8-2010

FOSINOPRIL, A POTENTIAL SUBSTRATE FOR MRP2, COMPETES WITH SEVERAL HIGH USE PHARMACEUTICALS FOR ELIMINATION

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FOSINOPRIL, A POTENTIAL SUBSTRATE FOR MRP2, COMPETES WITH SEVERAL HIGH USE PHARMACEUTICALS FOR ELIMINATION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

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by
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August 2010

Accepted by:
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Abstract

The multidrug-resistance associated protein 1 (MRP1) and multidrug-resistance associated protein 2 (MRP2) are membrane transporters responsible for the efflux of endogenous compounds, a variety of drugs, along with other xenobiotics. They are two of the most highly expressed transporters, but are localized on opposite sides of polarized membranes: MRP1 is on the basolateral side, while MRP2 is on the apical side. Since MRP1 and MRP2 can eliminate such a wide variety of compounds from cells, thereby reducing their half-life, assessing the transport of highly prescribed pharmaceuticals via MRP transporters either alone or in mixtures is important to determine the potential for drug-drug interactions. MDCK cells transfected with the MRP1 and MRP2 gene were used to assess drugs as potential substrates by examining their differential toxicity, accumulation, and efflux. Based upon initial screening assays, three high-use pharmaceuticals, fosinopril, loratadine, and desloratadine were assessed, along with the known substrates vincristine and methotrexate as positive controls. MRP1-overexpressing cells were 1.4-fold less sensitive to the toxicity of loratadine and 2-fold less sensitive to fosinopril. MRP2-overexpressing cells were 2.4-fold less sensitive to fosinopril than controls, while cells showed no difference in sensitivity to desloratadine. We then conducted accumulation/efflux assays to help substantiate that these compounds were indeed substrates for the MRP1 and MRP2 transporters. The positive control, vincristine, was transported out of the MRP1-overexpressing cells more rapidly, with 56% of the compound remaining compared to 85% of the vincristine remaining in the MDCK cells. The other positive control, methotrexate, accumulated 1.5-fold less in

MRP2 cells compared to controls. MRP1 and MRP2 only showed slight differences in their ability to transport loratadine. MRP1 cells retained 52% of the loratadine and 53% remained in MRP2 cells compared to 63% remaining in control cells. MRP2-overexpressing cells effluxed desloratadine significantly more rapidly, as 56% of the accumulated desloratadine remained in MRP2-overexpressing compared to 66% retention in the MDCK cells. More impressively, fosinopril only retained 13% in MRP2 cells while the MDCK cells retained 60%.

Since fosinopril appeared to be a good substrate for MRP2, we used it to perform mixture assays to assess the possibility of drug-drug interactions. Cells were incubated with fosinopril along with loratadine, desloratadine, or methotrexate to determine if the second drug would increase the retention of fosinopril. When fosinopril was combined with desloratadine, its retention was increased by 2-fold, with loratadine, its retention was increased by 4.7-fold, and with methotrexate the retention was increased by 2-fold. The increase in retention with multiple drugs likely indicates that a drug-drug interaction is occurring. We then dosed wild-type and Mrp2 knockout mice with methotrexate, a known Mrp2 substrate, and fosinopril. In mice lacking Mrp2, fosinopril and methotrexate levels are increased in the serum and the kidneys, which suggest that Mrp2 is needed to eliminate fosinopril. This also suggests that with the absence of Mrp2 there is a shift from the fecal to urinary elimination, which could potentially lead to toxic levels if no compensatory mechanism takes over. The body's ability to adapt to changes made by drug-drug interactions is vital for survival. Assessing the transport of highly

prescribed pharmaceuticals by MRP transporters is important to determine the potential for drug-drug interactions, and will aid clinicians in minimizing drug toxicity.

Key words: Multidrug resistance-associated protein 2, fosinopril, methotrexate

Dedication

I would like to dedicate the success of this project to my adviser, fellow graduate students, family and fiancée. Only with their help and patience could this have been done.

Acknowledgments

I would like to thank Dr. Lisa Bain for allowing me to join her lab at Clemson and for helping me develop my scientific education, techniques and patience. Without the amazing twist and humor that the late Mr. Witowich put on chemistry I would never had persuaded the field of science and chemistry; his friendship and wisdom will be missed forever. I would also like to thank Dr. Peter van den Hurk for the use of his instrumentation for analysis of my experiments. Thank you as well to my wonderful professors at Edinboro University: Dr. Lisa Unico, Dr. Naod Kebede, Dr. Hoffman, Dr. Smith, Dr. Fox and others, who inspired me to pursue graduate school and gave me the exceptional education to do well in my future. Thank you also goes to my fiancée Lindsay Bly for helping me center myself through all my trials, tribulations and always having faith in me. In addition, I want to thank my mother, father and sister for pushing me to become the person that I am today and for all their support though my life, their advice, encouragement and love is irreplaceable. Finally, thanks must go to my friends at Clemson who helped me get through these past two years; without their patience, help and knowledge this could not have come been done.

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1. Introduction:

A large variety of the anionic substrates are transported across the cellular membranes through the use of ABC transporters. ABC transporters are members of one of the largest super families, containing more than 48 members, which all require ATP to mediate transport (Dean, 2001). The MRP family (also known as ABCC), along with the cystic fibrosis regulator (ABCC7) makes up this MRP subfamily that contains 12 full transporters. Functioning in the elimination of many of the endogenous and xenobiotic compounds, some of the compounds transported by these proteins are bile salts, lipids, steroids, anticancer agents and drugs (Roger et al., 2006).

In order for many pharmaceuticals and physiological substrates to be excreted by the body, they first have to go through several biotransformation steps. Many times this multistep biotransformation process involves phase I and phase II enzymes. In the first phase, substances are hydrolyzed, reduced, or oxidized to add a functional group to the compound, which is oftentimes done by a family of enzymes called the cytochrome P450s. Following this first phase, one transformation substance will go through a conjugation reaction with a glucuronide, sulfate, or glutathione group added on by phase II enzymes. Adding on one of these cofactors make the lipophilic compounds more water-soluble and more likely to be excreted via the urine or the bile. This addition of a bulky hydrophilic group may also flag the compound to be substrate for MRP transporters.

Every year there are over 2.2 million people injured by prescription drugs alone (Watson et al., 2008). With the MRP's having such a diverse substrate specificity and ubiquitous location in the body, there are many situations in which potential drug-drug interactions could result. The most studied examples of drug-drug interactions involve situations where two drugs are metabolized by the same enzyme. P450s are one such superfamily of enzymes that are extremely important in the phase I metabolism of many drugs (reviewed in Omari et al, 2007). Certain drugs may increase the amount of P450s present and speed up the oxidation and clearance of a second drug. A good example of this type of drug-drug interaction is the induction of CYP2C9 by rifampin (Zakia, 2008). Among the substrates of CYP2C9 is the anticoagulant warfarin. If someone was depending on warfarin acting as a blood thinner, increasing its metabolism by the addition of a second drug could potentially result in a blood clot, stroke, or heart attack (Zakia, 2008). A search of the literature reveals that most published drug-drug interactions are mediated via P450s, as they describe how inactivation of CYP's could result in clinically relevant drug interactions and adverse drug reactions. Drug-drug interactions can result in a prolonged half-life of the compound or a higher concentration in the organs, which, if not put in check, can lead to toxic effects (Leucuta et al., 2006; Zhou et al., 2006). However, there are few studies investigating drug-drug interactions via phase II enzymes.

For the past few decades there have been many advances in the characterization and function of these transport proteins. Their role in the absorption, distribution,

metabolism and excretion of drugs, and endogenous compounds and other xenobiotics through the liver, kidneys and other tissues is very important (van de Water et al., 2005). Two of the biggest players involved in therapeutic compounds and toxicologically relevant drug resistance and in drug transport are the MRP1 and MRP2 proteins. They pump in opposite directions (basolateral vs. apical) and have been shown to contribute to drug elimination (Keppler et al., 1997; Kruh et al., 2001; Zhou et al., 2008).

MRP1 (ABCC1)

Multidrug resistance-associated protein 1 was first identified because of its ability to eliminate cytotoxic drugs from lung tumor cells (Cole et al., 1992). These cells were selected by being exposed to the anthracycline doxorubicin repeatedly (Cole et al., 1992). Although discovered with the use of a single drug, MRP1 cells are also resistant to a large variety of structurally unrelated anticancer compounds, such as vincristine, daunorubicin, and methotrexate and can transport physiological products, such as conjugated steroid hormones and bile salts. MRP1 can also transport drugs, such as flutamide, (an antiandrogen), ritonavir, (an antiviral), or difloxacin and grepafloxacin, (both antibiotics) (reviewed in Munoz et al., 2007; Sasabe et al., 2004; van de Water et al., 2005; Zaman et al., 1994). MRP1 expression is restricted to the basolateral side of certain tissues, including kidney, lung, intestine, brain, testis, macrophages, epithelial cells, and hepatocytes, although the expression of MRP1 in the liver is somewhat low (Flens et al., 1996; van de Water et al., 2005; Wijnholds et al., 1998). MRP1's highest expression can be found in the kidney, with expression in the distal tubule, collecting

duct tubule, and loop of Henle (Rothnie et al., 2008). MRP1 overexpression has been seen in a variety of solid tumors, such as in the lung, breast, and prostate, which shows to be problematic when pharmaceutical therapy is administered. The overexpression of the MRP1 increases the elimination of therapeutic compounds, thereby decreasing their effectiveness (Munoz et al., 2007).

MRP1's ability to transport a variety of conjugated substrates is confirmed by *in vitro* and *in vivo* studies using models lacking Mrp1. For example, one study showed that mouse cell lines lacking the Mrp1 gene were substantially more susceptible to drugs compared to wild-type mice cells lines. Some of the drugs examined were epipodophyllotoxins, whose toxicity increased 4 –7-fold in the knockout cell lines, camptothecins (3-fold increase), arsenite (4-fold increase), anthracyclines (6 –7 fold increase), and vincristine (28-fold increase in toxicity) (Allen et al., 2000). Another study showed that there is elevated intracellular accumulation and a decreased excretion of leukotriene C_4 (an MRP1 substrate). As a result, the secretion of this product decreased to about 1/4 of normal levels found in cultured bone marrow mast cells of Mrp $^{-/-}$ mice (Wijnholds et al., 1997). Mrp1$^{-/-}$ mice showed a decrease in body weight and fatalities at etoposide concentrations that were not normally fatal to wild-type mice (Wijnholds et al., 1997). In a study done on the drug grepafloxacin, an oral broad-spectrum quinoline antibacterial, the ratio of the drug found in the tissue compared to the drug found in the plasma was significantly higher in the heart, trachea, kidney, spleen, and brown fat of Mrp1$^{-/-}$ mice than the drug found in wild-type mice (Sasabe et al., 2004).

These findings demonstrate the importance of the MRP1 protein and its role in eliminating certain compounds.

MRP2 (ABCC2)

Although the function of the second member of the ABCC family had been known for years, the discovery of its gene did not occur until 4 years after the discovery of MRP1 (Borst et al., 2000). The MRP2 transporter was originally known as the canalicular multispecific organic anion transporter (cMOAT). TR-/GY rats were used for many years to study defects in the excretion of many negatively charged, amphiphilic organic anions, such as bilirubin-glucuronide (Borst et al., 2000), and are now known to have defective mutations in their ABCC2/cMOAT/MRP2 gene (Paulusma et al., 1996). The TR- rat's phenotype is similar to that of patients with Dubin-Johnson syndrome, which is characterized by jaundice, the result of chronic conjugated hyperbilirubinemia, caused by impaired hepatobiliary elimination of many organic compounds (Paulusma et al., 1996; Paulusma et al., 1997).

The Mrp2 protein product has been shown to be expressed on the apical membrane (Keppler et al., 1997). MRP2 tends to be more restricted in its tissue distribution than MRP1, being localized in the liver, gallbladder, kidney, small intestine, colon, placenta, and lung (Bandler et al., 2008). The coordinate induction of MRP2 and several phase II enzymes is, in part, regulated by a nuclear transcription factor termed the constitutive androstane receptor (CAR) (Johnson et al., 2002; Kast et al., 2002). MRP2

functions as a transporter of a variety of drugs and xenobiotics, such as bilirubin, sulfated bile salts, non-conjugated compounds, and cytotoxic agents: etoposide, vinblastine, cisplatin, methotrexate, doxorubicin, and epirubicin (Bandler et al., 2008; reviewed in van de Water et al., 2005). MRP2, along with MRP1 and MRP3, shows considerable overlap in the substrate specificity, which is supported by the fact that it has a high amino-acid homology (Bakos et al., 2000). The MRP2 was found to eliminate glutathione conjugates like leukotriene C4, S-(2, 4-dinitrophenyl)-glutathione, and N-ethylmaleimide glutathione, along with glutathione (reviewed in van de Water et al., 2005). Like MRP1, MRP2 transports GSH and GSSH, and therefore is also thought to help protect the cell from oxidative stress (Roger et al., 2006).

The MRP2 transporters have also shown to be expressed in various colorectal, hepatic, mesothelial, lung, ovarian, and leukemic cancer cells (Hoffmann et al., 2004). This leads to the assumption that MRP2 may be a potential obstacle in the use of certain chemotherapeutic agents. A clinical study on breast cancer indicated that the use of Ionafarnib, a MRP2 inhibitor, along with cisplatin and/or 5-fluorouracil, resulted in reduction of the chemotherapy dosage and could become less toxic (Hoffmann et al., 2004).

As stated previously, much of what we know about the MRP2 protein was performed on TR- rats. The TR- rats are compromised in their ability to effectively eliminative compounds via biliary secretion. In a study on TR- rats, it was found there

was dramatic reduction in the rats' ability to eliminate bromosulfophthalein by 15 %, dibromosulfophthalein by 50 %, and indocyanine green by 75%, compared to that of normal levels (Jansen et al., 1993). Although the original finding of the MRP2 transporter was done in rats, there have been many studies done with mice. For example, after the food-derived, oral administration of carcinogens PhIP (2-amino-1-methyl 6-phenylimidazo [4,5- *b*]pyridine) there was a 1.9-fold increase in the plasma levels in the Mrp2$^{-/-}$ mice versus wild-type mice. In the same study there was a 1.7-fold increase in plasma levels of oral-administered IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) in Mrp2$^{-/-}$ mice versus wild-type mice, suggesting that Mrp2 may be reducing exposure to these compounds (Vlaming et al., 2006). The Mrp2-deficient mice displayed decreased total bilirubin and glutathione in bile and increased levels of bilirubin in urine, indicating that urinary excretion may serve as an alternate excretion pathway for certain compounds in these mice (Vlaming et al., 2006). In a study on spiramycin, which is used to treat respiratory infections, it was found that there was a 10-fold decrease in the rate of biliary excretion of spiramycin in Mrp2 knockout mice (Xianbin et al., 2007). It has been shown that Mrp2 is one of the major regulators of biliary elimination of methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) in mice. In this study it was found the up-regulation of Mrp2, along with the absences of the breast cancer resistance protein, another important anionic transporter, mice reduced biliary excretions of MTX and/or 7OH-MTX, which resulted in increased plasma levels of 7OH-MTX. Levels of MTX in the kidneys also increased by 7-fold and liver levels of 7OH-MTX increased 90-fold (Vlaming et al., 2009).

Assessing the transport of highly prescribed pharmaceuticals via MRP transporters either alone or in mixtures is important to determine the potential for drug-drug interactions. In the present study, we examined fosinopril, an angiotensin converting enzyme (ACE) inhibitor, loratadine and desloratadine, both antihistamines, to determine if they were substrates for the MRP transporters, and if so, whether they could alter the transport and toxicity of one another. The pharmaceuticals of interest all have a high molecular weight, multiple rings, and are excreted from the body predominantly as conjugates. The aim of this study was to examine whether these drugs interact by inhibiting one another's excretion from cells via the multidrug resistance-associated proteins 1 and 2.

FOSINOPRIL, A POTENTIAL SUBSTRATE FOR MRP2, COMPETES WITH SEVERAL HIGH USE PHARMACEUTICALS FOR ELIMINATION

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Abstract

The multidrug-resistance associated protein 2 (MRP2) is a membrane-bound transporter responsible for the efflux of a variety of drugs and endogenous compounds. MDCK cells transfected with the human MRP2 gene were used to assess whether several highly used pharmaceuticals were potential substrates by examining their differential toxicity, accumulation, and efflux. Fosinopril, an ACE inhibitor, was 2.4-fold less toxic to the MRP2 transfected cells compared to mock transfected cells, suggesting that fosinopril is a potential MRP2 substrate. In addition, fosinopril was effluxed more rapidly, as the MRP2 cells only retained 13% of the dosed fosinopril after 20 minutes compared with 60% retained in the control cells. To determine whether fosinopril might contribute to a drug-drug interaction, fosinopril efflux was examined in combination with several other known or suspected MRP2 substrates. When fosinopril was coincubated with desloratadine, its retention was increased by 2-fold; with loratadine, its retention was increased by 4.7-fold, and with methotrexate, its retention was increased by 2-fold. The increases in retention with multiple drugs likely indicate that a drug-drug interaction is occurring. To further clarify whether fosinopril was a substrate for Mrp2, we dosed wild-type and Mrp2 knockout mice with the known Mrp2 substrate methotrexate and fosinopril. In mice lacking Mrp2, fosinopril and methotrexate levels were increased in the serum and the kidneys, which suggest that the lack of Mrp2 favors fosinopril excretion through the urine rather than the feces. Assessing the transport of highly prescribed pharmaceuticals by MRP transporters is important to determine the potential for drug-drug interactions, and will aid clinicians in minimizing drug toxicity.

Key words: Multidrug resistance-associated protein 2, fosinopril, methotrexate

1. Introduction:

Multidrug resistance-associated protein 1 and 2 (MRPs) are membrane-bound transporters that efflux a variety of drugs, other xenobiotics, as well as endogenous compounds (Homolya et al., 2003; Kruh et al., 2007; van de Water et al., 2005). Substrates for MRP1 include a large variety of structurally unrelated anticancer compounds such as doxorubicin, vincristine, daunorubicin, colchicines, and etoposide, physiological products, such as conjugated steroid hormones, leukotriene $C_{4,}$ and bile salts, and drugs such as grepafloxacin, (Allen et al., 2000; Bakos et al., 2000; Cole et al., 1994; Jedlitschky et al,. 1997; Sasabe et al., 2004; Zaman et al., 1994) saquinavir (Williams et al., 2002), chloroquine (Vezmar et al., 1998), and ethacrynic acid (Zaman et al., 1996). *In vivo* studies with mice lacking Mrp1 have confirmed some of these substrates. For example, *Mrp1*$^{-/-}$ mice have significant increases in the levels of the antibiotic grepafloxacin in the heart, trachea, kidney, spleen, and brown fat compared to wild-type mice (Sasabe et al., 2004). The secretion of leukotreine C_4 is diminished in cultured bone marrow mast cells of *Mrp1* $^{-/-}$ mice and these mice are hypersensitive to the toxicity of etoposide, which damages the mucosal layer of the tongue and cheek, and inhibits spermatogenesis (Wijnholds et al., 1997; Wijnholds et al., 1998).

MRP2 and MRP1 show considerable overlap in the substrate specificity, since they have a high amino acid homology (Bakos et al., 2000). MRP1 is expressed in most tissues, including the kidney, lung, intestine, brain, testis, macrophages, and hepatocytes, and it is localized to the basolateral side of polarized epithelial cells (Flens et al., 1996;

van de Water et al., 2005; Wijnholds et al., 1998). The MRP2 protein product is expressed on the apical membrane (Keppler et al., 1997), and tends to be a more restricted in its tissue distribution than MRP1, being localized in the liver, gallbladder, kidney, small intestine, colon, placenta, and lung (Bandler et al., 2008). Like MRP1, MRP2 also transports a variety of physiological substrates and drugs, such as bilirubin, sulfated bile salts, etoposide, methotrexate, adefovir, chloroquine, loperamide, naringenin, sulfusalazine, and tamoxifen (Bakos et al., 2000; Jedlitschky et al,. 1997; Pedersen et al., 2008). Other pharamaceuticals, such as saquinavir (Williams et al., 2002), docetaxel (Zimmermann et al., 2008), pravastatin (Matsushima et al., 2005), cyclosporin A (Masahiro et al., 2005), lopinavir (Agarwal et al., 2007), acetaminophen (Xiong et al., 2000), temocaprilat (Ishizuka et al., 1997), enalapril (Liu et al., 2006), and several antibiotics including cefbuperazone, cefoperazone, ceftriaxone, cefpiramide, benzylpenicillin, and moxifloxacin (Ahmed et al., 2008; Chio et al., 2009; Kato et al., 2008) are also substrates for the MRP2 transporter.

Because MRP2 has such diverse substrate specificity, it can alter the intracellular concentration of many therapeutically used agents and toxicologically relevant xenobiotics. For example, there was a 10-fold decrease in the rate of biliary excretion of spiramycin, an antibiotic, in Mrp2 knockout mice (Xianbin et al., 2007). Other studies showed a 1.7-1.9-fold increase in the plasma levels of the food-derived carcinogens IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) and PhIP (2-amino-1-methyl 6-phenylimidazo[4,5- *b*]pyridine), increases in serum levels of fexofenadine, and increases

in the serum levels of methotrexate in *Mrp2*$^{-/-}$ mice versus wild-type mice (Matsushima et al., 2008; Vlaming et al., 2006). Mrp2-deficient mice have decreased total bilirubin and glutathione in bile, and increased levels of bilirubin in urine, indicating that urinary excretion may serve as an alternate excretion pathway for certain compounds in these mice (Vlaming et al., 2006). Mrp2-deficient rats had an 8.5-fold increase in the serum concentration of ezetimibe, an anti-hyperlipidemic, which was associated with an increase in urinary elimination and decrease in fecal elimination (Oswald et al., 2006). Likewise, methotrexate serum concentrations were increased and biliary concentrations were decreased in mice lacking Mrp2 (Vlaming et al., 2006).

Since many drugs are substrates for the same transporter, there is an increased risk of serious adverse effects when these proteins are inhibited at the same time. It was shown that the coadministration of the anti-inflammatory drugs indomethacin and sulfasalazine, both substrates for MRP2, increased the absorption and decreased the elimination of sulfasalazine in the small intestine (Dahan et al., 2010). Additionally, a wide variety of nonsteroidal anti-inflammatory drugs, such as salicylate, ibuprofen, tolmetin, naproxen, sulindac, etodolac, indomethacin, piroxicam, ketoprofen, diclofenac, celecoxib, and phenylbutazone, all inhibit MRP2-mediated methotrexate transport *in vitro* (El-Sheikh et al., 2007). The pretreatment of dexomethasone in rats reduced the total clearance of methotrexate by 33%, suggesting that multiple substrates may decrease the ability of MRP2 to eliminate drugs (Fuksa et al., 2010). This coadministration of

MRP2 substrates or inhibitors could lead to the elongated or shorted effects of the drug, and these types of drug-drug interactions need to be further investigated.

Assessing the transport of highly prescribed pharmaceuticals via MRP transporters either alone or in mixtures is important to determine the potential for drug-drug interactions. In the present study, we examined fosinopril, an angiotensin converting enzyme (ACE) inhibitor, and loratadine and desloratadine, both antihistamines, to determine if they were substrates for the MRP transporters, and if so, whether they could alter the transport and toxicity of one another.

2. Methods:

2.1 Cell Culture

Marin-Darby canine kidney (MDCK) cells and MDCK cells transfected with the human MRP1 and MRP2 gene were a gift from Piet Borst (Netherlands Cancer Institute). Cells were grown in DMEM containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 37ºC in a 5% CO_2 atmosphere. The cells were passaged approximately every 4 days and were terminated at passage number 20.

2.2 Drug selection and cell viability assays

The drug index database (http://www.rxlist.com/top200.htm), which lists the 200 most-prescribed medications in the U.S, was scanned to determine potential MRP1 and MRP2 substrates based upon molecular weight, excretion into the feces, and the

formation of glucuronide conjugates. Based upon these criteria and preliminary experiments, fosinopril, loratadine, and desloratadine were selected to determine if they were substrates for the MRP transporters. Cell viability experiments were performed by seeding $6x10^3$ cells per well into 96 well plates. Cells were allowed to attach overnight and the next day, the medium was replaced with 100µL medium containing the drug of interest. The cells were incubated for 3 days with six increasing concentrations (n=5 wells per concentration) of vincristine (American Radiolabeled Chemicals, Saint Louis, MO), loratadine (Sigma, St. Louis, MO), fosinopril (Sequoia Research Products Ltd, Pangbourne, United Kingdom), desloratadine (Sigma), or methotrexate (American Radiolabeled Chemicals). The MTS assay (Promega, Madison, WI) was used to assess cell viability by spectrophotometry at 490nm. Each experiment was repeated two to three times. LC_{50} values were determined using dose response curves produced in Graphpad.

2.3 Cellular Accumulation and Efflux

Cells were seeded into a 6 well plates at $1.8x10^5$/well (4 wells/cell line) and were allowed to become confluent over 3 days. After confluence, the medium was aspirated off and pre-incubated with serum-free medium containing 100µM verapamil (Sigma) for 30 minutes. After pre-incubation, media was replaced with serum-free medium containing 100µM verapamil and 34.2µM desloratadine (the LC_{25}), 42µM loratadine (the LC_{25}), 100µM fosinopril (the LC_{95}), 500nM [^{3}H] vincristine (0.167µCi/well, American Radiolabeled Chemicals), or 1µM [^{3}H] methotrexate (0.167µCi/well, American Radiolabeled Chemicals). Cells were allowed to accumulate the drug for time periods

ranging for 0 to 6 hours. At the varying time point, cells were washed and scraped into 2 mL PBS. For [^{3}H] vincristine and [^{3}H] methorexate, the amount of compound remaining in the cells was determined by scintillation counting.

For desloratadine and loratadine, 400µL of 1M NaOH was added (Amini et al., 2004) and for fosinopril, 400µL of 1M HCl was added (Cui, 2007). All three drugs were extracted into butanol/hexane (2:98), evaporated under nitrogen, and then the extract was resuspended in methanol. The aqueous portion was used to determine protein concentration of the cells. Efflux experiments were carried out similarly, except after medium removal and washing, serum-free medium was placed back into each well, and the cells were allowed to efflux their accumulated drugs for 0-30 minutes. The amount of drug remaining in the cell was determined by a Waters HPLC equipped with a dual absorbance wavelength detector and a Hypersil C_{18} Column (ES Industries, West Berlin, NJ). For loratadine and desloratadine, detection was at 247nm, while detection for fosinopril was at 220nm. The mobile phase for loratadine and desloratadine was acetonitrile, 20mM dihydrogen phosphate, and triethylamine (43:57:0.02, v/v/v, pH 2.4) with a flow rate of 1mL/mim (El-Sherbiny, 2007). The mobile phase for fosinopril was acetonitrile, water, and ortho- phosphoric acid (4000:15:2, v/v/v) with a flow rate of 1mL/min (Kirschbaum, 1990).

2.4 Altered enzyme and gene expression in fosinopril dosed mice

Male FVB mice (n=10) were obtained from Taconic and individually housed until 9 weeks of age. All experiments were approved by the IACUC at Clemson University. Mice were gavaged for 3 days with water or 100mg/kg fosinopril (n=5 mice/group). The animals were euthanized 6 hours after the final gavage, and blood, livers, small intestines, gallbladders, and kidneys were collected. Serum was prepared from the blood and stored at -20^{o}C, while the organs were weighed and frozen at -80^{o}C until analysis. Serum was used to determine total bilirubin, ALT, and AST concentrations (Johnson & Johnson's VT250 Automatic Chemical Analyzer), while livers and kidneys were used to determine transporter and phase II enzyme expression by QPCR.

2.5 QPCR

RNA was isolated from liver and kidneys of the mice using TriReagent (Sigma-Aldrich, St. Louis, MO). Two micrograms RNA was incubated with 0.2mM deoxynucleotide mix, 2ng random hexamers, and 200U MMLV reverse transcriptase (Promega, Madison, WI) for 1 hour at 37°C to prepare cDNA. The expression of Mrp1, Mrp2, Mrp3, Mrp4, Bsep, UGT1A, and UGT2B was quantified by QPCR using SYBR green (Bio-Rad, Hercules, CA) and the appropriate primers (Table 1). Standards were prepared specifically for each gene to be analyzed using PCR and then purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). The concentration of the purified sample of cDNA was determined by spectrophotometry, and used to make serial dilutions. Samples and standards were run in triplicate, and GAPDH was used as a

housekeeper gene. Fold changes were determined by dividing gene levels of mice that received fosinopril by wild-type mice gene levels.

2.6 Drug disposition in Mrp2$^{-/-}$ mice

Male FVB (control) and Mrp2 knockout mice (n=10 of each strain) were obtained from Taconic and individually housed until 9 weeks of age. Five mice from each strain received 50mg/kg [^{3}H] methotrexate in saline via tail vein injection (Vlaming et al., 2006), and five mice from each strain received 50mg/kg [^{3}H] methotrexate via tail vein injection plus 100mg/kg fosinopril via gavage. Mice were euthanized 1 hour later, since the half-life of methotrexate in mice is ~30 minutes (Sani et al., 2010) and the half-life of fosinopril in humans is ~12 hours (reviewed in Murdoch et al., 1992). Blood was collected and spun to collect serum. Intact livers, kidneys, small intestines, colons, and gallbladders with contents were collected and homogenized in PBS. An aliquot of the homogenate or serum was used for scintillation counting to determine the amount of methotrexate in each organ.

2.7 Data Analysis

Samples from each group were averaged and significant differences were determined by ANOVA, followed by the appropriate post-hoc test, or by Student's t-test using Graphpad Prism Software (San Diego, CA).

3. Results:

3.1 Drug sensitivity

The sensitivity of MDCK cells transfected with MRP1 and MRP2 to 3 high-use pharmaceuticals, fosinopril, loratadine, and desloratadine was assessed, using the known substrates vincristine and methotrexate as positive controls. MRP1-overexpressing cells were 1.4-fold less sensitive to the toxicity of loratadine, while desloratadine showed no differences in sensitivity (Table 2). In contrast, MRP1-overexpressing cells are 2.0-fold and MRP2 are 2.4-fold less sensitive for fosinopril than controls (Table 2). These results suggest that loratadine and fosinopril are potential substrates for MRP transporters.

3.2 Drug efflux assays

Preliminary experiments were conducted to determine the appropriate accumulation time for each compound, which was 2 hours for fosinopril, 4 hours for vincristine, 1 hour for loratadine, and 6 hours for desloratadine and methotrexate. After cells were loaded with the drug, their rate of efflux was examined. Vincristine was effluxed out of the MRP1-overexpressing cells more rapidly, with 56% of the compound remaining in MRP1 cells after 20 minutes, compared to 85% remaining in the MDCK cells (Figure 1A). The other positive control, methotrexate, showed 1.5-fold less accumulation in MRP2 cells compared to controls (data not shown). For fosinopril, the MRP2 cells only retained 13 % of their accumulated fosinopril while the MDCK cells retained 60 % (Figure 1B). While MRP1 and MRP2 showed no real differences in their ability to efflux loratadine (Figure 1C), MRP2-overexpressing cells effluxed desloratadine significantly more rapidly, as 56% of the accumulated desloratadine

remained in MRP2-overexpressing cells after 20 minutes compared to 66% retention in the MDCK cells (Figure 1D).

Since fosinopril appeared to be a substrate for MRP2, we coincubated cells with fosinopril and either loratadine, desloratadine, or methotrexate to determine if the second drug would increase the retention of fosinopril. When fosinopril was combined with desloratadine, its retention was increased by 2-fold (Figure 2A), with loratadine, its retention was increased by 4.7-fold (Figure 2B), and with methotrexate, the retention was increased by 2-fold (Figure 2C).

3.4 mRNA expression and serum chemistry in mice dosed with fosinopril

FVB mice were treated with fosinopril for 3 days to determine whether phase II enzymes and transporters gene expression would be induced. There was a 2.1-fold increase in liver Bsep mRNA expression (Table 3), but no other changes in enzyme gene expression were seen. Additionally, there were no significant differences in serum AST, ALT, or total bilirubin (data not shown).

3.5 Altered drug disposition in Mrp2$^{-/-}$ mice

Preliminary drug disposition experiments indicated that fosinopril serum concentrations were below the HPLC detection limits. Therefore, [^{3}H]methotrexate was used to determine if fosinopril co-administration would alter methotrexate disposition. When Mrp2 was lacking, fosinopril increased the accumulation of methotrexate in the

liver by 2.1-fold and decreased methotrexate levels in the intestine by 1.5-fold (Figures 3A and B). While the gallbladder accumulated 2.9-fold less methotrexate when combined with fosinopril, there were no differences between the wild-type and knockout mice (Figure 3E). In both the serum and kidney, there was a significant 2.9-fold and 3-fold increase, respectively, in methotrexate accumulation in the Mrp2 knockout mice. The co-administration of fosinopril appeared to increase accumulation in the serum, although this was not quite statistically significant (Figure 3D). However, fosinopril coadministration did increase accumulation in the kidney (Figure 3C).

4. Discussion

The present study indicates that fosinopril is a substrate for the MRP2 transporter based upon reduced toxicity to and an increased rate of elimination from MRP2-overexpressing cells, as well as altered disposition in *Mrp2-/-* mice. Additionally, fosinopril coincubation with other substrates for the MRP2 transporter increases fosinopril retention and reduces its elimination, suggesting a potential for drug-drug interactions.

Fosinopril is an ACE inhibitor, which is used in patients with cardiovascular disease and hypertension. Approximately 3,155,000 prescriptions a year are written for fosinopril, making it one of the most highly prescribed medications for hypertension (http://www.rxlist.com/top200.htm). Our data indicates that fosinopril is a substrate for MRP2, since it is both 2-fold less toxic to MRP2-overexpressing cells and is eliminated

at a 4-fold faster rate in cells that overexpress MRP2 versus control cells. Like fosinopril, other ACE inhibitors such as enalapril and temocapril were previously shown to be substrates for the MRP2 transporter *in vitro* and *in vivo* (Ishizuka et al., 1997; Liu et al., 2006). For example, in EHBR rats, which do not express functional Mrp2 protein, the biliary excretion rate of enalapril is essentially 0 (Liu et al., 2006). Additionally, studies have shown that temocapril was taken up into Mrp2-expressing membrane vesicles to 3-fold higher levels than vesicles from EHBR rats, which lack Mrp2. Similarly, *in vivo* studies show that temocapril serum levels were approximately 2- to 5-fold higher in rats lacking Mrp2, which correlated with a 3-fold reduction in its biliary elimination rate (Ishizuka et al., 1997; Takada et al., 2004). Likewise, the current study indicates that fosinopril can increase methotrexate levels in the serum by 3- to 5-fold in mice lacking Mrp2, which is similar in magnitude to the above studies using rats lacking Mrp2. However, many other ACE inhibitors including benazepril, cilazapril, delapril, trandolapril, and imidapril are not substrates for Mrp2 (Ishizuka et al., 1997; Shionoiri et al., 2001).

Fosinopril, enalapril, and temocarpil are all known to be predominantly eliminated in the bile, while most other ACE inhibitors are eliminated in the urine (Murdoch and McTavish, 1992; Suzuki et al, 1993). Although the reasons for this high propensity towards biliary excretion are unclear, it has been suggested that the carboxyl groups within their chemical structures enable these three ACE inhibitors to be substrates for the MRP2 transporter (Ondetti, 1988).

Along with its transport by MRP2, studies have revealed that fosinopril can cause cholestatic jaundice and liver failure in a short period of time after starting treatment (Chou et al., 2008; Nunes et al., 2001). Similarly, enalapril has also been shown to induce cholestasis in some patients (Hartleb et al., 2002; Muela et al., 2002; Todd et al., 1990). These clinical findings add further credence to the ability of MRP2 to transport fosinopril, symptoms of jaundice and cholestasis are also seen in rats and humans that have mutations in MRP2 (Paulusma et al., 1996; Paulusma et al., 1997).

Next, we investigated changes in fosinopril elimination in the presence of a second drug. There are 2-, 5-, and 2-fold increased levels of fosinopril that remain in cells after coincubation with methotrexate, loratadine, and desloratadine, respectively. This indicates that the two drugs may be competing for elimination. Other studies have shown that dexomethasone can reduce the total clearance of methotrexate by 33% in rats, showing that the use of multiple pharmaceuticals that are substrates for the same transporter can decrease the clearance of the drugs (Fuksa et al., 2010). The coadministration of indomethacin and sulfasalazine, both substrates for MRP2, decreased the elimination of sulfasalazine in the small intestine by 7.5-fold (Dahan et al., 2010). Additionally, a wide variety of nonsteroidal anti-inflammatory drugs, such as salicylate, ibuprofen, tolmetin, naproxen, sulindac, etodolac, indomethacin, piroxicam, ketoprofen, diclofenac, celecoxib, and phenylbutazone all inhibit MRP2-mediated methotrexate transport *in vitro* (El-Sheikh et al., 2007). The drug-drug interaction that may result

could alter toxicity of one or more of the compounds, and may also increase or decrease the desired therapeutic effects.

Interestingly, the coincubation of loratadine and fosinopril resulted in a 5-fold increase in fosinopril retention compared to the 2-fold increase with either methotrexate or desloratadine. The reasons for this are unclear, but may be due to loratadine acting as an inhibitor of MRP2 rather than a substrate, as the efflux rate of loratadine alone was not very different between the three cell lines.

Next, [^{3}H]methotrexate and fosinopril were coadministered to *Mrp2-/-* mice, since methotrexate elimination is reduced in Mrp2 knockout mice (Vlaming et al., 2006). Our study indicated that when methotrexate was given alone, its levels were increased by 3-fold in the kidney and 2.9-fold in the serum of mice lacking Mrp2. This is consistent with what others have seen, with a 2.2-fold increase in methotrexate levels in the serum (Vlaming et al., 2006), and ~2-fold increase in levels of methotrexate in the kidney of Mrp2 knockout mice (Vlaming et al., 2008). These other studies also reported a significant shift from fecal to urinary elimination of methotrexate in mice lacking the Mrp2 transporter (Vlaming et al., 2008). Similar findings have been reported for other compounds in *Mrp2-/-* mice. For example, bilirubin levels are increased by 5-fold in the serum and 6.7-fold in the urine of mice lacking Mrp2 (Chu et al., 2006).

When fosinopril is given in combination with methotrexate in mice lacking Mrp2, fosinopril increased the accumulation of methotrexate in the liver by 2.1-fold. This change was expected because of the high expression of Mrp2 in the liver, and since Mrp2 is the predominant transporter that eliminates fosinopril and methotrexate (Keppler et al., 1997; Vlaming et al., 2006). Additionally, the 1.5-fold decrease of methotrexate in the intestines of mice lacking the Mrp2 protein, when given in combination with fosinopril, suggests that Mrp2 is involved in the biliary secretion of both methotrexate and fosinopril. Other studies have also reported a 2.3-fold decrease in intestinal levels of methotrexate (Vlaming et al., 2006; Vlaming et al., 2009). This increase in methotrexate concentrations in the liver, coupled with a decrease in intestinal concentrations, suggests that fosinopril coadministration is causing a reduction in fecal elimination. In the absence of Mrp2, there was a 3-fold and 2.9-fold increase in methotrexate levels in the serum and kidney, respectively, when methotrexate was coadministered with fosinopril. This again, suggests that when Mrp2 is lacking, fosinopril elimination shifts from the feces to the urine. Although we did not see changes in mRNA expression in FVB mice when treated with fosinopril, one possibility is that fosinopril is also a substrate for Mrp3, which resulted in its movement from the liver into the serum. It is known that MRP2, MRP3 and Bcrp can transport methotrexate and can all compensate for loss of one or more of the transporters in the liver (Doyle et al., 2003; Masuda et al., 1997; Vlaming et al., 2008; Vlaming et al., 2009; Volk et al., 2002; Zeng et al., 2001). Although MRP1, MRP4, MRP5 and P-gp can transport methotrexate *in vitro*, these other transporters do not appear to play a significant role in the elimination of methotrexate (Chen et al., 2002;

De Graaf et al., 1996; Weilinga et al., 2005; Zeng et al., 2001). From the present study, MRP1 does not appear to transport fosinopril and we attempted to minimize the effect of P-glycoprotein by using verapamil. However, we cannot exclude the possibility that Mrp3, Bcrp, Mrp4, and/or Bsep may also transport fosinopril.

In conclusion, MRP2 transports fosinopril and its efflux of fosinopril is reduced in the presence of other compounds, such as methotrexate. These findings and the further understanding of the ABC-transporters and their role in drug-drug interactions could eventually help minimize side effects and aid in the better treatment of patients.

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Table 1 – QPCR primer sets

Primer	Forward 5'to 3'	Reverse 5' to 3'	T_m (^oC)
Mrp1	taatggaagcagacaaggcccaga	agaggccagtgcagatacatggtt	**60**
Mrp2	atggtcctagacagcggcaagatt	ttcacactttcaatgccggcttcc	**60**
Mrp3	agaaccaagcatcaaggtcccaga	aaggttctcaccaaagccctcaga	**60**
Mrp4	caaagctgcaagccacatcctcat	agaccaaattgaggcctcggagaa	**60**
UGT1A	gaaattgctgaggctttgggcaga	agagtgtgtgatgaatgcccgagt	**60**
UGT2B	agttgagacaatgggccaag	gttgggtgaggaaactccaa	**61**
Bsep	gctggtcatcactggagccccc	gcagccctcctgtgcttggc	**60**
GAPDH	gccttccgtgttcctacc	gcctgcttcaccaccttc	**51**

Table 2. Drug sensitivity in cells overexpressing MRP1 and MRP2

LC_{50} Values			
Drugs	**MDCK**	**MRP1**	**MRP2**
Loratadine (μM)	87.1±9.0	124.3±13.6*	96.9±4.4
Desloratadine (μM)	44.5±5.9	48.0±9.2	45.7±5.4
Fosinopril (μM)	188.2±68.1	372.4±47.0*	452.2±77.6*
Vincristine (nM)	456.2±137.0	1373.9±143.0 *	494.9±113.6 *
Methotrexate (nM)	38.5±13.3	63.0± 26.1*	53.0± 9.5*

LC_{50} values $\pm$ SD are averaged from three to four separate experiments. Statistical differences between control and transfected cell lines (*) were determined by ANOVA followed by Tukey's ($p<0.05$).

Table 3. mRNA levels in liver and kidneys of mice treated with 100mg/kg fosinopril

Name	Liver (molecules)			Kidney (molecules)		
	Control	Fosinopril	Fold Change	Control	Fosinopril	Fold Change
Mrp1	9.4±2.3	12.3±5.6	1.3	109.6±39.9	194.5±197.7	1.8
Mrp2	1,378±325	1,286±366	0.9	1,317±229	2,462±2,002	1.9
Mrp3	774.8±301.6	815.8±550.1	1.1	28.6±6.0	55.6±59.7	1.9
Mrp4	275.6±146.4	335.9±283.8	1.2	11,589±1,359	15,945±13,280	1.4
Ugt1A	1,798±729	2,139±1,283	1.2	2,940±825	5,240±5,492	1.8
Ugt2B	65,511±32,965	83,463±58,067	1.3	80,868±24,831	92,016±55,931	1.1
Bsep	9,830±2641	20,490±11,651	2.1*	247.6±43.5	438.1±304.2	1.8

The number of molecules ± SD are the average of 4-5 mice, each run in triplicate.

Statistical differences (*) were determined by Student's t-test ($p<0.05$).

Figure 1. Efflux of drugs from MDCK, MRP1 and MRP2 cells.

MDCK (•), MRP1 (Δ) and MRP2 (O) cells were allowed to accumulate vincristine (A), fosinopril (B), loratadine (C), and desloratadine (D), and then efflux was measured by HPLC or scintillation counting as percent remaining in the cell. Data is presented as the average percentage efflux ± standard deviation (n=4-12) of the average of two to three combined trials. Statistical analysis was performed by ANOVA followed by Tukey's ($p<0.05$; A=MRP1 vs. MDCK; B=MRP2 vs. MDCK).

Figure 2. Fosinopril concentrations remaining in the MRP2 cells are increased when a second substrate is present.

Fosinopril was allowed to accumulate for 2 hours, and then efflux for twenty minutes. Fosinopril remaining in the cell was then measured by HPLC. Controls were dosed with 100uM fosinopril and mixtures with either 10, 15, 25uM desloratadine (A), loratadine (B), or 30, 50nM methotrexate (C), along with 100uM fosinopril, and then analyzed. Data is presented as the average percentage efflux ± standard deviation (n=4-8) of the average of two combined trials. Statistical differences between fosinopril alone and fosinopril plus the second drug (*) were determined by ANOVA followed by Tukey's ($p<0.05$).

Figure 3. Fosinopril alters [^{3}H] methotrexate disposition in FVB and FVB/Mrp2-/- mice.

FVB and Mrp2-/- mice (KO) were injected with 50mg/kg [^{3}H]methotrexate (MTX) with or without 100mg/kg fosinopril (Fos). The amount of [^{3}H] methotrexate remaining in the organs and serum was measured by scintillation counting; A (liver), B (small intestine), C (kidney), D (serum), E (gallbladder), F (colon). Data is presented as the average percentage remaining $\pm$ standard deviation (n=4-5 mice per group). Statistical differences (*) were determined by Student's t-test ($p<0.05$).

Figure 1.

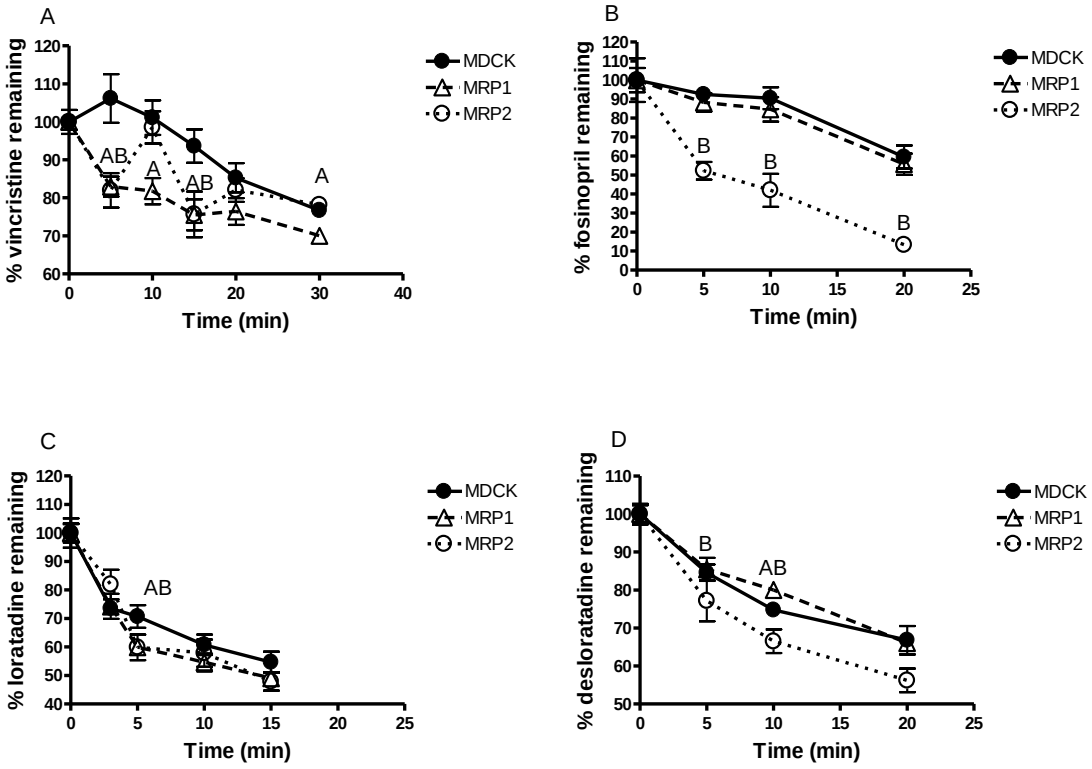

A
% vincristine remaining
Time (min)
MDCK
MRP1
MRP2
AB
A
AB
A
B
% fosinopril remaining
Time (min)
MDCK
MRP1
MRP2
B
B
B
C
% loratadine remaining
Time (min)
MDCK
MRP1
MRP2
AB
D
% desloratadine remaining
Time (min)
MDCK
MRP1
MRP2
B
AB

Figure 2.

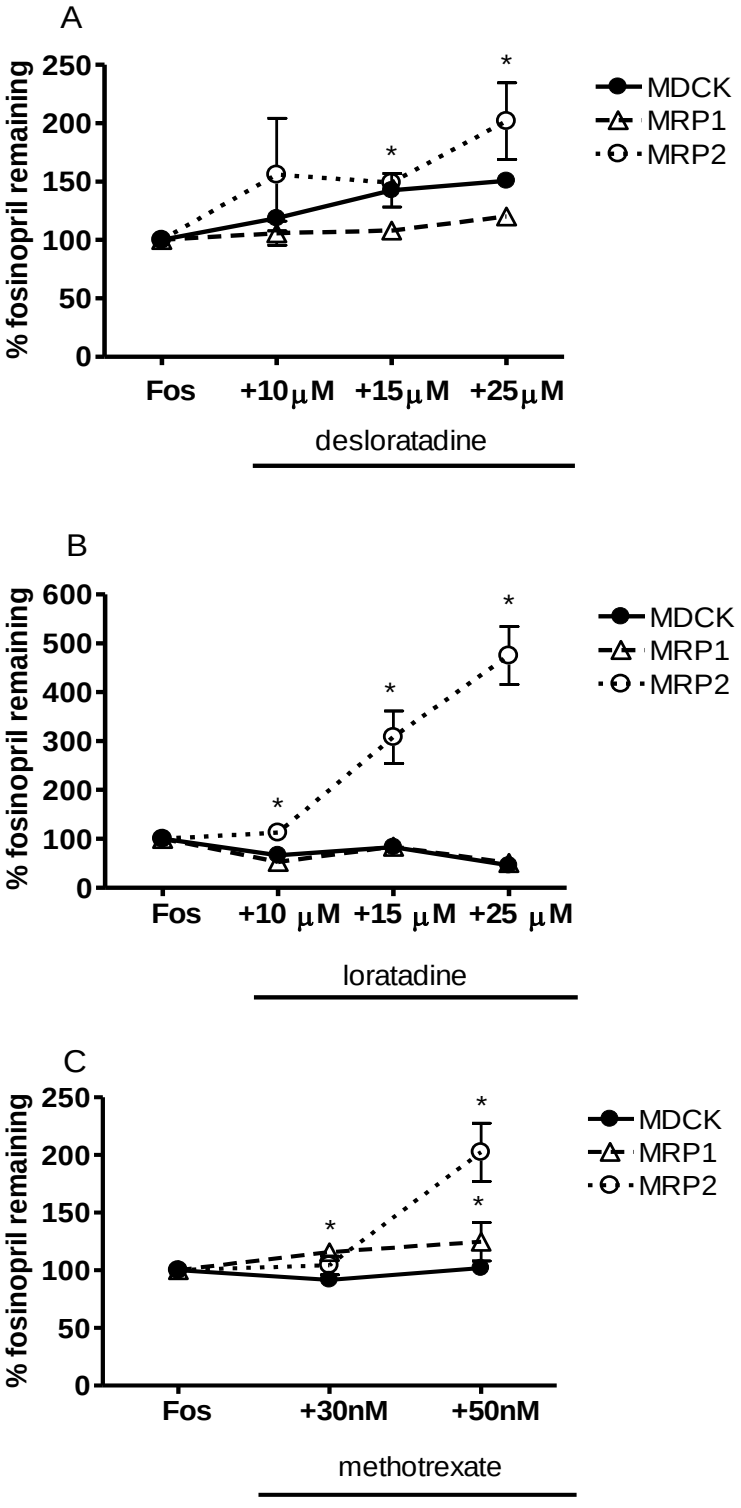
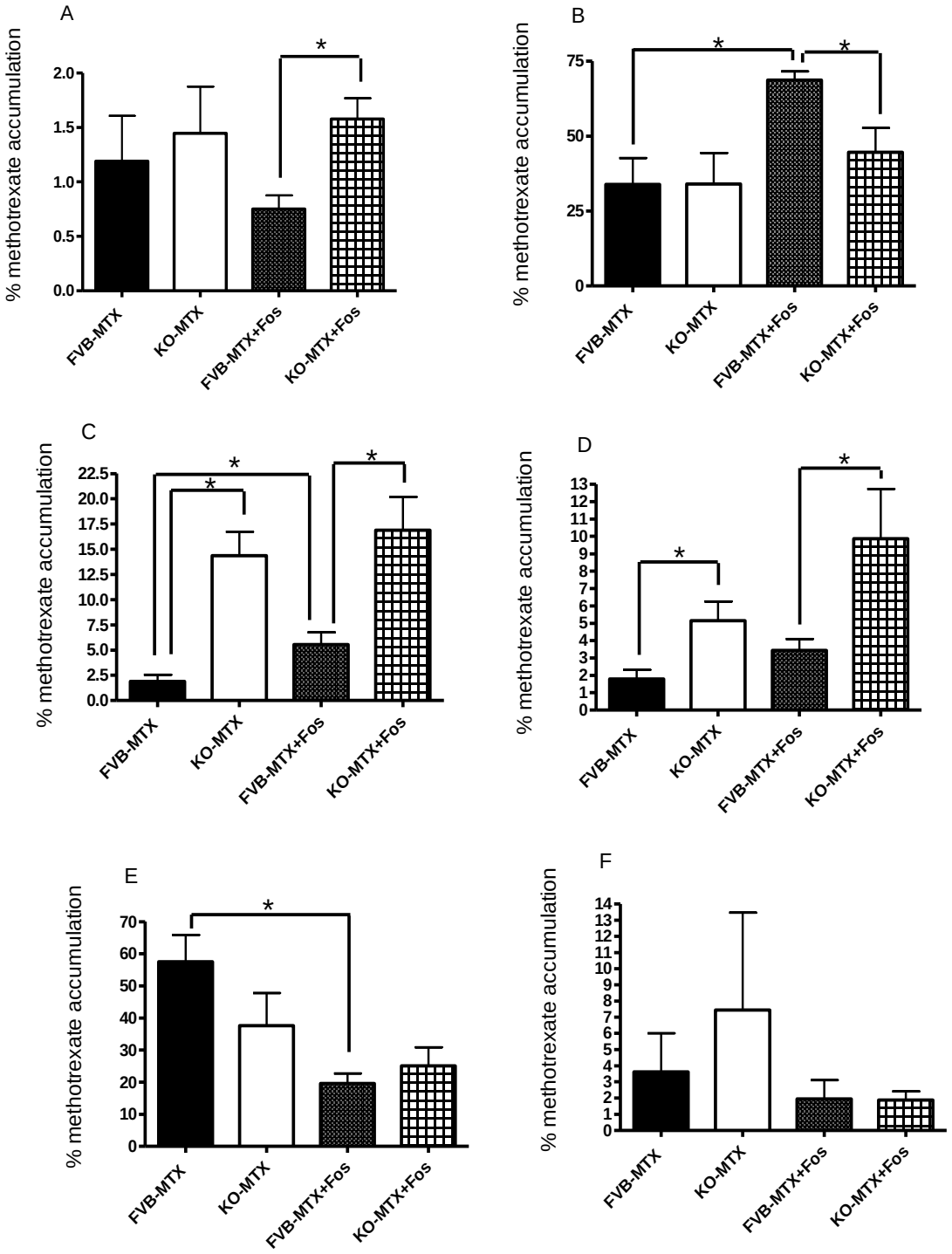

A
% fosinopril remaining
250
200
150
100
50
0
Fos
+10μM
+15μM
+25μM
desloratadine
MDCK
MRP1
MRP2
B
% fosinopril remaining
600
500
400
300
200
100
0
Fos
+10 μM
+15 μM
+25 μM
loratadine
MDCK
MRP1
MRP2
C
% fosinopril remaining
250
200
150
100
50
0
Fos
+30nM
+50nM
methotrexate
MDCK
MRP1
MRP2

Figure 3.


A
% methotrexate accumulation
2.0
1.5
1.0
0.5
0.0
*
FVB-MTX
KO-MTX
FVB-MTX+Fos
KO-MTX+Fos
B
% methotrexate accumulation
75
50
25
0
*
*
FVB-MTX
KO-MTX
FVB-MTX+Fos
KO-MTX+Fos
C
% methotrexate accumulation
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
*
FVB-MTX
KO-MTX
FVB-MTX+Fos
KO-MTX+Fos
D
% methotrexate accumulation
13
12
11
10
9
8
7
6
5
4
3
2
1
0
*
*
FVB-MTX
KO-MTX
FVB-MTX+Fos
KO-MTX+Fos
E
% methotrexate accumulation
70
60
50
40
30
20
10
0
*
FVB-MTX
KO-MTX
FVB-MTX+Fos
KO-MTX+Fos
F
% methotrexate accumulation
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
FVB-MTX
KO-MTX
FVB-MTX+Fos
KO-MTX+Fos

5. Conclusion

In the past few decades, the prescription of multiple pharmaceuticals has become commonplace in the United States. Every year there is more and more potential for injuries to occur, due to the lack of knowledge of certain drug-drug interactions. Some of the prominent mechanisms for toxicity and drug-drug interactions for many highly used pharmaceuticals are through the MRP transporters. Expanding our knowledge of these transporters and the interactions that may occur when confronted with multiple compounds is essential to avoid complications. We wanted to determine if certain highly used pharmaceuticals are substrates for MRP1 and/or MRP2, and if so, whether the co-administration of these compounds results in reduced elimination and altered toxicity. In conclusion, we have shown *in vitro* that the MRP2 transporter is one of the major transporters of some highly prescribed pharmaceuticals. We have demonstrated that when fosinopril is in the presence of other compounds, either substrates or potential inhibitors, concentrations can be increased significantly. This could lead to serious physiological damages and even fatalities. Our *in vivo* experiments further supported that MRP2 is a key eliminator of methotrexate and fosinopril. In these experiments we demonstrated that the co-administration of methotrexate and fosinopril can lead to increased concentration of drugs, and that the absence of the Mrp2 transporter leads to even higher concentration in the liver, kidney, and serum. This is one example of the type of drug-drug interactions that can occur when a combination of several pharmaceuticals are used.

It can be assumed that repeated use of more than one substrate for the MRP2 transporter could result in toxic levels of one or all drugs involved. The respective half lives of fosinopril, loratadine, desloratadine and methotrexate are 12, 12, 27 hours and 30 minutes (reviewed in Murdoch et al., 1992; Sani et al., 2010; www.merk.com). Usually, doses taken for adults are 10mg for fosinopril and loratadine once daily, 5mg every other day for desloratadine, and 15 to 30mg of methotrexate for a course of 5 days (www.merk.com; www.rxlist.com). Lethalilty in mice was found to occur at 2,600mg/kg fosinopril, 5000mg/kg loratadine, 250mg/kg desloratadine and at total dose of 1.5 grams of methotrexate (www.rxlist.com). With regular use, the chances of fosinopril, loratadine and desloratadine reaching toxic levels is unlikely, due the very high doses that would need to be reached to produce toxic effects. Due to the high dose treatment of methotrexate, the chances of toxic levels of methotrexate being reached in combination with one or more of the described drugs is very likely. The combination with fosinopil especially, has shown to increase the concentration of methotrexate remaining in the intestine, serum and kidneys of our wild-type mice. Special care should be taken to avoid the use of methotrexate and fosinopril, because of the potential for toxic levels to be reached.

To better explain how the body reacts to and regulates the potential high concentration of drugs in the presence of each other, studies to see if certain drugs can activate the ligand binding domain of the nuclear receptors, constitutive androstane receptor and pregnane X receptor could be conducted. These nuclear receptors mediate

the transcription of genes such as CYP3A4, glucuronosyltransferases, multidrug resistance-associated protein 2 and bile salt efflux protein (Kast et al., 2002; Johnson et al., 2002; Mackenzie et al., 2003), which are responsible for the metabolism of many endogenous compounds, therapeutic drugs, and xenobiotics such as bile salts, dexamethasone and phenobarbital (Byrne et al., 2002; Kast et al., 2002; Johnson et al., 2002). Activation of nuclear receptors increases the clearance of compounds from the body by increasing the levels of proteins responsible for elimination. Although our mRNA data does not support that fosinopril is a ligand for the nuclear receptors, we did not examine protein levels of glucuronosyltransferases or ABC transporters, which may indeed be upregulated. One way to quickly examine whether fosinopril activates PXR and CAR would be to examine Cyp2b expression, which is the best biomarker for activation by these nuclear receptors.

Mice lacking multiple genes is becoming a very useful tool in evaluating the metabolism of compounds. The use of a multiple gene knockout mice could aid in the explaining some of the potential compensatory mechanisms that may be occurring to reduce concentrations of drugs when one or more transporters are eliminated or impaired. For example, when only the Mrp2 protein is absent in mice, total bilirubin in plasma is increased by 5-fold (Chu et al., 2006; Vlaming et al., 2006), while mice lacking both the Mrp2 and Bcrp transporters have a 7.5-fold increase in plasma levels of bilirubin (Vlaming et al., 2009). These changes in substrate concentrations when more than one ABC transporters is absent can also be shown with the drug methotrexate. In the absence

of Mrp2 alone, plasma concentrations of methotrexate are increased 2.5-fold (Vlaming et al., 2006). When mice lacking the Bcrp transporter are dosed with methotrexate, there is a 1.6-fold increase in the concentration of methotrexate in the plasma, while mice lacking both the Mrp2 and Bcrp transporters have a 3.3-fold increase in plasma methotrexate concentrations (Vlaming et al., 2009). These studies indicate that in the absence of more than one transporter, the concentrations of compounds increase significantly. In our study, we only examined a single gene, Mrp2, and its effect on fosinopril disposition. The next step in our research should be to examine whether multiple transporters can eliminate fosinopril. Some potential transporters to examine would include Bsep, since its expression was upregulated in the livers of mice dosed with fosinopril. Another transporter to examine is Bcrp, since the double knockout of both Mrp2 and Bcrp significantly changes the disposition of methotrexate. Knowing whether these 2 proteins were upregulated and/or could transport fosinopril may help in explaining the some of our disposition results, such as why we see increased levels of methotrexate in the intestine of mice that received both methotrexate and fosinopril. The more we can uncover about these transporters and how they work in combination with each other will help in reducing complications and aid in the more effecting administration of therapeutic drugs.

As can be seen, the metabolism of many pharmaceuticals is a very complex system and the further study of these potentially dangerous drug-drug interactions that

occur between highly prescribed pharmaceuticals and the physiologic changes that result is an important area of study to minimize harm to patients.

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