lower activation marker expression in the untreated Mrp1-/- cells compared to untreated wildtype cells post activation. In the knock out cells treated with Sildenafil only the percentage of cells expressing CD54 was significantly decreased compared to untreated knock cells, CD86 expressing cells were lower but was not significant due to variability.. In terms of which transporter is more critical for DC maturation the data is inconclusive. That is to say the loss of Mrp1 had the same effect on the percentage cells that unregulated their activation markers as wildype cells treated with the Mrp4 inhibitor Sildenafil. Additionally when inhibiting Mrp4 in the knock cells the only the percentage of cells expressing CD54 was reduced. When mean intensities were examined, the number of activation markers expressed per cell, the only significant difference was in CD54 expression on the knockout cells. This is contrast to the previous work, which showed decreases in intensity in MHC II, CD 54, and CD86 when cells were exposed to MK571. That was however done in human derived DCs, and data in the literature suggest there may be a species differences in MRP requirements (van de Ven, de Jong et al. 2006; van de Ven, de Groot et al. 2009). Taken in its entirety this suggests that both Mrp1 and Mrp4 play a role in DC maturation and activation.

The ultimate test of DC maturation and activation is on the ability of DC to activate T cells and induce an adaptive immune response. We assayed the ability of DC to activate T cells in the presence of either MK571, or sildenafil. We discovered the same trend as above, compared to wildtype cells, the knock out cells were significantly less proficient at activating T cells. As well cells treated with MK571, the global inhibitor, were equally poor T cell activators compared to untreated wildtype cells.

The next phase was to try and elicit the mechanism by which transporters affect DC function. All of the immune related functions determined to date are effluxed from cells and have either autocrine or paracrine activity, these include both leukotrines and prostiaglands (Robbiani, Finch et al. 2000; Legler, Krause et al. 2006; Rius, Hummel-Eisenbeiss et al. 2008). Previous work proved established that the addition of exogenous leukotriene was able to rescued DC migration in cells in which Mrp1 was inhabited (Robbiani, Finch et al. 2000). Alone this lines we decided to try and recue DC maturation by adding the media from untreated control cells. When media from control cells, even with the addition of MK571, replaced the media in the treated cells, there was a significant increase in the expression of surface activation makers on the treated cells. However when we looked at the percentage of cells fully maturing to DC, there fewer cells expressing the DC maturation marker CD11c. It appeared that addition of exogenous media was able to increase activation marker expression on cells that matured to DC, but it was not able to increase the percentage of cells that matured to DC. This may be due to the 2 day lag time in maturation. The rescue cells did not receive control media for 2 days, this may have stalled any of the cells in the CD11c low and medium populations in an immature state. The addition of any potential maturation factors in the control media was unable to push them forward, pushing only the CD11c high population to full maturation. This may be corrected by staring the control cells used for the rescue media 2 days ahead of the reaming cultures, this would allow the rescue cells exposure to the control media and any beneficial factors from day zero.

4.5 Conclusion

In contrast to previous studies, that questioned the potential role of Mrp1 and/or Mrp4 in DC maturation/activation we have demonstrated that the transporters play a pivotal role. In the previous studies however only one transporter was examined, save the first study which unintentionally, thought only Mrp1 was being inhibited. In that case inhibitor used MK571, has inhibitory an effect on most of the Mrps, and they also saw alterations in DC differentiation. We therefore looked at the possibility that both Mrp1 and Mrp4 play a role in DC function. Our work shows that indeed both Mrps are required for appropriate dendritic cell maturation and activation. They appear to have overlapping function or there is a compensatory effect in the event one is lost. The attempt to determine if there is an autocrine molecule being pumped out to aid in maturation was however inconclusive. The role of Mrps in DC function requires further study including endeavoring to discern the molecules that may be involved in the maturation and activation process.

Referances

Akingbemi, B. T. (2005). "Estrogen regulation of testicular function." Reprod Biol Endocrinol **3**: 51.

Anakwe, O. O. and A. H. Payne (1987). "Noncoordinate regulation of *de novo* synthesis of cytochrome P450 cholesterol side-chain cleavage and cytochrome $P450_{17\alpha}$-hdryoxylase $C_{17\text{-}20}$ lyase in mouse Leydig cell cultures: relation to steroid production." Mol Endocrinol **1**: 595-603.

Andersen, M. E., R. S. Thomas, et al. (2005). "Dose-response modeling in reproductive toxicology in the systems biology era." Reprod Toxicol **19**(3): 327-337.

Ankley, G. T., D. C. Bencic, et al. (2009). "Dynamic nature of alterations in the endocrine system of fathead minnows exposed to the fungicide prochloraz." Toxicol Sci **112**: 344-353.

Aye, I. L., J. W. Paxton, et al. (2007). "Expression, localisation and activity of ATP binding cassette (ABC) family of drug transporters in human amnion membranes." Placenta **28**: 868-877.

Baker, P. J., J. H. Sha, et al. (1997). "Localisation and regulation of 17β-hydroxysteroid dehydrogenase type 3 mRNA during development in the mouse testis " Mol Cell Endocrin **133**: 127-133.

Bakos, E. and L. Homolya (2007). "Portrait of multifaceted transporter, the multidrug resistance-associated protein 1 (MRP1/ABCC1)." Pflugers Arch **453**: 621-641.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding." Anal Biochem **72**: 248-254.

Busygina, T. V., G. V. Vasiliev, et al. (2005). "Binding sites for transcription factor SF-1 in promoter regions of genes encoding mouse steroidogenesis enzymes 3betaHSDI and P450c17." Biochemistry (Mosc) **70**: 1152-1156.

Chen, Z. S., Y. Guo, et al. (2005). "Transport of bile acids, sulfated steroids, estradiol 17-β-D-glucuronide, and leukotriene C_4 by human multidrug resistance protein 8 (ABCC11)." Mol Pharmacol **67**: 545-557.

D'Souza, R., M. K. Gill-Sharma, et al. (2005). "Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats." Mol Cell Endocrinol **28**: 41-48.

Dallas, S., X. Zhu, et al. (2003). "Functional expression of the multidrug resistance protein 1 in microglia." J Pharmacol Exp Ther **307**: 282-290.

Deeley, R. G., C. Westlake, et al. (2006). "Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins." Physiol Rev **86**: 849-899.

Delbès, G., C. Levacher, et al. (2006). "Estrogen effects on fetal and neonatal testicular development." Reproduction **132**: 527-538.

Fevold, H. R., M. C. Lorence, et al. (1989). "Rat P450-17α from testis: characterization of a full-length cDNA encoding a unique steroid hydroxylase capable of catalyzing both Δ^4 and Δ^5-steroid-17, 20-lyase reactions." Mol Endocrinol **3**: 968-975.

Flens, M. J., G. J. R. Zaman, et al. (1996). "Tissue distribution of the multidrug resistance protein." Amer J Pathol **148**: 1237-1247.

Fluck, C. E., W. L. Miller, et al. (2003). "The 17,20-lyase activity of cytochrome P450c17 from human fetal testis favors the Δ^5 steroidogenic pathway." J Clin Endocrinol Metab **88**: 3762-3766.

Harlow, E. and D. Lane (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Hess, R. A. (2003). "Estrogen in the adult male reproductive tract: a review." Reprod Biol Endocrinol **9**: 52.

Honig, S. M., S. Fu, et al. (2003). "FTY720 stimulates multidrug transporter– and cysteinyl leukotriene–dependent T cell chemotaxis to lymph nodes." The Journal of Clinical Investigation **111**(5): 627-637.

Jeyaraj, D. A., G. Grossman, et al. (2005). "Altered bioavailability of testosterone in androgen-binding protein-transgenic mice." Steroids **70**: 704-714.

Johnson, D. R., R. A. Finch, et al. (2001). "The pharmacological phenotype of combined multidrug-resistance mdr1a/1b- and mrp1-deficient mice." Cancer Res **61**: 1469-1476.

Legler, D. F., P. Krause, et al. (2006). "Prostaglandin E2 Is Generally Required for Human Dendritic Cell Migration and Exerts Its Effect via EP2 and EP4 Receptors." J Immunol **176**(2): 966-973.

Leslie, E. M., R. G. Deeley, et al. (2005). "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense." Toxicol Appl Pharmacol **204**: 216-237.

Li, Q., Y. Kato, et al. (2005). "Multidrug resistance-associated protein 1 functions as an efflux pump of xenobiotics in the skin." Pharm Res **22**: 842-846.

Loe, D. W., K. C. Almquist, et al. (1996). "ATP-dependent 17β-estradiol 17-(β-D-glucuronide) transport by multidrug resistance protein (MRP)." J Biol Chem **271**: 9683-9689.

Maher, J. M., A. L. Slitt, et al. (2005). "Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice." Drug Metab Dispos **33**: 947-955.

Mathieu, A. P., R. J. Auchus, et al. (2002). "Comparison of the hamster and human adrenal P450c17 (17α-hydroxylase/17,20-lyase) using site directed mutagenesis and molecular modeling." J Steroid Biochem Mol Biol **80**: 99-107.

Mellon, S. H., N. A. Compagnone, et al. (1998). "Orphan receptors, proto-oncogenes and other nuclear factors regulate P450C17 gene transcription." Endocr Res **24**: 505-513.

Miki, Y., T. Nakata, et al. (2002). "Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues." J Clin Endocrinol Metab **87**: 5760-5768.

Nies, A. T., G. Jedlitschky, et al. (2004). "Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain." Neuroscience **129**: 349-360.

Ozbay, R., A. Leon, et al. (2006). "Cyclic adenosine 5'-monophosphate-dependent sphingosine-1-phosphate biosynthesis induces human CYP17 gene transcription by activating cleavage of sterol regulatory element binding protein 1." Endocrinology **147**: 1427–1437.

Patel, S. S., V. E. Beshay, et al. (2009). "Molecular mechanism for repression of 17{alpha}-hydroxylase expression and androstenedione production in granulosa cells." J Clin Endocrinol Metab **Epub**.

Payne, A. H. and L. L. Sha (1991). "Multiple mechanisms for regulation of 3ß-hydroxysteroid dehydrogenase/5-4 isomerase, 17-hydroxylase/C17–20 lyase cytochrome P450, and cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid levels in primary cultures of mouse Leydig cells." Endocrinology **129**: 1429–1435.

Peng, K.-C., F. Cluzeaud, et al. (1999). "Tissue and cell distribution of the multidrug resistance-associated protein (MRP) in mouse intestine and kidney." J Histochem Cytochem **47**(6): 757-768.

Qian, Y.-M., W.-C. Song, et al. (2001). "Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1." J Biol Chem **276**(9): 6404-6411.

Reichardt, H. M., K. H. Kaestner, et al. (1998). "DNA binding of the glucocorticoid receptor is not essential for survival." Cell **93**: 531–541.

Rius, M., J. Hummel-Eisenbeiss, et al. (2008). "ATP-Dependent Transport of Leukotrienes B4 and C4 by the Multidrug Resistance Protein ABCC4 (MRP4)." Journal of Pharmacology and Experimental Therapeutics **324**(1): 86-94.

Robbiani, D. F., R. A. Finch, et al. (2000). "The Leukotriene C4 Transporter MRP1 Regulates CCL19 (MIP-3², ELC) Dependent Mobilization of Dendritic Cells to Lymph Nodes." **103**(5): 757-768.

Sasabe, H., Y. Kato, et al. (2004). "Differential involvement of multidrug resistance-associated protein 1 and P-glycoprotein in tissue distribution and excretion of grepafloxacin in mice." J Pharmacol Exp Ther **310**: 648-655.

Sewer, M. B. and M. R. Waterman (2002). "Adrenocorticotropin/cyclic adenosine 3',5'-monophosphate-mediated transcription of the human CYP17 gene in the adrenal cortex is dependent on phosphatase activity." Endocrinology **143**: 1769–1777.

Shi, Y., M. D. Schonemann, et al. (2009). "Regulation of P450c17 expression in the early embryo depends on GATA factors." Endocrinology **150**: 946-956.

Simard, J., M.-L. Ricketts, et al. (2005). "Molecular biology of the 3ß-hydroxysteroid dehydrogenase/5-4 isomerase gene family " Endocr Rev **26**: 525-582.

Skazik, C., R. Heise, et al. (2008). "Differential expression of influx and efflux transport proteins in human antigen presenting cells." Experimental Dermatology **17**(9): 739-747.

Song, W. C. and M. H. Melner (2000). "Steroid transformation enzymes as critical regulators of steroid action in vivo." Endocrinology **141**: 1587-1589.

Stride, B. D., G. Valdimarsson, et al. (1996). "Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter." Mol Pharmacol **49**: 962-971.

Strott, C. A. (1996). "Steroid sulfotransferases." Endocr Rev **17**: 670-697.

Taniguchi, F., J. F. Couse, et al. (2007). "Estrogen receptor-alpha mediates an intraovarian negative feedback loop on thecal cell steroidogenesis via modulation of Cyp17a1 (cytochrome P450, steroid 17alpha-hydroxylase/17,20 lyase) expression." FASEB J **21**: 586-595.

Tong, M. H., L. K. Christenson, et al. (2004). "Aberrant cholesterol transport and impaired steroidogenesis in Leydig cells lacking estrogen sulfotransferase." Endocrinology **145**: 2487-2497.

Toppari, J. (2008). "Environmental endocrine disrupters." Sex Dev **2**: 260-267.

Tribull, T. E., R. H. Bruner, et al. (2003). "The multidrug resistance-associated protein 1 (MRP1/ABCC1) transports methoxychlor and protects the seminiferous epithelium from injury." Toxicol Lett **142**: 61-70.

Tsai-Morris, C. H., A. Khanum, et al. (1999). "The rat 17ß-hydroxysteroid dehydrogenase type III: molecular cloning and gonadotropin regulation." Endocrinology **40**: 3534-3542.

van de Ven, R., J. de Groot, et al. (2009). "Unimpaired immune functions in the absence of Mrp4 (Abcc4)." Immunology Letters **124**(2): 81-87.

van de Ven, R., M. C. de Jong, et al. (2006). "Dendritic Cells Require Multidrug Resistance Protein 1 (ABCC1) Transporter Activity for Differentiation." J Immunol **176**(9): 5191-5198.

van de Ven, R., G. L. Scheffer, et al. (2008). "A role for multidrug resistance protein 4 (MRP4; ABCC4) in human dendritic cell migration." Blood **112**(6): 2353-2359.

van der Deen, M., W. Timens, et al. (2007). "Reduced inflammatory response in cigarette smoke exposed Mrp1/Mdr1a/1b deficient mice." Respir Res **8**: 49.

van Tellingen, O., T. Buckle, et al. (2003). "P-glycoprotein and Mrp1 collectively protect the bone marrow from vincristine-induced toxicity in vivo." Br J Cancer **89**: 1776-1782.

Villeneuve, D. L., N. D. Mueller, et al. (2009). "Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor." Environ Health Perspect **117**: 624-631.

Wijnholds, J., G. L. Scheffer, et al. (1998). "Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage." J Exp Med **188**: 797-808.

Zelcer, N., G. Reid, et al. (2003). "Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4)." Biochem J **371**: 361-367.

Zhang, P., N. A. Compagnone, et al. (2001). "Developmental gonadal expression of the transcription factor SET and its target gene, P450c17 (17-hydroxylase/c17,20 lyase)." DNA Cell Biol **20**: 613–624.

Figure Legend.

Figure 4.2 PCR analysis of transporter mRNA expression.

Detection of Mrps in mature and activated mouse BM-dendtric cells. PCR amplification on reverse transcribed total DC mRNA using primers for Mrp1, Mrp2, Mrp3, Mrp4, and Mrp5. The data is one of three represented experiments.

Figure 4.3 The loss of Mrp1 and Mrp4 during DC activation reduces IL-12 production but does not alter activation marker expression.

A. IL-12 production measured by ELISA, by cells activated with LPS, 10 *ug*/ml, either media alone, DMSO control, LPS control, MK571 25 *u*M, and Sildenafil 20 *u*M. Data is expressed as the mean ± SEM of duplicate samples. B. Up regulation of activation markers CD54 and CD86 measured by flow cytometry. Data expressed as mean intensity ± SEM of duplicate samples. Statistical difference (*) determined using 2-way ANOVA, plus post-hoc test ($p \leq 0.05$). Data is one of three representative experiments.

Figure 4.4 The loss of function attenuates IL12 production from DCs derived from wild type and Mrp1-/- mice.

A. IL 12 production with inhibitors adds during DC maturation. LPS control, media, MK571, 25 *u*M, sildenafil, 20 *u*M. Data expressed as ng/ml in supernatant. B. Comparing IL12 produced by wild type and Mrp1-/- DCs to their respective LPS controls. Data expressed as a percent of control. Statistical difference (α=control media to ko media, *** wild type to wild type, # ko to ko), determined using 2-way ANOVE, plus post hoc test ($p \leq 0.05$)

Figure 4.5 Inhibition of Mrp function during DC maturation alters cell phenotype.A. Flow cytometer measure of the % of wild type cells expressing CD11c, MHC, CD54, CD80, and CD86. B. The % of Mpr1-/- DC expressing CD11c, MHC, CD54, CD80, and CD86. C. Comparison of wild type to Mrp1-/- DCs expression of activation markers. Statistical difference determined using 2-way ANOVE, plus post hoc test (* $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$)

Figue 4.6 The Mrp4 inhibitor Sidenafil down regulates DC activation markers CD54 in Mrp1-/- mice.

Flow cytometery measure of DC activation markers on wild type DC and Mrp1-/- DC in media alone or treated with Sildenafil 20 *u*M. Data expressed as mean intensity. Statistical differences determined using 2-way ANOVE, plus post hoc test (* $p \leq 0.05$)

Figure 4.7 The loss of Mrps 1 and 4 reduces BM- DC ability to activate allogenic T cells.

A. T cell proliferation measured by tritiated thymidine incorporation post activation by wild type or Mrp1-/- DCs either matured in media alone, or in the presence of either MK571(25*u*M) or Sildenafil (20*u*M). B. Allogeneic T cell proliferation in response to wild type and $Mrp1^{-/-}$ BM-DC compared to wild

type Con A control (BM-DC to T cell ration = 1:4). Data expressed as the mean ± SEM of duplicate wells. Statistical differences determined using 2-way ANOVE, plus post hoc test (*$p \leq 0.05$, **$p \leq 0.01$, *** $p < 0.001$).

Figure 4.8 Rescue of activation marker up regulation on activated DCs, but not the percentage of cells displaying CD11c phenotype.

A. Percentage of cells expressing CD11c was decreased when matured in the presence of MK571, measured by flow cytometery. Data expressed as percent of control cells. B. Upregulation of activation markers CD54, CD80 and CD86, measured by flow cytometery in rescued cells. Data expressed as mean intensity. Statistical differences determined using 2-way ANOVE, plus post hoc test (*$p \leq 0.05$)

Table1. Percentage of CD11c cells expressing DC activation markers.

Marker	FVB	FVB/Mrp1-/-	Difference
MHC	64.25	45.07	-19.17
MHC+ Sil	48.68	43.34	-5.31
CD54	49.90	33.32	-16.56 *
CD54 + Sil	29.93	4.18	-25.75 **
CD 80	42.92	27.16	-15.76 *
CD 80 + Sil	24.22	27.27	3.05
CD 86	36.24	22.80	-13.44 **
CD86 + Sil	21.93	13.63	-8.29

*P < 0.005, **P < 0.001

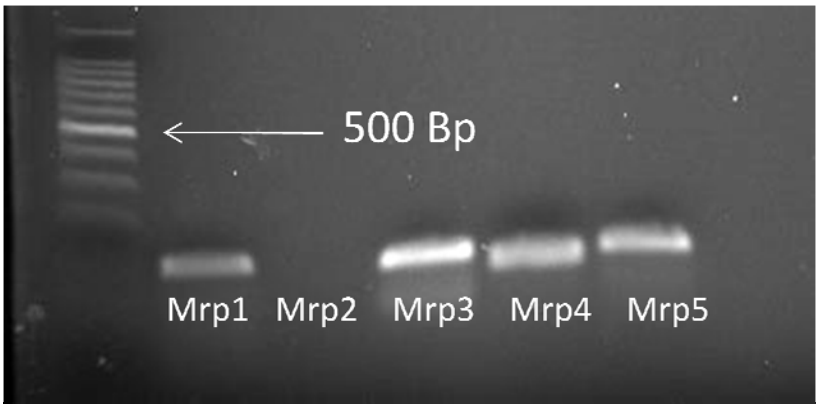
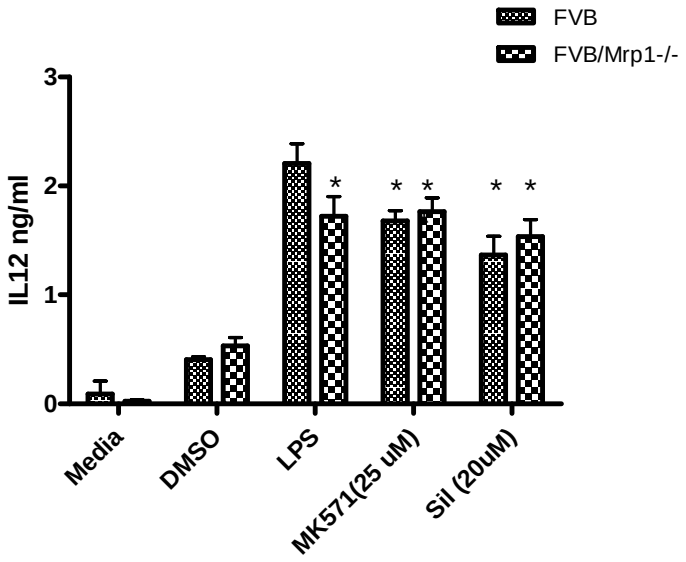


Figure 4.2

A.


FVB
FVB/Mrp1-/-
IL12 ng/ml
3
2
1
0
*
* *
* *
Media
DMSO
LPS
MK571(25 uM)
Sil (20uM)

B.

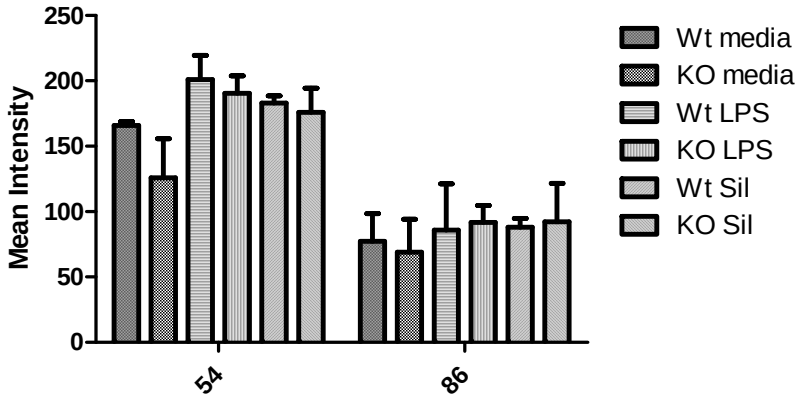
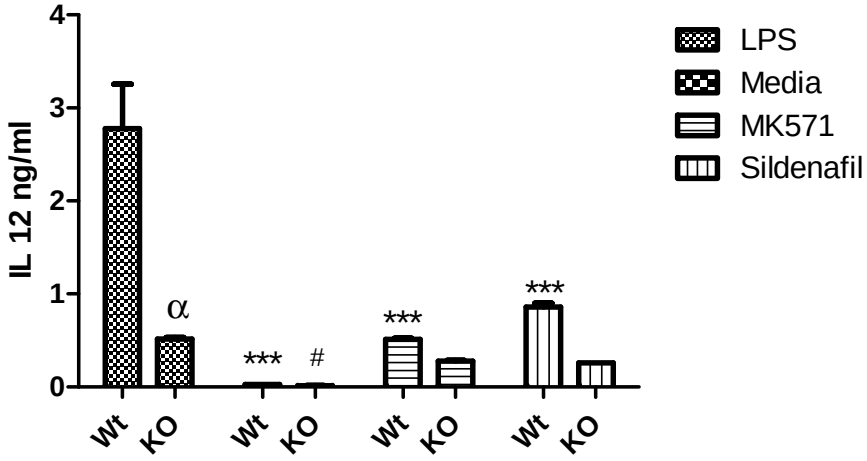

Mean Intensity
250
200
150
100
50
0
54
86
Wt media
KO media
Wt LPS
KO LPS
Wt Sil
KO Sil

Figure 4.

A.


IL 12 ng/ml
4
3
2
1
0
α
***
#
***
***
Wt
KO
Wt
KO
Wt
KO
Wt
KO
LPS
Media
MK571
Sildenafil

B.

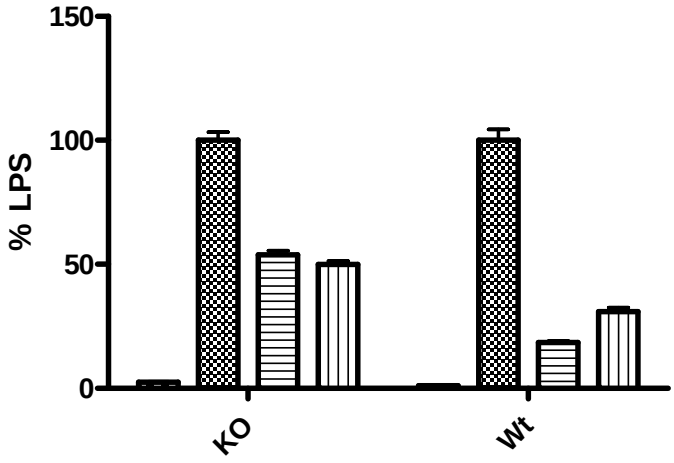

% LPS
150
100
50
0
KO
Wt

Figure 4.4

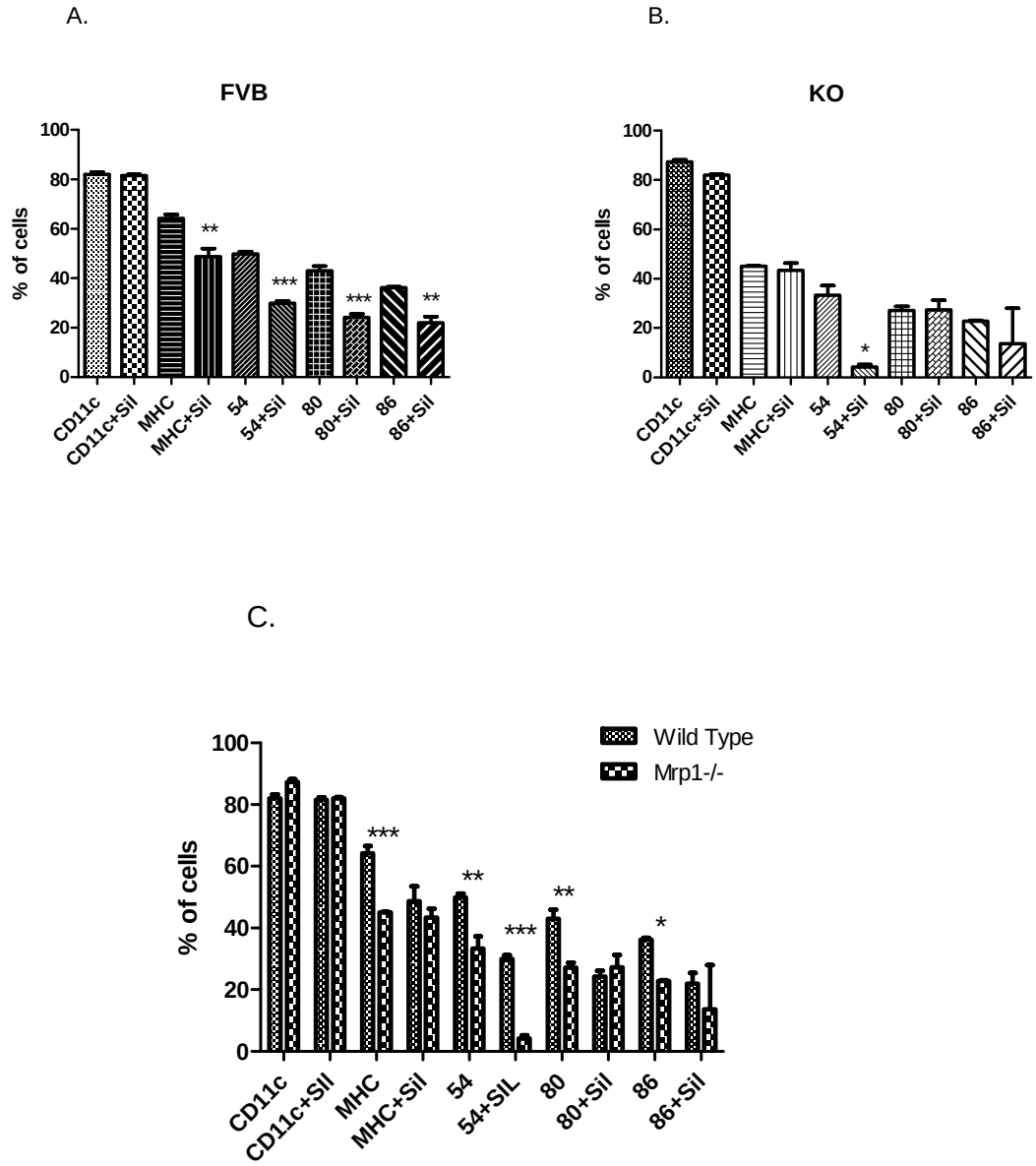

A.
FVB
% of cells
100
80
60
40
20
0
**
***
***
**
CD11c
CD11c+Sil
MHC
MHC+Sil
54
54+Sil
80
80+Sil
86
86+Sil
B.
KO
% of cells
100
80
60
40
20
0
*
CD11c
CD11c+Sil
MHC
MHC+Sil
54
54+Sil
80
80+Sil
86
86+Sil
C.
Wild Type
Mrp1-/-
% of cells
100
80
60
40
20
0
***
**
***
**
*
CD11c
CD11c+Sil
MHC
MHC+Sil
54
54+SIL
80
80+Sil
86
86+Sil

Figure 4.5

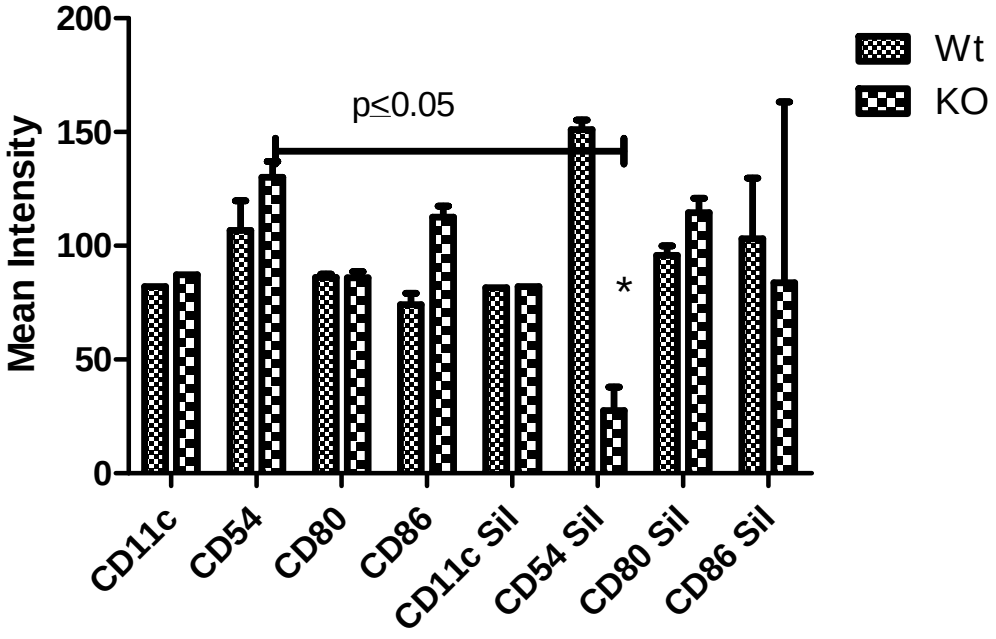
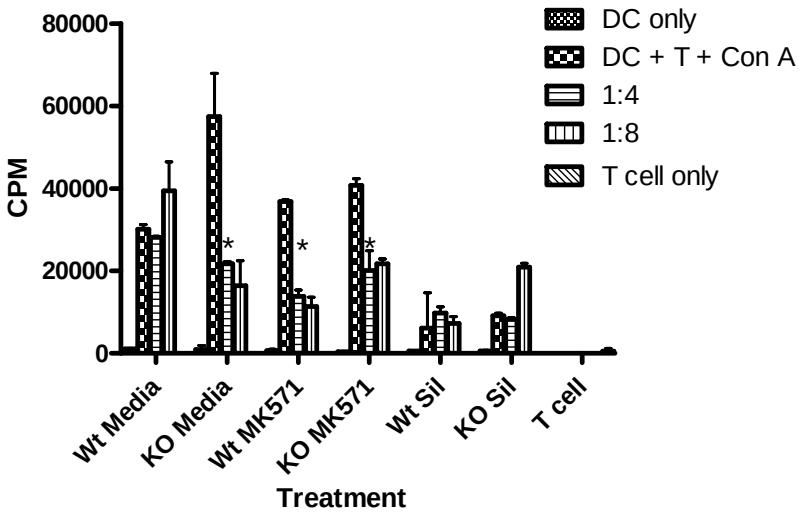

200
150
100
50
0
Mean Intensity
p≤0.05
*
Wt
KO
CD11c
CD54
CD80
CD86
CD11c Sil
CD54 Sil
CD80 Sil
CD86 Sil

Figure 4.6

A.


80000
60000
40000
20000
0
CPM
DC only
DC + T + Con A
1:4
1:8
T cell only
*
*
*
Wt Media
KO Media
Wt MK571
KO MK571
Wt Sil
KO Sil
T cell
Treatment

B.

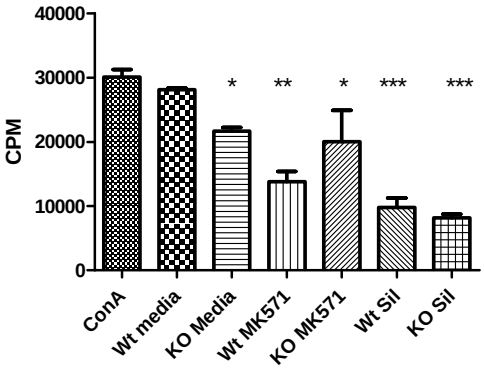

40000
30000
20000
10000
0
CPM
*
**
*
***
***
ConA
Wt media
KO Media
Wt MK571
KO MK571
Wt Sil
KO Sil

Figure 4.7

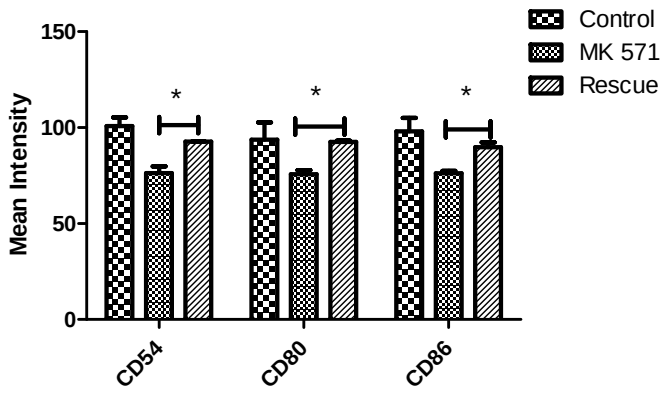

Control
MK 571
Rescue
Mean Intensity
150
100
50
0
*
*
*
CD54
CD80
CD86

Figure 4.8

Chapter 5: Extended discussion and Future directions

5.1 Discussion

Cancer is still a major health concern in the US, with an estimated 1,479,350 new cases in 2009 and an estimated 562,340 deaths (American Cancer Society, 2010). Cancer is the second leading cause of death in the United State averaging 599,888 deaths per year; only heart disease has a higher mortality rate with 631,636 deaths per year (CDC, 2010). From 1975 to 1990, there was an overall annual 0.5 percent increase in cancer mortalities, however, in the 1990s, the overall 5 year mortality rates began to began to fall. From 1990-1993, there was an overall 0.3 percent decrease in mortality, from 1993-2001 a 1.1 percent decrease, and from 2001-2006, a 1.6 percent decline in mortality (NCL, SEER, 2010). This decrease in mortality and subsequent increase in 5 year survival is in all probability due to advances in treatment and early detection. Although overall mortality rates have dropped, depending on the site of the primary neoplasm, the outlook is still for the patient may still be grim. Whereas 5 year survival rates for cancers of the testes and thyroid are above 90%, liver and pancreatic cancers' 5 year survival rates are 6% and 5% percent respectively (NIH, 2010).

The ultimate cause of refractory diseases in patients is due to the failure of chemotherapy. Even today, advances in chemotherapy progress at a sluggish pace. Statistical increases in chemotherapy efficacy are measured in terms of months. In a clinical trial comparing secondary treatments for prostate cancer with Cabaitaxel and mitoxantrone, after primary docetaxel treatment failure, there was a statistically significant median survival increase from 12.7 months to 15.1 months (Sartor et al, 2010). Although there was a statistically significant increase in in

median survival with the addition of the secondary treatment, few people would consider living an average 2.5 months longer in addition to suffering from the deleterious side effects of chemotherapy a significant improvement. As traditional chemotherapy agents indiscriminately kill healthy cells as well as cancer cells, and many new therapies are still taking this approach, most cancer patients have to suffer through the toxic side effects of chemotherapy. An alternative approach to standard chemotherapy treatment has been to overcome drug transporter mediated resistance, rather than trying to increase the innate toxicity of the chemotherapy agents. Inhibiting the activity of the efflux transporter responsible for extruding the chemotherapy agent, would thereby increase the intercellular retention time and cytotoxicity.

The quest to find a clinically effective MDR reversing agent has been ongoing for the past thirty years. The first ATP transporter to be characterized was P-glycoprotein (PgP) in 1979, and shortly thereafter, in 1981, the first attempt to use a drug as a PgP inhibitor was verapamil (Juliana and Lang, 1976, Tsuruo et al, 1981). This re-sensitized vincristine resistant leukemia cells (Tsuruo et al, 1981). In 1986, the immunosuppressant drug cyclosporine A was shown to reverse resistance to vincristine and daunorubicin in human T-cell lymphatic leukemia (Twentyman et al, 1987). Both verapamil and cyclosporine A were tested in clinical trials in an attempt to reverse MDR in patients. Unfortunately, neither of the compounds was able to clinically reverse MDR at the highest tolerable dosages (Wu et al, 2008). The reoccurring theme in all of the first generation inhibitors was low efficacy and high toxicity at clinical doses. Challenges arose with the search for second, third, and fourth generation drugs showing low toxicity and higher efficacy, as two additional families of ATP dependent transporters were discovered. In 1992, the first member of the second family of ATP dependent transporters, MRP1 was discovered and subsequently the newest ATP family member, breast cancer

resistance protein (BCRP) was discovered in 1998 (Cole et al, 1992, Doyle et al, 1998). The first MRP1 inhibitor discovered was the leukotriene LTD_4 antagonist MK571, developed in 1995 and in 1998 the fungal toxin fumitremorgin C was found to inhibit BCRP, thus beginning the race to find an inhibitor that could be used in the treatment of cancers that overexpressed these transporters (Gekeler et al, 1995, Rabindran et al, 1998). The challenge to date has been the synthesis of new compounds that are able to inhibit the growing array of drug resistance transporters but in addition have low toxicity. Currently, there have been greater than 100 compounds evaluated for the potential to inhibit transporter function (Choi, 2005). One question that still remains to be resolved is whether to inhibit a particular transporter with a single pump inhibitor or apply the use of a multi-pump compound to inhibit 2 or more transporters. The former would probably have fewer side effects, but may be less effective. This decision would depend on the phenotype of the tumor and the number and type of transporters being over expressed.

Either strategy has its own potential pitfalls. As we have shown in chapter two, inhibiting Mrp1 alone resulted in several unforeseen consequences and could be a double-edged sword in some tissues. The major P450s involved in vincristine phase I metabolism are CYP3A4 and CYP3A5 (Dennison et al, 2006). We found the loss of Mrp1 leads to increased Cyp3A4 activity in the mouse kidney. This increased activity may diminish the effectiveness of vincristine in treating renal tumors due to increased phase I metabolism of vincristine. Conversely, we also established a decrease in renal Mrp2 expression, resulting in a buildup of vincristine in the Mrp1 -/- mouse kidney, which may in turn exaggerate potential renal damage as a result of the chemotherapy. Mice lacking Mrp1 have been shown to have increased chemotherapy related damage to the seminiferous tubules, possibly leading to lower fertility (Wijnhold et al, 1998). As

chemotherapy alone can render males infertile, combining chemotherapy with an MRP1 or global MRP inhibitor if any of the drugs in the treatment régime were MRP1 substrates, the resulting damage would almost certainly cause male infertility (Drasga et al, 1983).

Although the effects described above as a result of inhibiting drug transporters to increase the efficacy of chemotherapy treatment are deleterious, anticancer therapy is usually relatively short in duration and the patient may thereby be spared any permanent damage. However MRP1, MRP4, and MRP5 are known to transport antiviral therapeutics as well as anticancer chemotherapy drugs (Fridland et al, 2000). HIV infection has been shown to increase the expression of MRP1, MRP4, and MRP5 in infected cells which translates into a reduction in the intracellular concentration of the antiviral cocktail. When trying to circumvent viral drug resistance, inhibiting the transporters may appear as a viable addition to highly active antiretroviral therapy (HAART), in HIV patients however, the result may again be unwanted side effects. As we illustrated in Chapter 3, Mrp1-/- mice have decreased levels of circulating testosterone. Long term MRP1 inhibition in a clinical setting may lead to chronic low testosterone, manifesting in decreased fertility and sex drive, depression, obesity, diabetes, cardiovascular diseases and possibly osteoporosis (Hoffman, 2008). Furthermore the long term inhibition of MRP1 and the resulting changes in steroid levels and ensuing alterations in metabolizing enzymes may have unforeseen systemic physiological and pharmacological consequences. The steroid related induction of Cyp3A4 in the kidney may potentiate the bioactivation of aflatoxin B1, an Mrp1 substrate, resulting in increased nephrotoxicity and an increased risk of renal carcinoma (Mathuria and Verma, 2008). Also, the widely used drug acetaminophen (APAP) can undergo Cyp3A4 bioactivation with the potential buildup of toxic metabolites resulting in severe APAP induce renal damage (Ma et al, 2009).

The long term inhibition of MRP1 in conjunction with antiviral therapy may not be a viable choice due to, but not limited to, the above unfavorable effects. Even the short term inhibition of MRP1 during chemotherapy may result in more serious side effects than just increased toxicity. As we discovered in chapter 4, both MRP1 and MRP4 have been implicated in DC maturation and activation. Within 24 hours from the time a DC acquires and processes a pathogenic antigen, it has gone through maturation and migration to a draining lymph node (He et al, 2006). If a patient were undergoing chemotherapy with an MRP1, MRP4 or a global MRP inhibitor, and were to be exposed to a unique pathogen, the resulting attenuation in DC activation would lead to a delay in the adaptive immune response. This, when added to an already crippled immune system due to the chemotherapy, could overwhelm the innate immune response resulting in increased morbidity or mortality. In this scenario, even a normally nonpathogenic organism could cause a potentially fatal infection.

In conclusion, reversal or prevention of ABC transporter mediated MDR in cancer patients undergoing chemotherapy is still the Holy Grail in clinical oncology. Prevention of the MDR phenotype would greatly increase the efficacy of existing chemotherapy drugs, potentiate therapy in intrinsically resistant cancers, and potentially eliminate refractory disease in cancer patients. Research is continuing with both known and newly synthesized compounds in an attempt develop an effective transporter inhibitor. There have been some promising *in vivo* and clinical successes, including verapamil, cyclosporine A, or PSC833 when administration prior to treating MDR resistant tumors in mice with the chemotherapy drug doxorubicin. Pretreatment increased doxorubicin accumulation in tumor cells from 68% to 94% (Shen et al, 2007). However, data found in the literature reports that the severe side effects caused by therapeutic levels of traditional transporter inhibitors greatly would hamper any viable clinical application. This

study reports potentially negative physiological consequences resulting from global transporter inhibition. There is, however, some promising research being done with nontraditional transporter inhibitors especially with RNA interference technologies, including short hairpin RNA (shRNA),and small interfering RNAs (siRNA) (Lage H , 2008). One such technology with promising results is Stealth^{TM} RNAi, which targeted Pgp (Xiao et al, 2008). The administration of Stealth RNAi in conjunction with the chemotherapy drug, Navelbine, increased the drug efficacy 9 fold (Xio et al, 2008). The advantage to these innovative treatments is they can be targeted to the tumor cells and have little, if any, affect on surrounding tissues.

Reversing the MDR phenotype is still a viable strategy in the treatment of drug resistant neoplasms. With the advent of tumor targeted therapies and the reduction of collateral damage to surrounding tissues and deleterious side effects, there is a light at the end of the tunnel in search for effective and safe anti cancer therapies.

5.2 Future Directions

Our preliminary data suggest that Mrp1 and/or Mrp4 are required for effective DC maturation and activation. The loss of either of the transporters negatively impacted the DC ability to properly mature and upon stimulation to be become efficiently activated. It appears thus far that both transporters play some role in DC function. We would like to continue using the Mrp1-/- mouse model to fully characterize the roles Mrp1 and Mrp4.play in DC function. Additionally our data shows that there is an as of yet undetermined MRP substrate that has been implicated in DC maturation/activation. Our data indicates that there is a compound that is exported out of the cell as the DC goes through maturation and activation. We would like to confirm this hypothesis, and confirm the existence of and characterize of the compound. If the existence of the MRP

substrate can be determined this may provide immunologist with a new tool for manipulating the immune response.

References

Adachi, M., et al., *Expression of MRP4 Confers Resistance to Ganciclovir and Compromises Bystander Cell Killing.* Journal of Biological Chemistry, 2002. **277**(41): p. 38998-39004.

Aleksunes, L.M., et al., *Coordinated Expression of Multidrug Resistance-Associated Proteins (Mrps) in Mouse Liver during Toxicant-Induced Injury.* Toxicol. Sci., 2006. **89**(2): p. 370-379.

Aleksunes, L.M., et al., *Differential Expression of Mouse Hepatic Transporter Genes in Response to Acetaminophen and Carbon Tetrachloride.* Toxicol. Sci., 2005. **83**(1): p. 44-52.

Ambudkar, S.V., et al., *BIOCHEMICAL, CELLULAR, AND PHARMACOLOGICAL ASPECTS OF THE MULTIDRUG TRANSPORTER.* Annual Review of Pharmacology & Toxicology, 1999. **39**(1): p. 361.

Armstrong, R.N., *Structure, Catalytic Mechanism, and Evolution of the Glutathione Transferases.* Chemical Research in Toxicology, 1997. **10**(1): p. 2-18.

Assem, M., et al., *Interactions between Hepatic Mrp4 and Sult2a as Revealed by the Constitutive Androstane Receptor and Mrp4 Knockout Mice.* J. Biol. Chem., 2004. **279**(21): p. 22250-22257.

Augustine, L.M., et al., *XENOBIOTIC AND ENDOBIOTIC TRANSPORTER MRNA EXPRESSION IN THE BLOOD-TESTIS BARRIER.* Drug Metab Dispos, 2005. **33**(1): p. 182-189.

Baldwin, W.S. and G.A. Leblanc, *The anti-carcinogenic plant compound indole-3-carbinol differentially modulates P450-mediated steroid hydroxylase activities in mice.* Chemico-Biological Interactions, 1992. **83**(2): p. 155-169.

Ballatori, N., et al., *Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology.* Molecular Aspects of Medicine. **30**(1-2): p. 13-28.

Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-252.

Banoglu, E., *Current Status of the Cytosolic Sulfotransferases in the Metabolic Activation of Promutagens and Procarcinogens.* Current Drug Metabolism, 2000. **1**(1): p. 1.

Bart, J., et al., *The distribution of drug-efflux pumps, P-gp, BCRP, MRP1 and MRP2, in the normal blood-testis barrier and in primary testicular tumours.* European Journal of Cancer, 2004. **40**(14): p. 2064-2070.

Blanchard, R.L., et al., *A proposed nomenclature system for the cytosolic sulfotransferase (SULT) superfamily.* Pharmacogenetics and Genomics, 2004. **14**(3): p. 199-211.

Bortfeld, M., et al., *Human multidrug resistance protein 8 (MRP8/ABCC11), an apical efflux pump for steroid sulfates, is an axonal protein of the CNS and peripheral nervous system.* Neuroscience, 2006. **137**(4): p. 1247-1257.

Buckley, D.B. and C.D. Klaassen, *Tissue- and Gender-Specific mRNA Expression of UDP-Glucuronosyltransferases (UGTs) in Mice.* Drug Metabolism and Disposition, 2007. **35**(1): p. 121-127.

Caux, C., et al., *Activation of human dendritic cells through CD40 cross-linking.* The Journal of Experimental Medicine, 1994. **180**(4): p. 1263-1272.

Che, X.-F., et al., *Reversal of P-glycoprotein mediated multidrug resistance by a newly synthesized 1,4-benzothiazipine derivative, JTV-519.* Cancer letters, 2002. **187**(1-2): p. 111-119.

Chen, Z.-S., et al., *Transport of Bile Acids, Sulfated Steroids, Estradiol 17-{beta}-D-Glucuronide, and Leukotriene C4 by Human Multidrug Resistance Protein 8 (ABCC11).* Mol Pharmacol, 2005. **67**(2): p. 545-557.

Chen, Z.-S., et al., *Characterization of the Transport Properties of Human Multidrug Resistance Protein 7 (MRP7, ABCC10).* Mol Pharmacol, 2003. **63**(2): p. 351-358.

Cheng, J., et al., *Rifampicin-Activated Human Pregnane X Receptor and CYP3A4 Induction Enhance Acetaminophen-Induced Toxicity.* Drug Metabolism and Disposition, 2009. **37**(8): p. 1611-1621

.

Choudhuri, S., Klaassen, Curtis E, *Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters.* International Journal of Toxicology, 2006. **25**: p. 28.

Cole, S.P.C., et al., *Overexpression of a Transporter Gene in a Multidrug-Resistant Human Lung Cancer Cell Line.* Science, 1992. **258**(5088): p. 1650-1654.

Cole, S.P.C., et al., *Pharmacological Characterization of Multidrug Resistant MRP-transfected Human Tumor Cells.* Cancer Res, 1994. **54**(22): p. 5902-5910.

Cui, Y., et al., *Drug Resistance and ATP-Dependent Conjugate Transport Mediated by the Apical Multidrug Resistance Protein, MRP2, Permanently Expressed in Human and Canine Cells.* Mol Pharmacol, 1999. **55**(5): p. 929-937.

Cui, Y.J., et al., *Compensatory Induction of Liver Efflux Transporters in Response to ANIT-Induced Liver Injury Is Impaired in FXR-Null Mice.* Toxicol. Sci., 2009. **110**(1): p. 47-60.

Dallas, S., et al., *Multidrug resistance protein 1-mediated transport of saquinavir by microglia.* NeuroReport, 2004. **15**(7): p. 1183-1186.

Danielson, P.B., *The Cytochrome P450 Superfamily: Biochemistry, Evolution and Drug Metabolism in Humans.* Current Drug Metabolism, 2002. **3**(6): p. 561-597.

Dauchy, S., et al., *Expression and transcriptional regulation of ABC transporters and cytochromes P450 in hCMEC/D3 human cerebral microvascular endothelial cells.* Biochemical Pharmacology, 2009. **77**(5): p. 897-909.

Dean, M., *ABC Transporters, Drug Resistance, and Cancer Stem Cells* Journal of Mammary Gland Biology and Neoplasia, 2009. **14**(1): p. 6.

Deeley, R.G. and S.P.C. Cole, *Substrate recognition and transport by multidrug resistance protein 1 (ABCC1).* FEBS Letters, 2006. **580**(4): p. 1103-1111.

Deeley, R.G., C. Westlake, and S.P.C. Cole, *Transmembrane Transport of Endo- and Xenobiotics by Mammalian ATP-Binding Cassette Multidrug Resistance Proteins.* Physiol. Rev., 2006. **86**(3): p. 849-899.

Denk, G.U., et al., *Multidrug resistance-associated protein 4 is up-regulated in liver but down-regulated in kidney in obstructive cholestasis in the rat.* Journal of Hepatology, 2004. **40**(4): p. 585-591.

Dong, H., et al., *Involvement of Human Cytochrome P450 2D6 in the Bioactivation of Acetaminophen.* Drug Metab Dispos, 2000. **28**(12): p. 1397-1400.

Dooley, T.P., et al., *Expression Profiling of Human Sulfotransferase and Sulfatase Gene Superfamilies in Epithelial Tissues and Cultured Cells.* Biochemical and Biophysical Research Communications, 2000. **277**(1): p. 236-245.

Drasga, R., et al., *Fertility after chemotherapy for testicular cancer.* J Clin Oncol, 1983. **1**(3): p. 179-183.

Dubin IN, J.F., *Chronic idiopathic jaundice with unidentified pigment in liver cells; a new clinicopathologic entity with a report of 12 cases.* Medicine, 1954. **33**(3): p. 52.

Eilers, M., U. Roy, and D. Mondal, *MRP (ABCC) Transporters-Mediated Efflux of Anti-HIV Drugs, Saquinavir and Zidovudine, from Human Endothelial Cells.* Experimental Biology and Medicine, 2008. **233**(9): p. 1149-1160.

Endres, C.J., et al., *The role of transporters in drug interactions.* European Journal of Pharmaceutical Sciences, 2006. **27**(5): p. 501-517.

Enokizono, J., H. Kusuhara, and Y. Sugiyama, *Regional Expression and Activity of Breast Cancer Resistance Protein (Bcrp/Abcg2) in Mouse Intestine: Overlapping Distribution with Sulfotransferases.* Drug Metabolism and Disposition, 2007. **35**(6): p. 922-928.

Evans, W.E. and M.V. Relling, *Pharmacogenomics: Translating Functional Genomics into Rational Therapeutics.* Science, 1999. **286**(5439): p. 487-491.

Evers R, K.M., van Deemter L, Janssen H, Calafat J, Oomen LC, Paulusma CC, Oude Elferink RP, Baas F, Schinkel AH, and Borst P, *Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA.* The Journal of clinical investigation., 1998. **101**(7): p. 9.

Falany, C.N., et al., *Regulation of hepatic sulfotransferase (SULT) 1E1 expression and effects on estrogenic activity in cystic fibrosis (CF).* The Journal of Steroid Biochemistry and Molecular Biology, 2009. **114**(1-2): p. 113-119.

Falany, J.L., et al., *Elevation of hepatic sulphotransferase activities in mice with resistance to cystic fibrosis.* Biochem. J., 2002. **364**(1): p. 115-120.

Flens, M., et al., *Tissue distribution of the multidrug resistance protein.* Am J Pathol, 1996. **148**(4): p. 1237-1247.

Francesco, L., et al., *Structural lung damage after chemotherapy: Fact or fiction?* Lung cancer (Amsterdam, Netherlands), 2010. **67**(3): p. 306-310.

Fujita K, N.K., Yamazaki T, Watanabe E, Shimada M, Yamazoe Y., *Enzymatic characterization of human cytosolic sulfotransferases; identification of ST1B2 as a thyroid hormone sulfotransferase.* Biological & pharmaceutical bulletin, 1999. **22**(5): p. 6.

Fujita, K.-i., et al., *Molecular Cloning and Characterization of Rat ST1B1 and Human ST1B2 cDNAs, Encoding Thyroid Hormone Sulfotransferases.* J Biochem, 1997. **122**(5): p. 1052-1061.

Gamage, N., et al., *Human Sulfotransferases and Their Role in Chemical Metabolism.* Toxicol. Sci., 2006. **90**(1): p. 5-22.

Gary D. Kruh, Y.G., Elizabeth Hopper-Borge, Martin G. and Zhe-Sheng Chen, *ABCC10,ABCC11,ABCC12.* Pflügers Archiv European Journal of Physiology, 2007. **453**(5): p. 9.

Gillet, J.-P., T. Efferth, and J. Remacle, *Chemotherapy-induced resistance by ATP-binding cassette transporter genes.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2007. **1775**(2): p. 237-262.

Gonzalez-Angulo AM, M.-V.F., Hortobagyi GN., *Overview of resistance to systemic therapy in patients with breast cancer.* Advances in experimental medicine and biology., 2007(608): p. 1-22.

Grant, C.E., et al., *Structural Determinants of Substrate Specificity Differences between Human Multidrug Resistance Protein (MRP) 1 (ABCC1) and MRP3 (ABCC3).* Drug Metab Dispos, 2008. **36**(12): p. 2571-2581.

Guengerich, F.P., *CYTOCHROME P-450 3A4: Regulation and Role in Drug Metabolism.* Annual Review of Pharmacology and Toxicology, 1999. **39**(1): p. 1-17.

Guengerich, F.P., *Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity.* Chemical Research in Toxicology, 2001. **14**(6): p. 611-650.

http.//./www.cancer.org/downloads/stt/CFF2009_EstCD_3.pdf

http.//www.cancercontrolplanet.cancer.gov/atlas/timeall.jsp?ac=1

http.//./www.seer.cancer.gov/csr/1975_2006/browse_csr.php?section=2&page=sect_02_table.01.html

http.//./www.cdc.gov/nchs/fastats/lcod.htm

http://rex.nci.nih.gov/NCI_Pub_Interface/raterisk/rates28.html

http.//./www.men.webmd.com/features/low-testosterone-explained-how-do-you-know-when-levels-are-too-low

http://www.sciencedaily.com /releases/2010/03/100326101108.htm HER2 posative breast cancer survival 14 months<2000<21 months.

Haimeur A., C.G., Deeley R.G. , Cole S.P.C., *The MRP-Related and BCRP / ABCG2 Multidrug Resistance Proteins: Biology, Substrate Specificity and Regulation.* Current Drug Metabolism 2004. **5**(1): p. 33.

Hartmann, G., A.K.Y. Cheung, and M. Piquette-Miller, *Inflammatory Cytokines, but Not Bile Acids, Regulate Expression of Murine Hepatic Anion Transporters in Endotoxemia.* Journal of Pharmacology and Experimental Therapeutics, 2002. **303**(1): p. 273-281.

Hayashi, A., et al., *Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein1 in mouse embryo fibroblasts.* Biochemical and Biophysical Research Communications, 2003. **310**(3): p. 824-829.

Hayes, J.D., J.U. Flanagan, and I.R. Jowsey, *GLUTATHIONE TRANSFERASES.* Annual Review of Pharmacology & Toxicology, 2005. **45**(1): p. 51-C-2.

He, D., et al., *Repression of CFTR activity in human MMNK-1 cholangiocytes induces sulfotransferase 1E1 expression in co-cultured HepG2 hepatocytes.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2008. **1783**(12): p. 2391-2397.

He, Y., et al., *Skin-Derived Dendritic Cells Induce Potent CD8+ T Cell Immunity in Recombinant Lentivector-Mediated Genetic Immunization.* 2006. **24**(5): p. 643-656.

Heath, W.R. and F.R. Carbone, *Dendritic cell subsets in primary and secondary T cell responses at body surfaces.* Nat Immunol, 2009. **10**(12): p. 1237-1244.

Hirohashi, T., et al., *Hepatic Expression of Multidrug Resistance-Associated Protein-Like Proteins Maintained in Eisai Hyperbilirubinemic Rats.* Mol Pharmacol, 1998. **53**(6): p. 1068-1075.

Hopper, E., et al., *Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily.* Cancer Letters, 2001. **162**(2): p. 181-191.

Hopper-Borge, E., et al., *Analysis of the Drug Resistance Profile of Multidrug Resistance Protein 7 (ABCC10): Resistance to Docetaxel.* Cancer Res, 2004. **64**(14): p. 4927-4930.

Hopper-Borge, E., et al., *Human Multidrug Resistance Protein 7 (ABCC10) Is a Resistance Factor for Nucleoside Analogues and Epothilone B.* Cancer Res, 2009. **69**(1): p. 178-184.

Hou, Y.-x., J.R. Riordan, and X.-b. Chang, *ATP Binding, Not Hydrolysis, at the First Nucleotide-binding Domain of Multidrug Resistance-associated Protein MRP1 Enhances ADP{middle dot}Vi Trapping at the Second Domain.* J. Biol. Chem., 2003. **278**(6): p. 3599-3605.

Huang, Y. and W. Sadée, *Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells.* Cancer letters, 2006. **239**(2): p. 168-182.

James I Elliott, S.R., Christopher F Higgins,, *Multidrug transporter activity in lymphocytes.* British Journal of Pharmacology, 2004. **143**(7): p. 899-907.

Jedlitschky, G., et al., *ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2.* Biochem. J., 1997. **327**(1): p. 305-310.

Jemal, A., et al., *Cancer Statistics, 2008.* CA Cancer J Clin, 2008. **58**(2): p. 71-96.

Johson, B.M., et al., *CHARACTERIZATION OF TRANSPORT PROTEINEXPRESSION IN MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN (MRP) 2-DEFICIENT RATS.* Drug Metabolism and Disposition, 2006. **34**(4): p. 556-562.

Jorajuria, S., et al., *Differential Expression Levels of MRP1, MRP4, and MRP5 in Response to Human Immunodeficiency Virus Infection in Human Macrophages.* Antimicrob. Agents Chemother., 2004. **48**(5): p. 1889-1891.

Jorajuria S, D.-B.N., Becher F, Martin S, Porcheray F, Garrigues A, Mabondzo A, Benech H, Grassi J, Orlowski S, Dormont D, Clayette P., *ATP binding cassette multidrug transporters limit the anti-HIV activity of zidovudine and indinavir in infected human macrophages.* Antiviral therapy, 2004. **9**(4): p. 9.

José E. Manautou, D.R.d.W., Cindy Kunne, Noam Zelcer, Michael Goedken, Piet Borst, Ronald Oude Elferink,, *Altered disposition of acetaminophen in mice with a disruption of the <I>Mrp3</I> gene.* Hepatology, 2005. **42**(5): p. 1091-1098.

Kamath, A.T., et al., *Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs.* Blood, 2002. **100**(5): p. 1734-1741.

Kamath, A.T., et al., *The Development, Maturation, and Turnover Rate of Mouse Spleen Dendritic Cell Populations.* J Immunol, 2000. **165**(12): p. 6762-6770.

Kang, H.S., et al., *Gene expression profiling reveals a regulatory role for ROR{alpha} and ROR{gamma} in phase I and phase II metabolism.* Physiol. Genomics, 2007. **31**(2): p. 281-294.

Klaassen, C.D. and L.M. Aleksunes, *Xenobiotic, Bile Acid, and Cholesterol Transporters: Function and Regulation.* Pharmacological Reviews, 2010. **62**(1): p. 1-96.

Klaassen, C.D. and H. Lu, *Xenobiotic Transporters: Ascribing Function from Gene Knockout and Mutation Studies.* Toxicol. Sci., 2008. **101**(2): p. 186-196.

Kleijmeer, M., et al., *MHC class II compartments and the kinetics of antigen presentation in activated mouse spleen dendritic cells.* J Immunol, 1995. **154**(11): p. 5715-5724.

KURODA, M., et al., *Increased hepatic and renal expressions of multidrug resistance-associated protein 3 in Eisai hyperbilirubinuria rats.* Journal of Gastroenterology and Hepatology, 2004. **19**(2): p. 146-153.

Lam, P., R. Wang, and V. Ling, *Bile Acid Transport in Sister of P-Glycoprotein (ABCB11) Knockout Mice&#x2020.* Biochemistry, 2005. **44**(37): p. 12598-12605.

Leslie, E.M., R.G. Deeley, and S.P.C. Cole, *Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters.* Toxicology, 2001. **167**(1): p. 3-23.

Leslie, E.M., R.G. Deeley, and S.P.C. Cole, *Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense.* Toxicology and Applied Pharmacology, 2005. **204**(3): p. 216-237.

Lewis, D.F., *P450 structures and oxidative metabolismof xenobiotics.* Pharmacogenomics, 2003. **4**(4): p. 387-395.

Longley, D.B. and P.G. Johnston, *Molecular mechanisms of drug resistance.* The Journal of Pathology, 2005. **205**: p. 275-292.

Lye Lin Ho, et al., *Androgen regulation of multidrug resistance-associated protein 4 (MRP4/ABCC4) in prostate cancer.* The Prostate, 2008. **68**(13): p. 1421-1429.

M Lohoff, S.P., F Sommer, M Roellinghoff, E Schmitt, G Gradehandt, P Rohwer, B D Stride, S P Cole and R G Deeley, *A multidrug-resistance protein (MRP)-like transmembrane pump is highly expressed by resting murine T helper (Th) 2, but not Th1 cells, and is induced to equal expression levels in Th1 and Th2 cells after antigenic stimulation in vivo.* The Journal of clinical investigation, 1998. **101**(3): p. 7.

Madon, J., et al., *Transport Function and Hepatocellular Localization of mrp6 in Rat Liver.* Mol Pharmacol, 2000. **57**(3): p. 634-641.

Maeno, K., et al., *Molecular Basis for Reduced Estrone Sulfate Transport and Altered Modulator Sensitivity of TM6 and TM17 Mutants of MRP1 (ABCC1).* Drug Metab Dispos, 2009: p. dmd.109.026633.

Mahaffey, C.M., et al., *Multidrug-resistant protein-3 gene regulation by the transcription factor Nrf2 in human bronchial epithelial and non-small-cell lung carcinoma.* Free Radical Biology and Medicine, 2009. **46**(12): p. 1650-1657.

Maher, J.M., et al., *Nrf2- and PPAR{alpha}-Mediated Regulation of Hepatic Mrp Transporters after Exposure to Perfluorooctanoic Acid and Perfluorodecanoic Acid.* Toxicol. Sci., 2008. **106**(2): p. 319-328.

Maher, J.M., et al., *Hormonal regulation of renal multidrug resistance-associated proteins 3 and 4 (Mrp3 and Mrp4) in mice.* Biochemical Pharmacology, 2006. **71**(10): p. 1470-1478.

Maher, J.M., et al., *Tissue distribution and induction of the rat multidrug resistance-associated proteins 5 and 6.* Life Sciences, 2006. **78**(19): p. 2219-2225.

Maher, J.M., et al., *TISSUE DISTRIBUTION AND HEPATIC AND RENAL ONTOGENY OF THE MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN (MRP) FAMILY IN MICE.* Drug Metabolism and Disposition, 2005. **33**(7): p. 947-955.

Maria Rius, A.T.N., Johanna Hummel-Eisenbeiss, Gabriele Jedlitschky, Dietrich Keppler,, *Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane.* Hepatology, 2003. **38**(2): p. 374-384.

Marschall, H.-U., et al., *Fxr-/- mice adapt to biliary obstruction by enhanced phase I detoxification and renal elimination of bile acids.* J. Lipid Res., 2006. **47**(3): p. 582-592.

Mathuria Neeta, V.J.R., *Ameliorative Effect of Curcumin on Aflatoxin-Induced Toxicity in Serum of Mice.* Acta Poloniae Pharmaceutica-Drug Research, 2008. **65**(3): p. 339-343.

McAleer, M.A., et al., *pABC11 (Also Known as MOAT-C and MRP5), a Member of the ABC Family of Proteins, Has Anion Transporter Activity but Does Not Confer Multidrug Resistance When Overexpressed in Human Embryonic Kidney 293 Cells.* J. Biol. Chem., 1999. **274**(33): p. 23541-23548.

McGrath, T. and M.S. Center, *Adriamycin resistance in HL60 cells in the absence of detectable P-glycoprotein.* Biochemical and Biophysical Research Communications, 1987. **145**(3): p. 1171-1176.

McGrath, T. and M.S. Center, *Mechanisms of Multidrug Resistance in HL60 Cells: Evidence That a Surface Membrane Protein Distinct from P-Glycoprotein Contributes to Reduced Cellular Accumulation of Drug.* Cancer Res, 1988. **48**(14): p. 3959-3963.

McLellan, A.D. and E. Kämpgen, *Functions of myeloid and lymphoid dendritic cells.* Immunology Letters, 2000. **72**(2): p. 101-105.

Meijerman, I., J.H. Beijnen, and J.H.M. Schellens, *Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer.* Cancer Treatment Reviews, 2008. **34**(6): p. 505-520.

Meng, S., et al., *Schisandrin B: A dual inhibitor of P-glycoprotein and multidrug resistance-associated protein 1.* Cancer letters, 2007. **246**(1): p. 300-307.

Ming, X. and D.R. Thakker, *Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil.* Biochemical Pharmacology. **79**(3): p. 455-462.

Mirski, S.E.L., J.H. Gerlach, and S.P.C. Cole, *Multidrug Resistance in a Human Small Cell Lung Cancer Cell Line Selected in Adriamycin.* Cancer Res, 1987. **47**(10): p. 2594-2598.

Montserrat Marí and Arthur I. Cederbaum, *Induction of catalase, alpha, and microsomal glutathione S-transferase in CYP2E1 overexpressing HepG2 cells and protection against short-term oxidative stress.* Hepatology, 2001. **33**(3): p. 652-661.

Nagata, K. and Y. Yamazoe, *Pharmacogenetics of Sulfotransferase.* Annual Review of Pharmacology & Toxicology, 2000. **40**(1): p. 159.

New, M.I. and P.C. White, *Genetic disorders of steroid hormone synthesis and metabolism.* Baillière's Clinical Endocrinology and Metabolism, 1995. **9**(3): p. 525-554.

Nies, A.T., M. Schwab, and D. Keppler, *Interplay of conjugating enzymes with OATP uptake transporters and ABCC/MRP efflux pumps in the elimination of drugs.* Expert Opinion on Drug Metabolism & Toxicology, 2008. **4**(5): p. 545-568.

Nishimura, M. and S. Naito, *Tissue-specific mRNA Expression Profiles of Human ATP-binding Cassette and Solute Carrier Transporter Superfamilies.* Drug Metabolism and Pharmacokinetics, 2005. **20**(6): p. 452-477.

Okada, K., et al., *Nrf2 counteracts cholestatic liver injury via stimulation of hepatic defense systems.* Biochemical and Biophysical Research Communications, 2009. **389**(3): p. 431-436.

Oldenhove, G., et al., *CD4+ CD25+ Regulatory T Cells Control T Helper Cell Type 1 Responses to Foreign Antigens Induced by Mature Dendritic Cells In Vivo.* The Journal of Experimental Medicine, 2003. **198**(2): p. 259-266.

Patrick T. Ronaldson, Y.P., Reina Bendayan,, *Regulation of ABC membrane transporters in glial cells: Relevance to the pharmacotherapy of brain HIV-1 infection.* Glia, 2008. **56**(16): p. 1711-1735.

Payen, L., et al., *Functional Interactions Between Nucleotide Binding Domains and Leukotriene C4 Binding Sites of Multidrug Resistance Protein 1 (ABCC1).* Mol Pharmacol, 2005. **67**(6): p. 1944-1953.

Peng, K.-C., et al., *Tissue and Cell Distribution of the Multidrug Resistance-Associated Protein (MRP) in Mouse Intestine and Kidney.* J. Histochem. Cytochem., 1999. **47**(6): p. 757-768.

Petrick, J.S. and C.D. Klaassen, *Importance of Hepatic Induction of Constitutive Androstane Receptor and Other Transcription Factors That Regulate Xenobiotic Metabolism and Transport.* Drug Metabolism and Disposition, 2007. **35**(10): p. 1806-1815.

Pratt, S., et al., *The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites.* Molecular Cancer Therapeutics, 2005. **4**(5): p. 855-863.

Qadri, I., et al., *Interaction of hepatocyte nuclear factors in transcriptional regulation of tissue specific hormonal expression of human multidrug resistance-associated protein 2 (abcc2).* Toxicology and Applied Pharmacology, 2009. **234**(3): p. 281-292.

Radominska-Pandya, A., S. Bratton, and J.M. Little, *A Historical Overview of the Heterologous Expression of Mammalian UDP-Glucuronosyltransferase Isoforms Over the Past Twenty Years.* Current Drug Metabolism, 2005. **6**(2): p. 141-160.

Radominska-Pandya, A., et al., *STRUCTURAL AND FUNCTIONAL STUDIES OF UDP-GLUCURONOSYLTRANSFERASES*.* Drug Metabolism Reviews, 1999. **31**(4): p. 817.

Randolph, G.J., *Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators.* Seminars in Immunology, 2001. **13**(5): p. 267-274.

Rappa, G., et al., *Evidence That the Multidrug Resistance Protein (MRP) Functions as a Co-Transporter of Glutathione and Natural Product Toxins.* Cancer Res, 1997. **57**(23): p. 5232-5237.

Reisman, S.A., et al., *Altered Disposition of Acetaminophen in Nrf2-null and Keap1-knockdown Mice.* Toxicol. Sci., 2009. **109**(1): p. 31-40.

Ren, X.-Q., et al., *Function of the ABC Signature Sequences in the Human Multidrug Resistance Protein 1.* Mol Pharmacol, 2004. **65**(6): p. 1536-1542.

Riordan, J.R., et al., *Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines.* Nature, 1985. **316**(6031): p. 817-819.

Rissoan, M.-C., et al., *Reciprocal Control of T Helper Cell and Dendritic Cell Differentiation.* Science, 1999. **283**(5405): p. 1183-1186.

Rius, M., et al., *PROSTANOID TRANSPORT BY MULTIDRUG RESISTANCE PROTEIN 4 (MRP4/ABCC4) LOCALIZED IN TISSUES OF THE HUMAN UROGENITAL TRACT.* The Journal of Urology, 2005. **174**(6): p. 2409-2414.

Robbiani, D.F., et al., *The Leukotriene C4 Transporter MRP1 Regulates CCL19 (MIP-3[beta], ELC)-Dependent Mobilization of Dendritic Cells to Lymph Nodes.* Cell, 2000. **103**(5): p. 757-768.

Rodríguez-Ibáñez, M., et al., *Transintestinal secretion of ciprofloxacin, grepafloxacin and sparfloxacin: in vitro and in situ inhibition studies.* European Journal of Pharmaceutics and Biopharmaceutics, 2003. **55**(2): p. 241-246.

Russel, F.G.M., J.B. Koenderink, and R. Masereeuw, *Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules.* Trends in Pharmacological Sciences, 2008. **29**(4): p. 200-207.

Sakamoto, S., et al., *Identification of the Transporters Involved in the Hepatobiliary Transport and Intestinal Efflux of Methyl 1-(3,4-Dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) Glucuronide, a Pharmacologically Active Metabolite of S-8921.* Drug Metab Dispos, 2008. **36**(8): p. 1553-1561.

Sallusto, F. and A. Lanzavecchia, *Understanding dendritic cell and T‐lymphocyte traffic through the analysis of chemokine receptor expression.* Immunological Reviews, 2000. **177**(1): p. 134-140.

Schinkel, A.H. and J.W. Jonker, *Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview.* Advanced Drug Delivery Reviews, 2003. **55**(1): p. 3-29.

Schuetz, E.G., et al., *Altered Expression of Hepatic Cytochromes P-450 in Mice Deficient in One or More mdr1 Genes.* Molecular Pharmacology, 2000. **57**(1): p. 188-197.

Shen, F., et al., *Quantitation of Doxorubicin Uptake, Efflux, and Modulation of Multidrug Resistance (MDR) in MDR Human Cancer Cells.* Journal of Pharmacology and Experimental Therapeutics, 2008. **324**(1): p. 95-102.

Shi, L.-x., et al., *Reversal effect of tyroservatide (YSV) tripeptide on multi-drug resistance in resistant human hepatocellular carcinoma cell line BEL-7402/5-FU.* Cancer letters, 2008. **269**(1): p. 101-110.

Shimizu, H., et al., *Characterization of the mouse Abcc12 gene and its transcript encoding an ATP-binding cassette transporter, an orthologue of human ABCC12.* Gene, 2003. **310**: p. 17-28.

Soontornmalai, A., M.L.H. Vlaming, and J.M. Fritschy, *Differential, strain-specific cellular and subcellular distribution of multidrug transporters in murine choroid plexus and blood-brain barrier.* Neuroscience, 2006. **138**(1): p. 159-169.

Srinivas, R.V., et al., *Human Immunodeficiency Virus Protease Inhibitors Serve as Substrates for Multidrug Transporter Proteins MDR1 and MRP1 but Retain Antiviral Efficacy in Cell Lines Expressing These Transporters.* Antimicrob. Agents Chemother., 1998. **42**(12): p. 3157-3162.

Stanley, L.A., et al., *Drug transporters: Gatekeepers controlling access of xenobiotics to the cellular interior.* Drug Metabolism Reviews, 2009. **41**(1): p. 27-65.

Steinbrink, K., et al., *Induction of tolerance by IL-10-treated dendritic cells.* J Immunol, 1997. **159**(10): p. 4772-4780.

Stephen, L. and S.M. William, *Cellular and virological mechanisms of HBV drug resistance.* Journal of hepatology, 2006. **44**(2): p. 422-431.

Strassburg, C.P., M.P. Manns, and R.H. Tukey, *Differential Down-Regulation of the UDP-Glucuronosyltransferase 1A Locus Is an Early Event in Human Liver and Biliary Cancer.* Cancer Res, 1997. **57**(14): p. 2979-2985.

Strassburg, C.P., et al., *Differential Expression of the UGT1A Locus in Human Liver, Biliary, and Gastric Tissue: Identification of UGT1A7 and UGT1A10 Transcripts in Extrahepatic Tissue.* Mol Pharmacol, 1997. **52**(2): p. 212-220.

Tamai, I., et al., *Limited Distribution of New Quinolone Antibacterial Agents into Brain Caused by Multiple Efflux Transporters at the Blood-Brain Barrier.* J Pharmacol Exp Ther, 2000. **295**(1): p. 146-152.

Tammur, J., et al., *Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12.* Gene, 2001. **273**(1): p. 89-96.

Teng, S. and M. Piquette-Miller, *Regulation of Transporters by Nuclear Hormone Receptors: Implications during Inflammation.* Molecular Pharmaceutics, 2008. **5**(1): p. 67-76.

Toshinori Kamisako, et al., *Transport of monoglucuronosyl and bisglucuronosyl bilirubin by recombinant human and rat multidrug resistance protein 2.* Hepatology, 1999. **30**(2): p. 485-490.

Toyoda, Y., et al., *MRP class of human ATP binding cassette (ABC) transporters: historical background and new research directions.* Xenobiotica, 2008. **38**(7-8): p. 833-862.

Tribull, T.E., R.H. Bruner, and L.J. Bain, *The multidrug resistance-associated protein 1 transports methoxychlor and protects the seminiferous epithelium from injury.* Toxicology Letters, 2003. **142**(1-2): p. 61-70.

Tukey, R.H. and C.P. Strassburg, *Human UDP-Glucuronosyltransferases: Metabolism, Expression, and Disease.* Annual Review of Pharmacology & Toxicology, 2000. **40**(1): p. 581.

Ueda, K., et al., *The human multidrug resistance (mdr1) gene. cDNA cloning and transcription initiation.* J. Biol. Chem., 1987. **262**(2): p. 505-508.

Urquhart, B.L., R.G. Tirona, and R.B. Kim, *Nuclear Receptors and the Regulation of Drug-Metabolizing Enzymes and Drug Transporters: Implications for Interindividual Variability in Response to Drugs.* J Clin Pharmacol, 2007. **47**(5): p. 566-578.

van de Ven, R., et al., *Dendritic Cells Require Multidrug Resistance Protein 1 (ABCC1) Transporter Activity for Differentiation.* J Immunol, 2006. **176**(9): p. 5191-5198.

Vos, T.A., et al., *Up-regulation of the multidrug resistance genes, <I>Mrp1</I> and <I>Mdr1b</I>, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Spgp, in endotoxemic rat liver.* Hepatology, 1998. **28**(6): p. 1637-1644.

Wagner, M., et al., *Role of farnesoid X receptor in determining hepatic ABC transporter expression and liver injury in bile duct-ligated mice.* Gastroenterology, 2003. **125**(3): p. 825-838.

Wang, Y., et al., *Autocrine production of interleukin-6 confers cisplatin and paclitaxel resistance in ovarian cancer cells.* Cancer letters. **In Press, Corrected Proof**.

Wielinga, P., et al., *The Human Multidrug Resistance Protein MRP5 Transports Folates and Can Mediate Cellular Resistance against Antifolates.* Cancer Res, 2005. **65**(10): p. 4425-4430.

Wijnholds, J., et al., *Multidrug Resistance Protein 1 Protects the Oropharyngeal Mucosal Layer and the Testicular Tubules against Drug-induced Damage.* J. Exp. Med., 1998. **188**(5): p. 797-808.

Wijnholds, J., et al., *Multidrug Resistance Protein 1 Protects the Oropharyngeal Mucosal Layer and the Testicular Tubules against Drug-induced Damage.* J. Exp. Med., 1998. **188**(5): p. 797-808.

Williams, G.C., et al., *Direct Evidence that Saquinavir Is Transported by Multidrug Resistance-Associated Protein (MRP1) and Canalicular Multispecific Organic Anion Transporter (MRP2).* Antimicrob. Agents Chemother., 2002. **46**(11): p. 3456-3462.

Wooden, S.L., et al., *Cutting Edge: HLA-E Binds a Peptide Derived from the ATP-Binding Cassette Transporter Multidrug Resistance-Associated Protein 7 aSnd Inhibits NK Cell-Mediated Lysis.* J Immunol, 2005. **175**(3): p. 1383-1387.

Xiao, H., et al., *In vivo Reversal of P-Glycoprotein-Mediated Multidrug Resistance by Efficient Delivery of Stealth^{TM} RNAi.* Basic & Clinical Pharmacology & Toxicology, 2008. **103**(4): p. 342-348.

Xue-Qing, Y., et al., *Multidrug Resistance Associated Proteins as Determining Factors of Pharmacokinetics and Pharmacodynamics of Drugs.* Current Drug Metabolism, 2007. **8**(8): p. 787-802.

Yabuuchi, H., et al., *Multiple Splicing Variants of Two New Human ATP-Binding Cassette Transporters, ABCC11 and ABCC12.* Biochemical and Biophysical Research Communications, 2001. **288**(4): p. 933-939.

Yamazoe, Y., et al., *Sulfotransferase catalyzing sulfation of heterocyclic amines.* Cancer Letters, 1999. **143**(2): p. 103-107.

Yang, R., et al., *Nucleotide dissociation from NBD1 promotes solute transport by MRP1.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2005. **1668**(2): p. 248-261.

Yoshitomi, S., et al., *Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology.* Toxicology in Vitro, 2001. **15**(3): p. 245-256.

Zamek-Gliszczynski, M.J., et al., *Evaluation of the Role of Multidrug Resistance-Associated Protein (Mrp) 3 and Mrp4 in Hepatic Basolateral Excretion of Sulfate and Glucuronide Metabolites of Acetaminophen, 4-Methylumbelliferone, and Harmol in Abcc3-/- and Abcc4-/- Mice.* J Pharmacol Exp Ther, 2006. **319**(3): p. 1485-1491.

Zelcer, N., et al., *Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4).* Biochem. J., 2003. **371**(2): p. 361-367.

Zhang, Y.-H., et al., *Silencing MRP4 by small interfering RNA reverses acquired DDP resistance of gastric cancer cell.* Cancer letters. **In Press, Corrected Proof**.

Zhou SF, W.L., Di YM, Xue CC, Duan W, Li CG, Li Y., *Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development.* Current medicinal chemistry., 2008. **15**(20): p. 59.

Zollner, G., et al., *Role of nuclear bile acid receptor, FXR, in adaptive ABC transporter regulation by cholic and ursodeoxycholic acid in mouse liver, kidney and intestine.* Journal of Hepatology, 2003. **39**(4): p. 480-488.

Curriculum vitae

Jeffrey Charles Sivils was born in Columbus, Ohio and grew up in El Paso, Texas. The middle son of Jimmy and Marcia Sivils, he received his Bachelor of Science in Biology from The University of Texas at El Paso in 2000. From the spring of 2001 to the fall of 2002 he was a staff scientist for Systems and Processes Engineering Corporation (SPEC) in Austin Texas. In the spring of 2003, he entered the Graduate Program at The University of Texas at El Paso (UTEP) and was employed as a research assistant under Dr. Lisa Bain. His research focused on the compensatory mechanism in drug metabolizing enzymes in response to the loss of multidrug resistance associated 1(Mrp1). He transferred to the UTEP PhD Program in the fall of 2005, where he continued his research with Mrp1. From 2006 to 2007, he was an Assistant Instructor for Topics in Study of Life and Anatomy and Physiology II. From the summer of 2006 to summer of 2008, he was awarded the National Science Foundation Graduate Teaching Fellow in K-12 Education, where he was a science mentor in the El Paso Independent School District for grades 6 and 7. In addition, during the period from 2006 thru 2008, he was a research assistant in the Department of Emergency Medicine at Texas Tech Health science Center El Paso. From June 2008 to September 2009, he was awarded the Howard Hughes Medical Institute Undergraduate Science program graduate mentor, where he mentored two senior undergraduate students. From October 2009 to his graduation he was a research assistant for Dr. Kristine Garza. He presented posters at The Society of Toxicology annual meetings in 2005 and 2009 and at the regional American Society of Microbiology meetings in 2009 and 2010. In 2010 he published “Mice Lacking Mrp1 have Reduced Testicular Steroid Hormone Levels and Alterations in Hormone Metabolism” in General and Competitive Endocrinology. He was offered and accepted a position at Operational Technologies biotechnology division located in San Antonio Texas.

Permanent address:

Jeffrey Charles Sivils

436 Crown Point

El Paso, Texas 79912

Funding Sources
NIH ES012417-01
NIH-SCORE-5S06GM008012-35
BBRC-2G12RR008124
-DNA core
-Tissue culture core
-Molecular charaterization core