6-8-2016 12:00 AM

Evaluating the role of intestinal transporters in fruit juice-drug interactions

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Graduate Program in Physiology and Pharmacology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

Abstract

Fruit juice-drug interactions (FJDIs) involving non-metabolized oral medications result in decreased drug exposure that may lead to reduced therapeutic efficacy. The effect is thought to be mediated by inhibition of the intestinal drug transporters organic anion transporting polypeptide 1A2 and 2B1 (OATP1A2 and OATP2B1) by fruit juice constituents, however the exact mechanisms remain controversial. We tested the hypothesis that fruit juices limit the absorption of fexofenadine through interactions with specific intestinal transporters. *In vitro* transport and fruit juice inhibition studies using fexofenadine, a medication involved in FJDIs, revealed that in addition to previously implicated transporters, organic cation transporter 1 (OCT1) and organic solute transporter alpha/beta (OSTα/β) are potential novel targets of fruit juice components. Pharmacokinetic interaction studies in wild-type and mOatp2b1 knockout mice demonstrated that mOatp2b1 is not involved in fexofenadine absorption or FJDIs *in vivo*. These findings provide new insights into the mechanism of FJDIs.

Keywords

Fruit juice-drug interaction, grapefruit juice, apple juice, fexofenadine, drug transporter, OATP1A2, OATP2B1, OCT1, OSTα/β, P-gp, flavonoids

Co-Authorship Statement

Mandy MJ Li designed, performed and analyzed all experiments and data. Sara Mansell-Gallien assisted with animal studies. Dr. Rommel G Tirona assisted with transport experiments.

Acknowledgments

Firstly, I would like to sincerely thank my supervisor, Dr. Rommel Tirona. As a third year undergraduate student, I approached Rommel with the hope of becoming his Accelerated MSc student. Despite the fact that I was young, the program was new and it was dangerously close to the application deadline, he graciously took me on as a student. Since that first day, Rommel has continued to show incredible belief in me and has gone above and beyond as a supervisor. I can undoubtedly say that without his vast knowledge, unwavering support, understanding and patience, I would not have maneuvered through graduate school as well as I had.

I would also like to thank the members of my advisory committee, Dr. Nica Borradaile, Dr. George Dresser and Dr. Wei-Yang Lu for their insight and mentorship.

I am grateful to have gone through this program alongside Alvin Tieu and Jacob Poirier. I could always depend on them for support as they truly understood this unique experience.

To past and present members of the Personalized Medicine Lab, Dr. Richard Kim, Dr. Ute Schwarz, Dr. Crystal Engelage, Dr. Wendy Teft, Dr. Sarah Woolsey, Dr. Michael Knauer, Cameron Ross, Markus Gulilat, Dr. Aze Wilson, Adrienne Borrie, Laura Russell and Cheynne McLean, thank you all for your friendship and guidance. A special thanks to Sara Mansell-Gallien, for making 5 am mouse experiment mornings worth it.

To my roommates and fellow graduate students Michelle Kim and Lisa Choi, without you guys, the last two years would not have been filled with fun, late nights, trips to the gym and if not more, trips to fast food places. Thank you guys for always being encouraging and for teaching me what it means to “treat yourself”.

Finally, I would like to thank my friends and family for their unconditional support, love and encouragement.

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List of Abbreviations

ADE	Adverse drug event
AJ	Apple juice
ANOVA	Analysis of variance
ASBT	Apical sodium-dependent bile acid transporter
ATP	Adenosine triphosphate
AUC	Area under the plasma-concentration time curve
AUC_{0-3}	Area under the plasma concentration-time curve from 0 to 3 hours
BCRP	Breast cancer resistance protein
cDNA	Complementary deoxyribonucleic acid
C_{max}	Peak plasma concentration
CYP	Cytochrome P450
DAPI	4', 6-Diamidino-2-phenylindole
DHB	6'7-Dihydroxybergamottin
DMEM	Dulbecco's modified eagle medium
E_1S	Estrone 3-sulfate
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FJDI	Fruit juice-drug interaction
GFJ	Grapefruit juice
HCP1	Heme carrier protein 1
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
KHB	Krebs-Henseleit buffer
KO	Knockout
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
m/z	Mass-to-charge ratio
mAsbt	Mouse apical sodium-dependent bile acid transporter
mBcrp	Mouse breast cancer resistance protein
MDR1	Multidrug resistance protein 1
mHcp1	Mouse heme carrier protein 1

Min	Minute
mMrp	Mouse multidrug resistance-associated protein
mOatp	Mouse organic anion-transporting polypeptide
mOct	Mouse organic cation transporter
mOctn	Mouse organic cation/carnitine transporter
mOstα/β	Mouse organic solute transporters alpha and beta
mPept1	Mouse peptide transporter 1
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
OATP	Organic anion-transporting polypeptide
OCT	Organic cation transporter
OCTN	Organic cation/carnitine transporter
OJ	Orange juice
OSTα/β	Organic solute transporters alpha and beta
P-gp	P-glycoprotein
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% Tween-20
PCR	Polymerase chain reaction
PEPT1	Peptide transporter 1
qPCR	Quantitative real-time polymerase chain reaction
RT	Room temperature
SDS	Sodium dodecyl sulfate
SLC	Solute carrier
SLCO	Solute carrier organic anion
$t_{1/2}$	Half-life
TCA	Taurocholic acid
TEA	Tetraethylammonium
T_{max}	Time to reach peak plasma concentration
UHPLC-MS/MS	Ultra-high pressure liquid chromatography-tandem mass spectrometry
WT	Wild-type

1 Introduction

1.1 Adverse Drug Events

Patient safety during drug treatment has become a top priority for health care systems worldwide. Adverse drug events (ADEs), defined as unwanted effects due to the use or misuse of medications, are an important cause of patient harm and suboptimal therapy (Baker *et al.*, 2004; Nebeker *et al.*, 2004). Alarmingly, ADEs happen virtually on a daily basis in hospitals and the community (Nebeker *et al.*, 2004). Such events may result in increased doctor visits, longer hospital stays, reduced quality of life and even death (Baker *et al.*, 2004). Lazarous *et al.,* (1998) reported that approximately 6.7% of all hospitalized patients experience a serious adverse drug reaction. Of particular concern is that these reactions have risen to become the fourth leading cause of death (Lazarou *et al.*, 1998). Not only do ADEs pose additional patient health risks and impede effective disease management, they also constitute a substantial economic burden on the Canadian health care system. During the 2010-2011 period, 1 in 200 seniors was hospitalized for an adverse drug reaction (https://www.cihi.ca/en). In 2008 alone, it was estimated that ADEs occurring in hospitals cost approximately $5.6 billion (Hohl *et al.*, 2011). It should be noted that a staggering 37-51% of ADEs are avoidable (Baker *et al.*, 2004).

1.2 Drug Interactions

A major cause of ADEs are drug interactions which are common and in many cases, preventable (Roughead *et al.*, 2010). Drug interactions can be defined as changes in the effect of an administered drug when it is taken together with another drug or dietary substance or with specific disease states (http://www.fda.gov). This occurrence can result in increased or decreased therapeutic activity of a medication, or lead to unanticipated side effects (http://www.fda.gov). While it is often considered that drug interactions can

cause harm, it is necessary to consider that in some therapeutic contexts, drug interactions are intended. The true incidence of unintended drug interactions in the population is difficult to quantify. Some reasons for the lack of such statistics include the notion that only 1 in 20 ADEs are ever reported and that ADEs are less commonly captured in non-institutional settings (Hazell and Shakir, 2006). Importantly, ADEs that result in loss of drug efficacy such as certain fruit juice-drug interactions, which form the subject of this thesis, are rarely identified and reported.

1.2.1 Pharmacokinetic and Pharmacodynamic Mechanisms of Drug Interactions

Pharmacokinetics is the study of drug concentrations in various body compartments as a function of time. This area of pharmacology also examines how the body processes a medication. Consequently, pharmacokinetic drug interactions can be defined as those which occur when an illness or co-consumed substance alters drug disposition by changing a drug's *absorption* into circulation, its *distribution* into tissues, its *metabolism* by enzymes into polar compounds, or its *excretion* out of the body, generally via urine or feces (Buxton and Benet, 2011). Drug interactions of this nature will ultimately affect the concentration or duration of medications at their site of action and the associated therapeutic or toxic responses.

Pharmacodynamics on the other hand, links drug concentration at the site of action with the degree of therapeutic effect elicited (Blumenthal and Garrison, 2011). This branch of pharmacology is focused on the cellular targets of medications, commonly, receptors (Blumenthal and Garrison, 2011). Therefore, pharmacodynamic drug interactions typically arise when another substance or pre-existing disorder alters the receptor

signalling pathway affected by the drug, resulting in synergistic, antagonistic or novel responses. Generally, greater clinical effects of drugs are associated with increasing drug concentrations at the target site.

1.2.2 Types and Clinical Consequences of Drug Interactions

Drug interactions can compromise the safe and effective use of medicinal agents. There are 4 main types of drug interactions, namely, drug-drug, herb-drug, disease-drug and food-drug interactions (http://www.fda.gov). Examples of each category and the clinical consequences associated are found in **Table 1.1**. Despite the fact that the many mechanisms are well-characterized, in clinical practice, it is often difficult to recognize potential drug interactions, making them hard to avoid. The prevalence of polypharmacy and individuals presenting with comorbidities, especially in the elderly patient population, mean that drug-drug and disease-drug interactions occur frequently (Maher *et al.*, 2014). It therefore becomes easy to miss possible interactions when prescribing medications. Indeed, a recent population-based study estimated that 15% of older adults are at risk for a major drug-drug interaction (Qato *et al.*, 2016). Furthermore, herbs, other natural products and dietary supplements are regularly used but are often considered safe and frequently overlooked as substances that may impact drug activity (Hussain, 2011). As such, patients fail to mention these products to their physicians and similarly, physicians often do not consider these remedies as possible contraindications to prescription medications. Co-ingestion of oral medications with food is commonly used as a tool to improve patient adherence to drug therapy, but at the potential risk of increasing the probability of food-drug interactions (Marek and Antle, 2008). Because diets are complex and have only recently been considered as mediators of drug

interactions, the mechanisms underlying most food-drug effects have not been fully explored. A better understanding of the mechanisms mediating food-drug interactions can aid in the implementation of practical strategies to avoid ADEs and improve pharmacotherapy management. The focus of this thesis is on food-drug interactions, in particular those involving fruit juices.

Table 1.1 Examples of clinically relevant drug interactions.

Type of Drug Interaction	Drug	Interacting Substance/Disease	Clinical Consequence	Reference
Drug-Drug	Warfarin	Amiodarone	Increase bleeding risk	Heimark *et al.* (1992)
Disease-Drug	Methotrexate	Chronic kidney disease	Neutropenia	Ellman and Ginsberg (1990); Chatham *et al.* (2000)
Herb-Drug	Cyclosporine	St. John's Wort	Organ transplant rejection	Ruschitzka *et al.* (2000); Breidenbach *et al.* (2000)
Food-Drug	Felodipine	Grapefruit juice	Hypotension	Bailey *et al.* (1989); Bailey *et al.* (1991)

1.3 Fruit Juice-Drug Interaction (FJDI)

1.3.1 Discovery

One third of Canadians drink fruit juices on a daily basis (http://www.statcan.gc.ca). This statistic is concerning from a therapeutic point of view as fruit juices are now well-documented components of the diet that are involved in food-drug interactions. The effect was serendipitously discovered by Bailey *et al.* (1989) during an interaction study between ethanol and felodipine, an oral antihypertensive, in which grapefruit juice (GFJ) was used to mask the flavor of ethanol. Interestingly, the co-ingestion of GFJ with felodipine in both the control and experimental groups resulted in a clinically significant rise in plasma drug levels in comparison to previously reported values (Bailey *et al.*, 1989). This prompted a follow-up study where volunteers were administered felodipine with and without GFJ (Bailey *et al.*, 1991). In this study, co-administration with the fruit juice led to a 3-fold increase in the area under the plasma drug concentration vs. time curve (AUC), a reflection of systemic drug exposure, which was presumed to result from increased felodipine oral bioavailability rather than reduced systemic clearance (Bailey *et al.*, 1991). Oral bioavailability refers to the fraction of an orally administered dose that makes it into systemic circulation unchanged, following absorption across the gastrointestinal wall and metabolism by gut and hepatic enzymes, to become therapeutically available. The dramatic rise in plasma felodipine levels led to an exacerbation of its clinical effect (greater drop in diastolic blood pressure) and increased the incidence of adverse side effects (more frequent headaches) (Bailey *et al.*, 1991).

1.3.2 Drug Metabolizing Enzyme-Mediated Mechanisms

Since the initial discovery, the effect of GFJ on the pharmacokinetics of numerous other

drugs have been evaluated. Clinically relevant interactions have been identified across numerous drug classes including calcium channel blockers, protease inhibitors, statins and immunosuppressants such as nifedipine, saquinavir, atorvastatin and cyclosporine, respectively (Bailey *et al.*, 1991; Ducharme *et al.*, 1995; James, 1995; Yee *et al.*, 1995; Reddy *et al.*, 2011).

Many GFJ-drug interactions involve medications that are substrates of cytochrome P450 3A4 (CYP3A4), an enzyme responsible for the intestinal and hepatic oxidative metabolism of xenobiotic compounds. In most cases, increased drug bioavailability with GFJ co-consumption was only seen when the therapeutic compound was given orally, but not intravenously. Furthermore, the time required for the plasma drug concentration to decline by one half, the half life ($t_{1/2}$), is generally not affected by grapefruit juice intake. Together, these findings suggested that GFJ caused specific inhibition of CYP3A4 that occurred at the level of the intestine. Moreover, it would appear that GFJ does not have an inhibitory effect on the metabolic activity of hepatic CYP3A4 as reflected by the lack of change to $t_{1/2}$ and systemic clearance.

In support of this mechanism, Lown *et al.* (1997) demonstrated using small bowel biopsies that GFJ ingestion for 6 days resulted in a 62% decrease in enterocyte CYP3A4 protein concentration. The effect was observed as early as 4 hours post juice administration (Lown *et al.*, 1997). Interestingly, CYP3A4 messenger ribonucleic acid (mRNA) levels in intestinal epithelial cells were unchanged with GFJ consumption (Lown *et al.*, 1997). Thus, GFJ appeared to impact the enzyme post-transcriptionally, through a mechanism involving rapid CYP3A4 protein degradation. Studies by Edwards *et al.* (1996) and Schmiedlin-Ren *et al.* (1997) reinforced this idea by showing that

specific components of GFJ known as furanocoumarins are mechanism-based inactivators of CYP3A4 *in vitro*. A key GFJ furanocoumarin, bergamottin, was found to be metabolized to 6'7-dihydroxybergamottin (DHB), a suicide inhibitor of intestinal CYP3A4 (Lin *et al.*, 2012). Duration of the GFJ effect was dependent on the *de novo* synthesis rate of the CYP3A4 enzyme and the natural turnover of intestinal enterocytes. The intestinal specificity of this metabolic interaction was confirmed in a study that showed hepatic CYP3A4 activity, as determined by erythromycin breath tests, was unaffected by GFJ (Lown *et al.*, 1997). This finding implies that liver exposure to GFJ furanocoumarins is limited.

1.3.3 Non-Metabolic FJDIs

With subsequent studies, it has become increasingly evident that fruit juice-mediated effects on drug disposition cannot be attributed solely to inhibition of metabolism. The pharmacokinetics of numerous minimally-metabolized drugs have also shown to be influenced by GFJ co-ingestion (**Table 1.2**). Intriguingly, reductions in oral bioavailability and plasma concentrations are seen for some non-metabolized drugs when taken with GFJ which contrasts with increased systemic drug concentrations for CYP3A4-metabolized medications. The duration of GFJ effects was another difference observed for interactions with non-metabolized drugs. Due to the requirement for *de novo* CYP3A4 synthesis after GFJ exposure, the bioavailability of metabolized drugs administered is significantly impacted up to 26 hours post juice intake (Lundahl *et al.*, 1995; Greenblatt *et al.*, 2003). However, with non-metabolized medications, the effect of GFJ on drug disposition dissipated within 2-4 hours after juice administration (Glaeser *et al.*, 2007). Furthermore, apple juice (AJ) and orange juice (OJ) elicit minimal changes to

the pharmacokinetics of CYP3A4 substrate drugs. On the other hand, these juices reduce the systemic exposure of minimally-metabolized therapeutic agents to a degree similar to that observed with GFJ (Dresser *et al.*, 2002). Therefore, it would appear that there are drug metabolizing enzyme-independent mechanisms involved in certain FJDIs, perhaps related to modulation of membrane drug transport. Moreover, the specific constituents in fruit juices that affect the pharmacokinetics of non-metabolized drugs may differ from the furanocoumarins implicated in metabolic GFJ interactions.

Table 1.2 Clinical effects of grapefruit juice (GFJ) and apple juice (AJ) on oral drug bioavailability that cannot be explained by changes in drug metabolism.

Drug	Juice	Effect on Oral Bioavailability (Compared to Water)	Reference
Antihistamine			
Fexofenadine	GFJ	Decrease	Banfield *et al.* (2002); Dresser *et al.* (2002); Dresser *et al.* (2005); Bailey *et al.* (2007); Glaeser *et al.* (2007); Won *et al.* (2013)
	AJ	Decrease	Dresser *et al.* (2002); Imanaga *et al.* (2011); Akamine *et al.* (2014)
Cardiovascular Drugs			
Acebutolol	GFJ	Decrease	Lilja *et al.* (2005b)
Aliskiren	GFJ	Decrease	Tapaninen *et al.* (2010); Rebello *et al.* (2012)
	AJ	Decrease	Tapaninen *et al.* (2011)
Atenolol	AJ	Decrease	Jeon *et al.* (2013)
Celiprolol	GFJ	Decrease	Lilja *et al.* (2003); Ieiri *et al.* (2012)
Digoxin	GFJ	Increase*	Becquemont *et al.* (2001)
Talinolol	GFJ	Decrease	Schwarz *et al.* (2005)
Chemotherapy Drug			
Etoposide	GFJ	Decrease	Reif *et al.* (2002)
Hormone			
Levothyroxine	GFJ	Decrease	Lilja *et al.* (2005a)

*Effect was significant between 0-24 hours post drug administration. Effect was not significant between 0-48 hours post drug administration.

1.4 Fexofenadine, a Prototypical Non-Metabolized Drug That is Subject to FJDIs

Fexofenadine, commonly known by its trade name Allegra, is a second generation H1 histamine receptor antagonist used for the treatment of seasonal allergies and chronic hives (Smith and Gums, 2009). Available as oral fexofenadine hydrochloride tablets, the typical doses for adults are 60 mg twice daily or 180 mg once daily (http://products.sanofi.ca). Following oral administration, fexofenadine exists as a zwitterion during transit through the small and large bowel (Chen, 2007) (**Figure 1.1**). Indeed, it is categorized as a Class III drug under the Food and Drug Administration biopharmaceutical classification system, meaning that it has high water solubility and low membrane permeability. Hence, oral fexofenadine absorption from the gut lumen is presumed to be mediated not by passive diffusion but by the opposing activities of intestinal uptake and efflux transporters located in the apical membrane of the gut mucosa (Chen, 2007). The antihistamine is absorbed relatively quickly into circulation as the time needed to reach peak plasma concentration (T_{max}) occurs between 1-3 hours after administration (Smith and Gums, 2009). Fexofenadine undergoes minimal breakdown by metabolic enzymes such as CYP3A4 and is mainly eliminated unchanged in the urine (10%) and feces (80%) (Smith and Gums, 2009). Bioavailability has been estimated to be approximately 33% for the oral dose (Smith and Gums, 2009). As fexofenadine experiences negligible metabolism, the oral bioavailability, tissue distribution and clearance is largely dependent on the activity of drug transporters in the gut, liver and kidney. For this reason, fexofenadine has been administered to study subjects as a "probe drug" to assess the *in vivo* transport activity of solute carrier proteins (Flynn *et al.*, 2011).

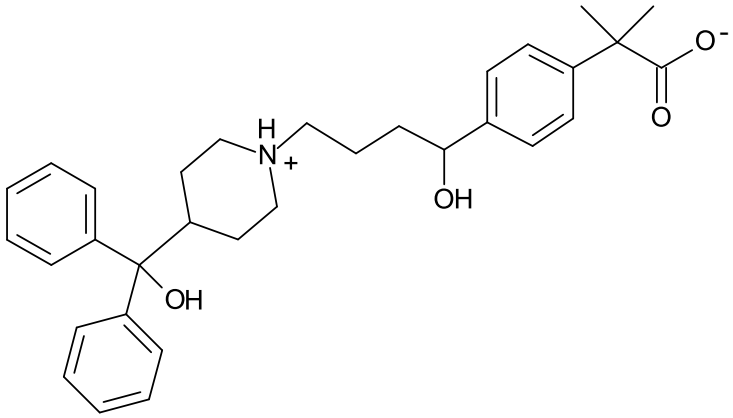


Figure 1.1 Chemical structure of fexofenadine.

1.5 Role of Intestinal Transporters in Fexofenadine and Other FJDIs

In 2002, Dresser *et al.* demonstrated the first example of a FJDI whose mechanism was independent of CYP enzymes. In that seminal report, fexofenadine was co-administered with 1.2 L of GFJ, AJ or water over 3 hours to a cohort of healthy volunteers. Interestingly, concomitant ingestion of either fruit juice reduced plasma drug exposure to 23-33% of that with water (Dresser *et al.*, 2002). Fexofenadine renal clearance and $t_{1/2}$ were not affected by the fruit juices, suggesting minimal impact of juices on excretory organs such as the liver and kidney and localizing the interaction to the site of drug absorption (Dresser *et al.*, 2002). A similar finding was reported by Banfield and colleagues (2002) when they administered fexofenadine to subjects who consumed 240 mL of double-strength GFJ, 3 times daily for 2 days prior to, during and 2 hours after drug administration. Given the unusual amount of GFJ consumed in these early studies, the clinical relevance of the GFJ-fexofenadine interaction was demonstrated in a subsequent study involving co-administration of drug with a more reasonable volume (300 mL) of GFJ (Dresser *et al.*, 2005).

As eluded to previously, the effect of GFJ on plasma fexofenadine exposure was short-lived as no reduction in AUC was observed when GFJ was administered 4 hours prior to fexofenadine intake (Glaeser *et al.*, 2007). Additionally, unlike enzyme-mediated FJDIs, other components of fruit juices known as flavonoids, not furanocoumarins, were considered probable players of this interaction (will be discussed in further detail in **Section 1.9**). This was compellingly demonstrated when naringin, a major flavonoid found in GFJ, was orally administered at concentrations equivalent (1200 μM, 300 mL)

to that found in GFJ, together with fexofenadine, plasma drug levels were reduced significantly but not to the same extent as with GFJ (Bailey *et al.*, 2007). Furthermore, modified GFJ lacking furanocoumarins (commercially produced to minimize metabolic drug interactions) elicited the same change to fexofenadine AUC as regular GFJ (Won *et al.*, 2013).

Since these initial reports with fexofenadine, other non-metabolized medications across several drug classes have been shown to be affected by fruit juices (**Table 1.2**). Generally, there is decreased systemic drug exposure for these oral medications when given with juices. Such interactions are expected to result in reduced clinical effects, subjecting patients to suboptimal disease treatment and/or management.

Given the common pharmacokinetic effect of fruit juices on the oral absorption of many non-metabolized drugs, it is generally considered that the mechanism of these food-drug interactions involves modulation of intestinal drug transporters. Indeed, a number of intestinal drug transporters that are responsible for facilitating or impeding drug absorption have become proposed targets for fruit juice-drug interactions. At present however, the exact transporters involved and the molecular mechanisms still remain unclear owing to the lack of compelling data from *in vitro* and preclinical models that are all consistent with the clinical observations. Ideally, the mechanisms for clinical FJDIs for non-metabolized drugs will be firmly established by *in vitro* studies that demonstrate that the affected drug is a transport substrate of a membrane transporter known to be expressed in the human intestine. Furthermore, *in vitro* studies should show that drug transport by these membrane proteins is inhibited or stimulated by fruit juices and their specific constituents. In conjunction with *in vitro* studies, mechanisms for FJDIs will be

persuasively established in experiments with animal models, including transporter knockout (KO) mice, that will shed additional *in vivo* insights to explain the clinical problem.

1.6 Intestinal Apical Uptake Transporters

1.6.1 Potential Mechanistic Role in FJDI

The oral absorption of hydrophilic and charged drugs such as fexofenadine are less likely to result from passive diffusion mechanisms but are largely dependent on the actions of drug transporters localized on the apical (luminal) and basolateral membranes of intestinal enterocytes. Vectorial movement of fexofenadine across the enterocyte would require uptake transport on the apical membrane coupled with efflux transport on the basolateral membrane for entry into the portal circulation. Inhibition of uptake transporters located on the apical membrane of intestinal epithelial cells is the leading mechanism that would be consistent with the observed reduction of fexofenadine bioavailability by juices. A number of drug uptake transporters are expressed on the enterocyte apical membrane including those in the organic anion transporting polypeptide (OATP), organic cation transporter (OCT, OCTN), peptide transporter (PEPT), bile acid and heme transporter families of solute carriers (**Figure 1.2**). A few of these transporters are already known to facilitate fexofenadine cellular uptake *in vitro*, however the entire complement of relevant intestinal transporters has not been established. Moreover, *in vitro* studies have demonstrated that some of these transporters are inhibited by fruit juices or their constituents.

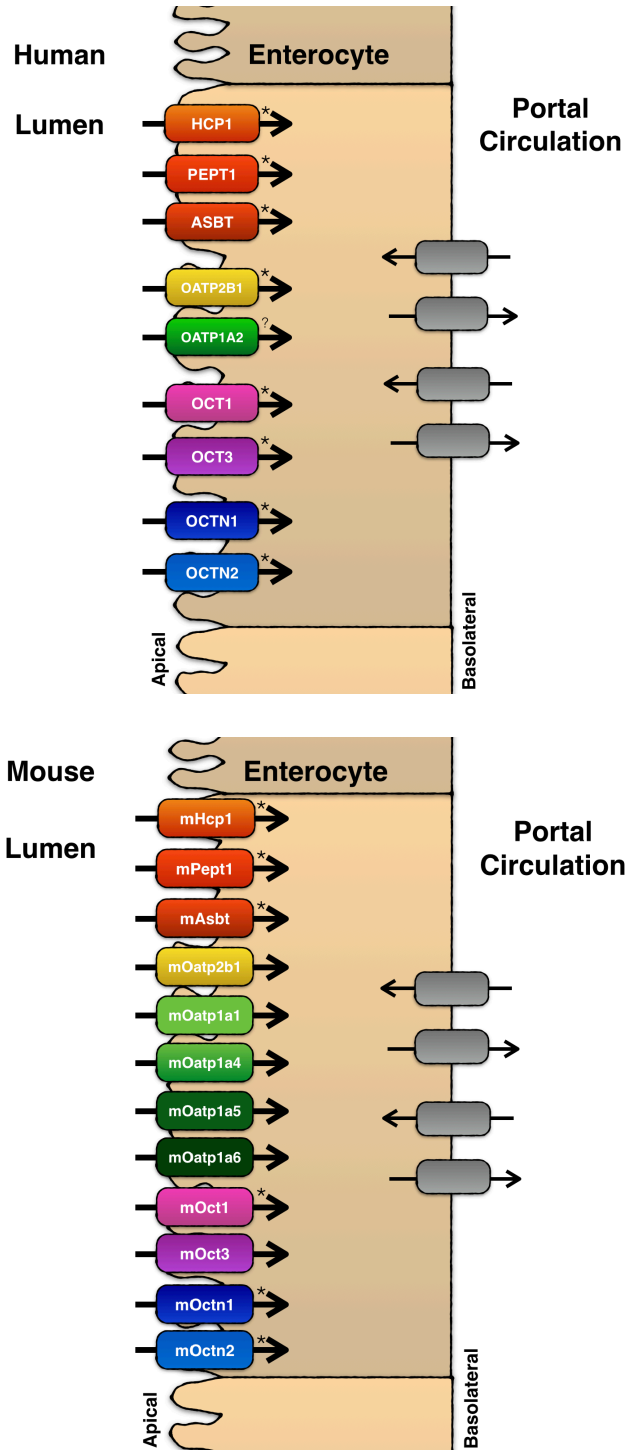


Figure 1.2 Potential intestinal uptake transporters involved in fruit juice-drug interactions in humans and in mice. *Confirmed and ?controversial localization to the apical membrane of enterocytes.

1.6.2 Organic Anion-Transporting Polypeptides (OATPs)

Organic anion-transporting polypeptides (OATPs) are a superfamily of integral membrane uptake transporters belonging to the solute carrier organic anion (*SLCO*) gene superfamily (Hagenbuch and Meier, 2003). They are responsible for the sodium-independent cellular influx of a wide spectrum of cationic, neutral and anionic compounds (Hagenbuch and Meier, 2003). These solute carriers facilitate the movement of endogenous molecules including bilirubin, bile acids, prostaglandins, thyroid and steroid hormones throughout the body (Tirona and Kim, 2014). Moreover, OATPs have been shown to transport several clinically relevant xenobiotics including antihistamines, beta blockers, statins and antidiabetic agents (Dresser *et al.*, 2002; Satoh *et al.*, 2005; Ho *et al.*, 2006; Ieiri *et al.*, 2012). OATP transport activity typically favors a low pH environment and is mediated by exchange with intracellular compounds such as bicarbonate and glutathione (Nozawa *et al.*, 2004; Meier-Abt *et al.*, 2005; Leuthold *et al.*, 2009; Eechoute *et al.*, 2011). Some OATPs are ubiquitously expressed while others are tissue-selective (Tirona and Kim, 2014). Additionally, OATP cellular expression is commonly polarized to either the apical or basolateral membranes, depending on the tissue epithelium (Tirona and Kim, 2014). Notably, OATP1A2 and OATP2B1 are two isoforms found in the luminal membrane of human small bowel epithelial cells (Kobayashi *et al.*, 2003; Glaeser *et al.*, 2007). Numerous substrate drugs, such as fexofenadine are thought to rely on the transport activity of these two OATPs for absorption (Tamai and Nakanishi, 2013). **For these reasons, OATP1A2 and OATP2B1 have been the principal suspect transporters involved in FJDIs.**

1.6.2.1 OATP1A2

With the first clinical demonstration of the fruit juice-fexofenadine interaction, OATP1A2 was proposed to be the primary culprit (Dresser *et al.*, 2002). In support of this claim, *in vitro* studies performed by Dresser *et al.* (2002) reconfirmed that fexofenadine was a substrate of OATP1A2 (Cvetkovic *et al.,* 1999). Importantly, GFJ, AJ and the juice flavonoids naringin and hesperidin, at relevant concentrations, limited uptake of the antihistamine by OATP1A2 in cellular models (Dresser *et al.*, 2002; Bailey *et al.*, 2007). Remarkably however, it was not known in 2002 whether OATP1A2 is expressed in the intestine. In a follow-up study by the same research group, the mRNA expression of various transporters was determined in human duodenal biopsies. OATP1A2 transcript, among other uptake transport proteins, was detected consistently in small bowel mucosa (Glaeser *et al.*, 2007). Subsequent immunohistochemical and immunofluorescent staining supported OATP1A2 localization to the apical membrane of enterocytes (Glaeser *et al.*, 2007). Its prominent role in the fruit juice interaction was further emphasized after an *in vitro* screen of transport proteins identified OATP1A2 as the only intestinally-expressed uptake transporter capable of mediating significant fexofenadine cellular influx (Glaeser *et al.*, 2007). Moreover, in line with the short-lived effect of GFJ on fexofenadine plasma concentration, intestinal OATP1A2 mRNA and protein levels were not affected by juice exposure (Glaeser *et al.*, 2007).

Although these findings had strongly implicated OATP1A2 in FJDIs, its mechanistic role *in vivo* remains highly debated, as controversy surrounds whether the transporter is expressed in the small intestine at all. While numerous research groups have detected levels of OATP1A2 mRNA and protein in the gut (Su *et al.*, 2004; Ballestero *et al.*, 2006;

Glaeser *et al.*, 2007; Maubon *et al.*, 2007), there is equal evidence demonstrating limited or absent expression of both transcript and protein at the site of fexofenadine absorption (Nishimura and Naito, 2005; Hilgendorf *et al.*, 2007; Meier *et al.*, 2007; Eechoute *et al.*, 2011; Drozdzik *et al.*, 2014). Therefore, current focus has shifted to another OATP isoform as the main facilitator of the fruit juice-fexofenadine effect.

1.6.2.2 OATP2B1

There has been increasing support for the role of OATP2B1 in transporter-mediated FJDIs. Unlike OATP1A2, its expression in the small intestine is well documented and undisputed, as high levels of both mRNA and protein have been consistently reported in the literature (Kobayashi *et al.*, 2003; Nishimura and Naito, 2005; Meier *et al.*, 2007; Drozdzik *et al.*, 2014). Although Glaeser *et al.* (2007) detected OATP2B1 mRNA transcript in duodenal biopsies, their original *in vitro* evaluation did not demonstrate fexofenadine transport by this solute carrier. Since then however, there have been several accounts of OATP2B1-mediated fexofenadine uptake in other cellular models, solidifying fexofenadine as a now well-accepted substrate of the transporter (Nozawa *et al.*, 2004; Imanaga *et al.*, 2011; Shirasaka *et al.*, 2011; Shirasaka *et al.*, 2013b; Akamine *et al.*, 2014; Akamine, 2015). Similar to OATP1A2, fexofenadine co-incubation with GFJ and AJ significantly reduced its cellular influx by OATP2B1 *in vitro* (Imanaga *et al.*, 2011; Shirasaka *et al.*, 2013b). While flavonoid inhibition of OATP2B1 transport activity of various drugs have been demonstrated, specific attenuation of fexofenadine transport by these fruit juice components has not yet been reported in OATP2B1 cellular expression systems (Shirasaka *et al.*, 2013a).

In contrast to OATP1A2, there is some *in vivo* support for the role of OATP2B1 in FJDIs. The *SLCO2B1* c.1457C>T genetic polymorphism is associated with reduced OATP2B1 transport function *in vitro* (Nozawa *et al.*, 2002). Interestingly, individuals harboring this allele displayed lower fexofenadine plasma exposure than wild-type (WT) volunteers (Imanaga *et al.*, 2011). Moreover, although AJ diminished fexofenadine AUC in both WT and c.1457C>T allele carriers, the effect was less pronounced in those carrying *SLCO2B1* variant alleles (Imanaga *et al.*, 2011). Therefore, it would appear that fexofenadine is an *in vivo* substrate of OATP2B1 and that AJ may act on this drug transporter as a functional inhibitor.

1.6.2.3 Mouse Orthologs of OATPs

There are 4 mouse orthologs of human OATP1A2, namely mOatp1a1, mOatp1a4, mOatp1a5 and mOatp1a6 (van de Steeg *et al.*, 2010). Interestingly, these 4 transporters have low to undetectable mRNA expression along the gastrointestinal tract (Cheng *et al.*, 2005; Fu *et al.*, 2016). Unfortunately, substrate specificities of individual mOatp1a transporters have not been well characterized, particularly with respect to fexofenadine. Additionally, *in vitro* effects of fruit juices on mOatp1a transporters have never been reported.

In contrast to OATP1A2, OATP2B1 is highly conserved between species as a single orthologous isoform. Approximately 77% of the protein sequence is shared between the human and the mouse ortholog (Lan *et al.*, 2009). Importantly, mouse Oatp2b1 (mOatp2b1) mRNA has been detected at high levels in the small bowel of mice, suggesting analogous expression to the human transporter, but protein expression has not been confirmed experimentally (Cheng *et al.*, 2005; Fu *et al.*, 2016). It is currently known

whether fexofenadine is a substrate for mOatp2b1 or if it can be inhibited by fruit juices.

1.6.3 Organic Cation Transporters (OCTs)

Organic cation transporter 1 (OCT1), an isoform of the polyspecific transporter proteins of solute carrier family 22 (*SLC22A1*), is an uptake protein responsible for the cellular influx of cationic compounds such as endogenous acetylcholine (Mimura *et al.*, 2015). Notably, it transports numerous drug substrates including antidiabetic and antiviral medications (Jonker *et al.*, 2003). This solute carrier was originally localized to the basolateral membrane of intestinal epithelial cells (Muller *et al.*, 2005). However, more recent findings have provided evidence to support the expression of OCT1 in the apical membrane of human enterocytes (Han *et al.*, 2013). *In vitro* studies have shown the inhibition of OCT1 transport activity by AJ flavonoids, quercetin and phloretin (Mimura *et al.*, 2015). However, fexofenadine appears to be a very weak transporter substrate of OCT1 *in vitro* (Glaeser *et al.*, 2007).

OCT3 is another isoform found on the apical membrane of enterocytes (Muller *et al.*, 2005). Similarly, it mediates the absorption of organic cations and has a wide spectrum of xenobiotic drug substrates. It is not known whether fexofenadine is a substrate of OCT3 or if fruit juices affect transport activity.

1.6.3.1 Mouse Orthologs of OCTs

The mouse ortholog of OCT1 has been localized to the apical membrane of the small intestine (Han *et al.*, 2013). Mouse Oct1 (mOct1) and human OCT1 possess similar substrate specificity. For example, metformin, an antidiabetic drug and tetraethylammonium, an experimental compound, are substrates of both transporters

(Lozano *et al.*, 2013). The mRNA transcript of the mouse ortholog of OCT3 (mOct3) has been measured at significant levels in the small intestine of mice but its polarized expression in enterocytes remains unknown (Fu *et al.*, 2016). Fexofenadine transport by mOct1 and mOct3 and inhibition by fruit juices have not been previously reported.

1.6.4 Other Apical Uptake Transporters

Organic cation/carnitine transporters 1 and 2 (OCTN1 and OCTN2) may also be involved in FJDIs. They are located in the luminal membrane of intestinal epithelial cells (Lahjouji *et al.*, 2001; Sugiura *et al.*, 2010). An important endogenous substrate of OCTN transporters is carnitine, an amino acid derivative involved in lipid metabolism (Lahjouji *et al.*, 2001). OCTNs also have several drug substrates such as verapamil and doxorubicin, antihypertensive and chemotherapeutic medications, respectively (Pochini *et al.*, 2013). Finally, peptide transporter 1 (PEPT1), apical sodium-dependent bile acid transporter (ASBT) and heme carrier protein 1 (HCP1; *SLC46A1*) are uptake transporters found in the luminal membrane of the gut epithelia (Ziegler *et al.*, 2002; Shayeghi *et al.*, 2005; Balakrishnan and Polli, 2006). Due to the localization of these transporters, they may also play a role in FJDIs. However, fexofenadine transport ability and the effect of fruit juices these carrier proteins have not been studied.

The mouse orthologs of OCTN1, OCTN2, PEPT1, ASBT and HCP1 are mOctn1, mOctn2, mPepT1, mAsbt and mHcp1, respectively. Similarly, they are located in the luminal membrane of mouse enterocytes (Hakansson *et al.*, 2002; Shayeghi *et al.*, 2005; Kato *et al.*, 2006; Chen *et al.*, 2010; Sugiura *et al.*, 2010). Again, fexofenadine transport by these proteins and the effect of fruit juices on their activity are unknown.

1.7 Intestinal Apical Efflux Transporters

1.7.1 Potential Mechanistic Role in FJDI

Other possible explanations for FJDIs include transport stimulation or gene expression induction of efflux transporters located on the apical membrane of enterocytes (**Figure 1.3**). Drugs that traverse the apical membrane of intestinal epithelial cells are pumped back out into the gut lumen by these transporters, limiting absorption. Increased activity of efflux transporters would further impede drug absorption and reduce systemic exposure.

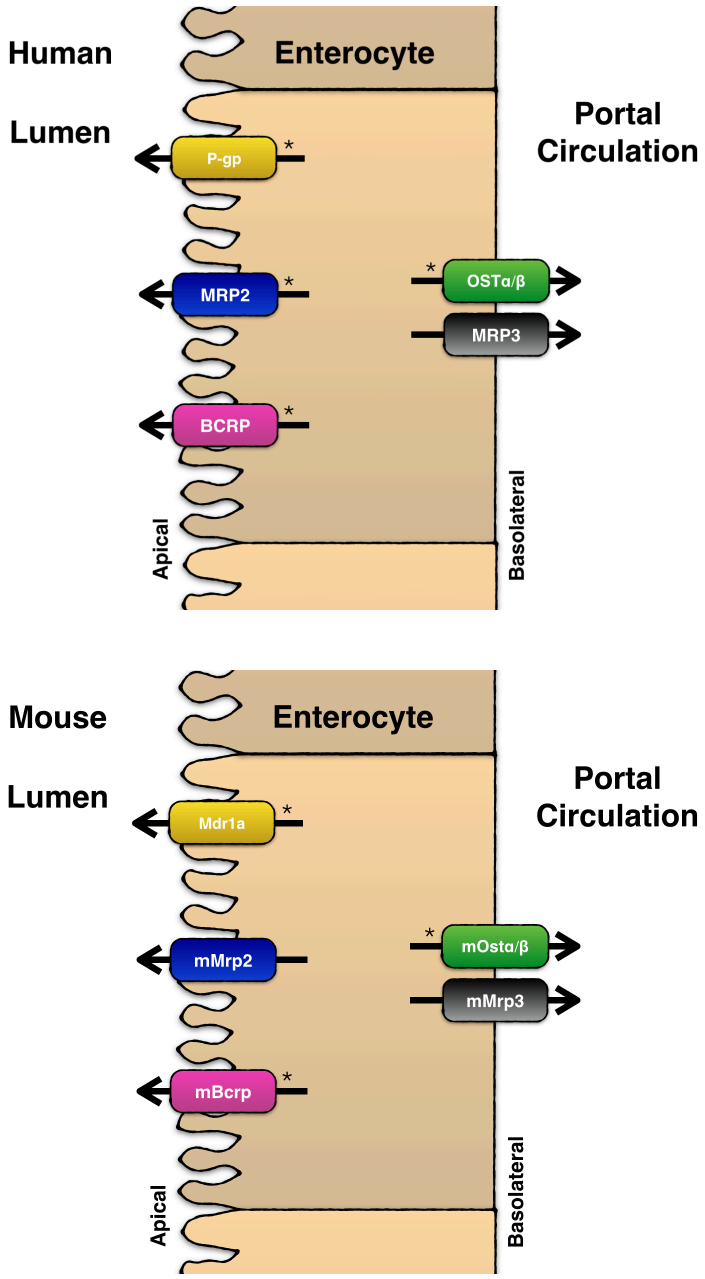


Figure 1.3 Potential intestinal efflux transporters involved in fruit juice-drug interactions in humans and in mice. *Confirmed localization to the apical or basolateral membrane of enterocytes.

1.7.2 P-Glycoprotein (P-gp)

P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1), is a member of the adenosine triphosphate (ATP)-binding cassette superfamily of membrane-bound efflux transporters (Lin and Yamazaki, 2003). It is recognized for mediating the cellular exclusion of numerous xenobiotic compounds including but certainly not limited to, digoxin, a cardiac glycoside, cyclosporine, an immunosuppressant and pertinently, fexofenadine, an antihistamine (Cvetkovic *et al.*, 1999; Lin and Yamazaki, 2003). P-gp is expressed in a polarized manner in the epithelial cells of numerous tissues. For example, it is found in the blood side of brain capillary endothelial cells and apical membrane of kidney proximal tubule epithelial cells (Lin and Yamazaki, 2003). Importantly, this efflux transporter is localized to the brush boarder of enterocytes, allowing it to limit the intestinal absorption of substrate oral medications (Thiebaut *et al.*, 1987; Glaeser *et al.*, 2007). Indeed, co-administration of P-gp inhibitor drugs such as ketoconazole, itraconazole and lopinavir/ritonavir increases the plasma concentrations of fexofenadine in humans (Yasui-Furukori *et al.*, 2005; Shimizu *et al.*, 2006; Uno *et al.*, 2006). Moreover in humans, inducing intestinal P-gp expression by treatment with the herbal medication, St. John's Wort, reduced the bioavailability of fexofenadine (Dresser *et al.*, 2003). Due to its broad substrate specificity and clear importance in modulating intestinal drug absorption, the involvement of P-gp in FJDIs must be considered.

Several *in vitro* studies examining the effect of GFJ and its components on P-gp transport have led to contradictory findings. For instance, while Soldner *et al.* (1999) found stimulation of P-gp transport by GFJ, Takanaga *et al.* (1998), Honda *et al.* (2004) and de Castro *et al.* (2007) all demonstrated inhibition of P-gp activity by GFJ and/or its

components. Various other groups have also proposed concentration- and exposure time-dependent effects of fruit juices on P-gp mediated efflux (Mitsunaga *et al.*, 2000; Panchagnula *et al.*, 2005). At present, the evidence favors the inhibitory action of juices on this efflux transporter.

In the case that P-gp function was impaired clinically, significant efflux transport inhibition would have led to a rise in plasma fexofenadine exposure. Since oral bioavailability is attenuated in the fruit juice-fexofenadine interaction, it is commonly thought that inhibition of uptake transporters contributes more significantly to fexofenadine plasma drug levels than inhibition of intestinal efflux transport. In support of this idea, *in vitro* studies have shown that the GFJ flavonoid, naringin was a more potent inhibitor of intestinal uptake transporters than P-gp (Dolton *et al.*, 2012). Furthermore, Dresser *et al.* (2002) found that while 5% GFJ and AJ significantly inhibited OATP transport activity, P-gp mediated efflux activity was not altered in cellular expression models.

1.7.2.1 Mouse Orthologs of P-gp

There are 2 mouse orthologs of human P-gp, namely Mdr1a and Mdr1b. Both transporters are found in the liver and kidney, while Mdr1a is distinctly expressed in the luminal membrane of small bowel epithelial cells (Panwala *et al.*, 1998; Lin and Yamazaki, 2003). Thus, in terms of intestinal absorption, Mdr1a plays a larger role in drug disposition than Mdr1b. Little information exists on the *in vitro* transport activity of Mdr1a. However, *in vivo* studies in Mdr1a KO mice suggest that fexofenadine is a substrate of the efflux transporter (Cvetkovic *et al.*, 1999; Tahara *et al.*, 2005). Again, the effect of fruit juices on Mdr1a function is unknown.

1.7.3 Other Apical Efflux Transporters

Other apically-localized intestinal efflux transporters exist and their roles in FJDIs should be investigated. One notable example is the intestinal brush boarder solute carrier, multidrug resistance-associated protein 2 (MRP2) (Fromm *et al.*, 2000). Fexofenadine is a substrate of this solute carrier *in vitro* (Ming *et al.*, 2011). Moreover, much like P-gp, GFJ has been shown to inhibit MRP2 transport activity in cellular models (Honda *et al.*, 2004). Finally, components of GFJ and AJ have also been shown to inhibit the transport activity of another apical efflux transporter, breast cancer resistance protein (BCRP), *in vitro* (Fleisher *et al.*, 2015). However, fexofenadine does not appear to be a good substrate of BCRP as no significant difference in fexofenadine pharmacokinetics was observed in individuals with a BCRP functional polymorphism (Akamine *et al.*, 2010).

Significant levels of mouse MRP2 (mMrp2) mRNA transcript along the small intestine have been reported (Fu *et al.*, 2016). The efflux transporter is also expressed in the bile canalicular membrane of hepatocytes (Kikuchi *et al.*, 2002). Therefore, MRP2 is considered a key transporter mediating the excretion of xenobiotics into bile and subsequent elimination in feces. Although its role in the intestinal absorption of fexofenadine has not been elucidated, the antihistamine appears to be an *in vivo* substrate of the transporter based on altered biliary excretion of the drug in mMrp2 KO mice (Tian *et al.*, 2008). Mouse BCRP (mBcrp) has been confirmed to the apical membrane of gut epithelial cells (Shimizu *et al.*, 2011). Similar to the human ortholog, fexofenadine does not appear to be a substrate of mBcrp *in vivo* (Tian *et al.*, 2008). The impact of fruit juices on the transport activities of mMrp2 and mBcrp is not known.

1.8 Intestinal Basolateral Efflux Transporters

1.8.1 Potential Mechanistic Role in FJDI

Fruit juice inhibition of efflux transporters found on the basolateral membrane of intestinal epithelial cells could also lead to decreased drug exposure (**Figure 1.3**). These transporters are responsible for moving intracellular compounds across the basolateral membrane of enterocytes, into circulation. Inhibition of efflux transporters would prevent this final step in intestinal absorption.

1.8.2 Organic Solute Transporters Alpha and Beta (OSTα/β)

Organic solute transporters alpha and beta (OSTα/β) form a heteromeric efflux structure located in the basolateral membranes of human hepatocytes, kidney proximal tubule cells and importantly, enterocytes (Ballatori *et al.*, 2005). The primary endogenous substrates of OSTα/β are bile acids, which require this transporter for efficient enterohepatic recirculation (Seward *et al.*, 2003). Following cellular influx, typically accomplished by apically expressed uptake transporters such as ASBT, OSTα/β mediates absorption of bile acids by moving intracellular compounds across the basolateral membrane of enterocytes (Seward *et al.*, 2003). OSTα/β is a bidirectional transporter that facilitates movement of its substrates down their concentration gradients (Ballatori *et al.*, 2005). Due to their important role in intestinal bile acid reabsorption, it remains conceivable that OSTα/β is involved in fexofenadine absorption. Therefore, if fexofenadine is a substrate, the effect of fruit juices on OSTα/β function should be investigated.

1.8.2.1 Mouse Orthologs of OSTα/β

The mouse orthologs of OSTα and OSTβ (mOstα and mOstβ) are located in the basolateral membrane of mouse small bowel epithelia (Ballatori *et al.*, 2005). Again, fexofenadine transport and fruit juice effect on mOstα/β have never been examined.

1.8.3 Other Basolateral Efflux Transporters

MRP3 is another efflux transporter expressed in the basolateral membrane of gut epithelial cells. The mRNA transcripts of this carrier molecule and its mouse ortholog, mMrp3, are readily detected along the small intestine (Taipalensuu *et al.*, 2001; Rost *et al.*, 2002). Fexofenadine is a substrate of both MRP3 and mMrp3, as determined by *in vitro* and *in vivo* studies, respectively (Matsushima *et al.*, 2008; Ming *et al.*, 2011). Currently, the effect of fruit juices on MRP3 and mMrp3 activities is not known.

1.9 Grapefruit Juice and Apple Juice Components That May Be Responsible for FJDIs

In the original report by Dresser and colleagues (2002), AJ co-administration produced a slightly greater reduction in fexofenadine plasma concentration than GFJ. Similarly for the minimally-metabolized renin inhibitor drug, aliskiren, AJ co-ingestion led to a more significant drop in oral bioavailability than with GFJ (Tapaninen *et al.*, 2010; Tapaninen *et al.*, 2011). Differences in the magnitudes of effect among fruit juices on drug absorption may relate to the concentrations of juice components and their interactions with gut drug transporters. Flavonoids are plant secondary metabolites that have been identified as the probable fruit juice constituents responsible for pharmacokinetic drug interactions that involve non-metabolized medications (Bailey *et al.*, 2007; Shirasaka *et al.*, 2013a). These phytochemicals provide pigmentation, protect against ultraviolet

damage, participate in growth regulation and act as chemical defenses against plant pathogens (Yao *et al.*, 2004; Petrussa *et al.*, 2013). As components of our diet, flavonoids are considered natural antioxidants with protective roles in cancer, cardiovascular disease, metabolic disorders and allergies (Yao *et al.*, 2004).

With over 5000 identified flavonoids, the polyphenolic compounds are characterized by their 15 carbon backbone (C_6-C_3-C_6) (Yao *et al.*, 2004) (**Figure 1.4**). They can be further subdivided into the pertinent classes flavanone, flavonol and dihydrochalcone. Naringenin and its glycosylated forms naringin (naringenin 7-*O*-neohesperidoside) and narirutin (naringenin 7-*O*-rutinoside) and hesperitin and its glycosylated forms hesperidin (hesperetin 7-*O*-rutinoside) and neohesperidin (hesperetin 7-*O*-neohesperidoside) are common flavanones found in citrus fruits (Ross *et al.*, 2000; Yao *et al.*, 2004). The flavonols quercetin and kaempferol and the dihydrochalcones phloretin and its glycosylated form phloridzin (phloretin-2'-O-glucoside) are found in onions, broccoli and apples (Yao *et al.*, 2004; Shao *et al.*, 2008).

Concentrations of flavonoids are variable between different fruit juices and brands. For example, naringin, narirutin and hesperidin are highly concentrated in GFJ but not AJ, while phloridzin, hesperitin and quercetin are readily detected in AJ but not GFJ (Ross *et al.*, 2000; Shirasaka *et al.*, 2013a). Between juice brands, flavonoid concentrations are affected by the source of the fruit and the techniques used to process and prepare the juice (Yao *et al.*, 2004). The predominant flavonoids are found at micromolar to millimolar concentrations in fruit juices (Zhang, 2007).

There have been two fexofenadine pharmacokinetic studies in humans involving co-administered flavonoids. In one study, relevant doses of naringin caused a reduction in fexofenadine oral absorption with a magnitude of effect about half that found for GFJ (Bailey *et al.*, 2007). This finding suggests an important role for naringin in the fexofenadine-GFJ interaction. In the other study, when subjects were co-treated with quercetin (500 mg), surprisingly they experienced greater plasma fexofenadine concentrations suggesting that this flavonol does not play a decisive role in the fexofenadine-AJ interaction (Kim *et al.*, 2009).

In vitro, GFJ, AJ and the specific flavonoids naringin and hesperidin have inhibitory effects on OATP1A2 activity (Dresser *et al.*, 2002; Bailey *et al.*, 2007). In OATP2B1-expressing cells in culture, transport activity was inhibited by AJ and GFJ (Shirasaka *et al.*, 2013b). Additionally, the flavonoids naringin, naringenin, hesperidin, hesperetin, phloridzin, phloretin, quercetin and kaempferol all demonstrated inhibitory effects on *in vitro* OATP2B1 transport activity (Shirasaka *et al.*, 2013a). For OCT1 mediated transport, AJ flavonoids quercetin and phloridzin but not the GFJ flavonoid naringin attenuated activity *in vitro* (Mandery *et al.*, 2012; Mimura *et al.*, 2015). Overall, variable flavonoid profiles may explain the *in vitro* discrepancies between AJ- and GFJ-drug interactions and suggest that their affects on the oral absorption of drugs may differ.

It should be noted that furanocoumarins, a class of phytochemicals found distinctly in GFJ, are thought to be responsible for drug metabolizing enzyme-mediated FJDIs (Guo and Yamazoe, 2004). However, major furanocoumarins bergamottin and its metabolite DHB do not elicit inhibitory effects on OATP mediated transport *in vitro* (Bailey *et al.*, 2007; Shirasaka *et al.*, 2013a).

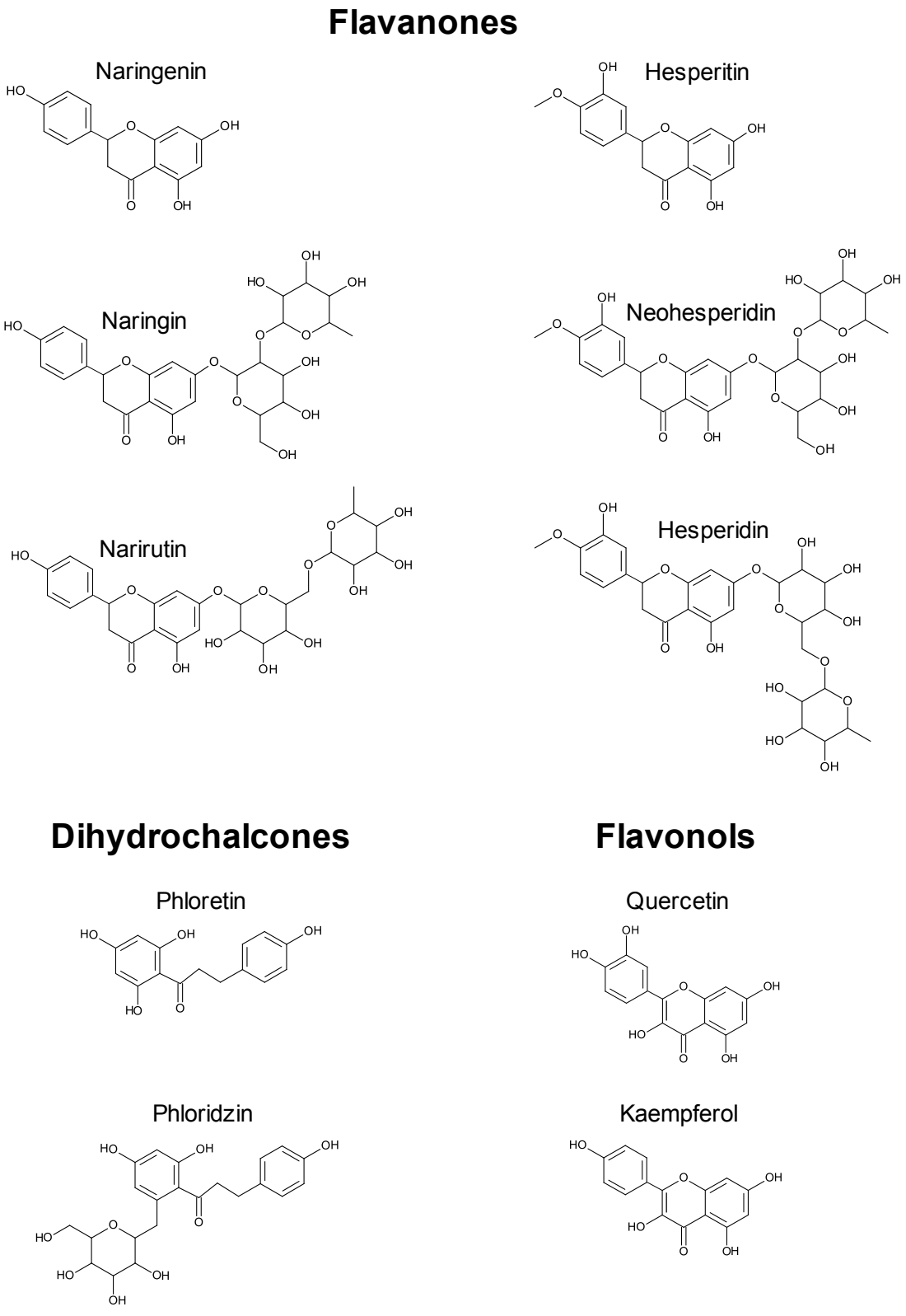


Figure 1.4 Common flavonoids found in grapefruit juice and apple juice.

1.10 Preclinical Fruit Juice-Fexofenadine Studies

Animal models may provide additional insights to elucidate the *in vivo* mechanisms of FJDIs. Fexofenadine pharmacokinetics have been reported in rodent, porcine and primate models (Cvetkovic *et al.*, 1999; Tahara *et al.*, 2005; Petri *et al.*, 2006; Ogasawara *et al.*, 2007; van de Steeg *et al.*, 2010). In rats and monkeys, co-administration with P-gp inhibitors including cyclosporine A and ketoconazole, increased the bioavailability of oral fexofenadine, a result consistent with human studies (Ogasawara *et al.*, 2007; Ujie *et al.*, 2008). In P-gp KO mice (Mdr1a$^{-/-}$ and Mdr1a/b$^{-/-}$), there was 4-5 times greater fexofenadine plasma levels than WT mice after oral administration (Cvetkovic *et al.,* 1999; Tahara *et al.,* 2005). In WT mice, oral fexofenadine bioavailability is only 2.38% and much less than that measured in humans (33%) (Tahara *et al.*, 2005; Smith and Gums, 2009). These findings suggest that the expression and activity of mouse orthologs of P-gp play more substantial roles in limiting intestinal fexofenadine absorption than human P-gp. As mentioned previously, there are 4 mouse orthologs of human OATP1A2, namely mOatp1a1, mOatp1a4, mOatp1a5 and mOatp1a6 (van de Steeg et al. 2010). Another difference exists for liver-specific human OATP1B1 and OATP1B3 transporters which have a single mouse ortholog, mOatp1b2. These species differences have prompted the development of an Oatp1a/1b cluster KO mouse model which is deficient in all mOatp1a transporters as well as mOatp1b2. Interestingly, intestinal fexofenadine absorption in the Oatp1a/1b cluster KO mice was not significantly different than WT mice (van de Steeg *et al.*, 2010). This result, suggests minimal roles are played by intestinal mouse Oatp1a transporters in oral fexofenadine absorption.

Interestingly, there is only a single reported study investigating the fruit juice-

fexofenadine pharmacokinetic interaction in preclinical species. In 2005, Kamath and colleagues showed in Sprague-Dawley rats that fexofenadine co-ingestion with AJ (28 mL/kg) resulted in a 28% reduction in oral drug exposure. While the direction of effect on fexofenadine bioavailability in rats was consistent with that seen in humans, there was a greater drop in AUC (78% reduction) with comparably less AJ volume (approximately 17mL/kg) in the clinical study (Dresser *et al.*, 2002). Nevertheless, this finding suggests that the rat model can recapitulate the human fruit juice-fexofenadine interaction.

The mouse may be a convenient preclinical model to further study the mechanisms of FJDIs. Surprisingly, this model has never been reported previously as a tool to establish the intestinal drug transporters involved. In this regard, it would be interesting to test whether fruit juice-fexofenadine interactions occur in WT mice and compare effects to mice with genetic deficiencies in intestinal fexofenadine transport proteins. Given that intestinal Mdr1a acts more significantly in mice than humans to limit oral drug absorption, a fruit juice-fexofenadine study in Mdr1a$^{-/-}$ mice may unmask the roles of intestinal uptake transporters in the food-drug effect by eliminating dominant efflux mechanisms. Studies in the Oatp1a/1b cluster KO mouse model have already indicated a limited role for intestinal mOatp1a transporters in the bioavailability of fexofenadine. It is possible that another intestinal mouse Oatp, namely, mOatp2b1, contributes to fexofenadine absorption. Our laboratory has recently developed a novel mOatp2b1 KO (Oatp2b1$^{-/-}$) mouse model. With this mouse model, it may be possible to convincingly demonstrate or rule out an *in vivo* role for mOatp2b1 in fruit juice-fexofenadine interactions.

2 Hypothesis and Specific Aims

2.1 Statement of the Problem and Overall Hypothesis

Canada's Food Guide endorses the consumption of fruit juice as a means to help meet the daily-recommended intake of fruits and vegetables (http://www.hc-sc.gc.ca). Indeed, fruit juices have become a common component of diet. However, in recent years, there has been a growing appreciation that fruit juices interact with drugs in a manner that raises serious concerns for patient safety. Particularly troublesome are fruit juice interactions involving medications metabolized by CYP3A4, that result in marked elevations in plasma drug concentrations and increased risk of drug toxicity. Less appreciated is the fact that non-metabolized medications also interact with fruit juices, but in these cases the outcome is a reduction in plasma drug concentrations and potential loss of efficacy. The mechanisms underlying fruit juice interactions with non-metabolized drugs remain poorly understood. In recent years, attention has been directed towards roles of intestinal drug transporters in this food-drug effect. In this regard, OATP1A2 and OATP2B1, have become the primary suspect mediators of FJDIs, despite that *in vitro* studies are not entirely consistent with the clinical effect. This raises the possibility that there exist other, yet to be discovered transporters involved. Moreover, essentially all support for specific mechanisms of clinical fruit juice interactions with non-metabolized drugs have been obtained from *in vitro* studies. Convincing *in vivo* studies that support proposed mechanisms are currently lacking.

The overall hypothesis is that GFJ and AJ limit the absorption of fexofenadine through their interactions with specific intestinal transporters.

As numerous oral medications on the market today rely on intestinal transport proteins for absorption, individuals taking substrate drugs, which include commonly prescribed

cardiovascular medications, may inadvertently be at risk for reduced drug efficacy due to FJDIs. Understanding the molecular mechanisms behind fruit juice-non-metabolized drug interactions will provide a basis for implementation of safer and more effective pharmacotherapy, reducing the incidence of preventable ADEs and improving therapeutic outcomes. Furthermore, potential fruit juice-drug interactions can be specifically investigated for drugs in development prior to market release, allowing for more rational drug design that minimizes the incidence of suboptimal therapy.

2.2 Specific Aim 1

To determine candidate intestinal transporters and their mouse orthologs involved in the fruit juice-fexofenadine interaction.

While OATP1A2 and OATP2B1 have been proposed as key players of this food-drug effect, the entire spectrum of intestinal transporters involved has not been fully elucidated. Furthermore, current studies have largely focused on apically expressed membrane proteins and have failed to consider the involvement of transporters located on basolateral membrane of intestinal epithelial cells. Due to its physiochemical properties and minimal breakdown by metabolic enzymes, fexofenadine serves as a prototypical probe drug for transporter function. As such, the antihistamine was used throughout this thesis for the study of enzyme independent-FJDIs. Additionally, GFJ and AJ have been demonstrated to elicit both similar and differing *in vivo* effects on oral drug bioavailability and *in vitro* modulation of drug transporter function, possibly due to their distinct flavonoid compositions. Therefore, we sought to identify human intestinal transporters involved in the GFJ and AJ-fexofenadine interaction. Moreover, we aimed to

characterize the transport function of their murine orthologs as a prelude to *in vivo* pharmacokinetic studies in mice.

We hypothesize that in addition to the previously suspected transporters, OATP1A2 and OATP2B1, other intestinal transport proteins involved in the fruit juice-fexofenadine interaction exist.

To test this hypothesis, we performed an *in vitro* screen of a panel of intestinal transporters and their murine orthologs for fexofenadine transport using a cellular expression system. The flavonoid profiles of GFJ and AJ were measured and the effect of each fruit juice on identified human and mouse fexofenadine transport proteins were subsequently evaluated by *in vitro* fexofenadine transport inhibition studies. We expected to find novel fexofenadine transporters that may be involved in the FJDI and that GFJ and AJ would elicit fruit juice-specific effects on transporter activity.

2.3 Specific Aim 2

To establish whether the mouse can serve as an *in vivo* model for the fruit juice-fexofenadine interaction and to investigate the role of mOatp2b1 in this food-drug effect *in vivo*.

While clinical studies have localized the FJDI to the site of absorption and *in vitro* studies have implicated several intestinal transporters in the effect, there remains some disconnect between current findings and a paucity of supportive evidence from preclinical models. Interestingly, there have been no reports to date which have evaluated mice as a preclinical model for FJDIs. Moreover, while many drug transporter knockout mouse models exist, surprisingly they have not yet been used to gain a better

understanding of the *in vivo* roles of proposed transport proteins involved in FJDIs. Therefore, we aimed to first characterize the mouse as an *in vivo* model of FJDIs. With the recent development of a novel Oatp2b1$^{-/-}$ mouse model, the *in vivo* role of mOatp2b1 in FJDIs was evaluated.

We hypothesize that mice will faithfully recapitulate the human fruit juice-fexofenadine interaction. Furthermore, the FJDI will be mediated through inhibition of mOatp2b1 by GFJ *in vivo*.

To test this hypothesis, we conducted fruit juice-fexofenadine pharmacokinetic interaction studies in WT and Oatp2b1$^{-/-}$ mice. We expected that fruit juice co-administration would result in a reduction in fexofenadine oral bioavailability in WT mice. It was also expected that the plasma levels of fexofenadine would be lower in Oatp2b1$^{-/-}$ mice in comparison to WT mice. We also reasoned that a reduction in systemic fexofenadine levels would occur in WT but not in KO mice with fruit juice co-ingestion.

3 Materials and Methods

3.1 Materials

[^{3}H]-Fexofenadine (78Ci/mmol, 99.7% radiochemical purity) was custom synthesized by Quotient Bioresearch (Fordham, Cambridgeshire, UK) by non-specific labeling. Unlabeled fexofenadine, fexofenadine-d6 and phloridzin were purchased from Toronto Research Chemicals (Toronto, ON). Concentrated (4x) GFJ (Minute Maid, 100% Grapefruit Juice Frozen Concentrate) and concentrated (4x) AJ (No Name, Apple Juice, Frozen Concentrate) were acquired from Walmart (London, ON). The same GFJ and AJ stocks were used for all fruit juice experiments. All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO).

3.2 Transporter Expression Plasmids

OATP1A2, OATP2B1, OCT1, OCT2, OCT3, ASBT, PEPT1, HCP1, P-gp and BCRP expression plasmids were generated as previously described (Cvetkovic *et al.*, 1999; Kim *et al.*, 2001; Tirona *et al.*, 2003; Ho *et al.*, 2006; Urquhart *et al.*, 2008; Urquhart *et al.*, 2010; Posada *et al.*, 2015). Constructs for OCTN1, OCTN2, MRP2, OSTα, OSTβ, mOatp1a1, mOatp1a4, mOatp1a6, mOatp2b1, mOct3, mOstα and mOstβ were developed in the Personalized Medicine Lab, London Health Sciences Centre – University Hospital (unpublished data). mOct1 complementary deoxyribonucleic acid (cDNA) was amplified from a mouse small intestine cDNA library (BioChain Institute, Inc.; Newark, CA) using primers 5'-GGGCGCACCATGCCCACGTTCGACCAGGCA-3' (forward) and 5'-ACAGGGGCCTCAGACATCAGAAGTAGAAAC-3' (reverse) by polymerase chain reaction (PCR) with Expand Long Template Polymerase Chain Reaction System (Roche Applied Science; Indianapolis, IN). The resulting amplicon was cloned into pcDNA3.1/V5-His-TOPO expression plasmid (Invitrogen; Burlington, ON).

3.3 Cell Culture

Human cervical adenocarcinoma (HeLa) cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific; Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (Invitrogen), at 37°C, 5% CO_2.

3.4 Transport and Inhibition Studies

3.4.1 Transient Transfection

HeLa cells were grown on 12-well plates (seeding density of 2.5 x 10^5 cells/well) at 37°C, 5% CO_2. After 48 hours, cells were transfected by blank expression plasmids (control) (Invitrogen) or expression plasmids containing transporter cDNA inserts using Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions. In brief, plasmid expression vectors were incubated with Lipofectamine 3000 and P3000 reagents in Opti-MEM, a reduced serum media (Invitrogen), for 15 minutes (min) at room temperature (RT). The DNA-lipid mixture (1 µg DNA/well) was then added to the culture medium. Cells were incubated for 16 hours at 37°C, 5% CO_2 prior to transport experiments.

3.4.2 Transport Study

Transport studies were conducted to identify human and mouse fexofenadine transporters according to methods described previously (Cvetkovic *et al.,* 1999). [^{3}H]-Fexofenadine and the unlabeled drug (0.1 µM) were dissolved in phosphate buffered saline (PBS) (Roche Applied Science) when evaluating OATP2B1 and mOatp2b1 transport or Krebs-

Henseleit buffer (KHB) (1.2 mM $MgSO_4 \cdot 7H_2O$, 0.96 mM KH_2PO_4, 4.83 mM KCl, 118 mM NaCl, 1.53 mM $CaCl_2 \cdot 2H_2O$, 23.8 mM $NaHCO_3$, 12.5 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], 5 mM glucose in water) for all other transporters at pH 6 with the exception of human and mouse OCT and OCTN transporters where pH 7.5 was used. The drug dose (400 µL) was applied to cultured cells for 30 min at 37°C, 5% CO_2. Thereafter, cells were washed three times rapidly with ice-cold PBS. To quantify cellular drug accumulation, cells were lysed using 1 mL of 1% sodium dodecyl sulfate (SDS) in water (v/v) and intracellular radioactivity was measured by liquid scintillation spectrometry (Tri-Carb 3900TR; Perkin Elmer; Waltham, MA). To evaluate OATP2B1- and mOatp2b1- mediated fexofenadine uptake, unlabeled fexofenadine (100 µM) was dissolved in PBS at pH 6 and applied to culture cells for 30 min. Cells were subsequently washed with ice-cold PBS and lysed using 400 µL of acetonitrile spiked with internal standard (fexofenadine-d6, 10 ng/mL). Intracellular fexofenadine was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (described in **Section 3.5**).

3.4.3 Inhibition Study

Inhibition studies were conducted to evaluate the *in vitro* effects of fruit juices on transporter mediated fexofenadine uptake. Concentrated (4x) GFJ and AJ were diluted to normal strength (1x) with KHB. [^{3}H]-Fexofenadine and unlabeled fexofenadine (0.1 µM) were dissolved in 5% normal strength GFJ or AJ in KHB (v/v) and exposed to cultured cells for 30 min. Juice and control dose solutions were all adjusted to pH 6. Under these conditions, 5% GFJ and 5% AJ are sufficient to elicit inhibitory effects on drug transport while avoiding cytotoxicity (Dresser *et al.,* 2002). After incubation with the drug dose,

cells were washed, lysed and intracellular radioactivity was quantified by liquid scintillation spectrometry. To examine the effect of GFJ on OATP2B1 and mOatp2b1 mediated fexofenadine transport, unlabeled fexofenadine (100 μM) was dissolved in 5% GFJ in PBS (v/v) at pH 6 and incubated with the expression systems for 30 min. Cells were washed, lysed and intracellular fexofenadine was quantified by LC-MS/MS.

3.5 Fexofenadine LC-MS/MS Quantification

3.5.1 Sample Preparation

Cell lysates (in acetonitrile spiked with internal standard) from transport and inhibition studies were centrifuged for 10 min at 13,500 rpm in a microcentrifuge. An aliquot of the resulting supernatant (40 μL) was diluted with 160 μL of 0.1% formic acid in water (v/v). Standard curve samples were created by adding increasing concentrations of fexofenadine diluted in water which were subsequently processed in a similar fashion as cell lysate samples.

Plasma samples (5 μL) were spiked with internal standard (fexofenadine-d6, 5 μL, 200 ng/mL), precipitated with acetonitrile (15 μL) and centrifuged for 10 min at 13,500 rpm. The resulting supernatant (20 μL) was diluted with 125 μL of 0.1% formic acid in water (v/v). Standard curve samples were created by spiking fexofenadine into blank human ethylenediaminetetraacetic acid（EDTA) plasma (Bioreclamation IVT; Baltimore, MD) for subsequent processing similar to plasma samples.

3.5.2 LC-MS/MS Conditions

Analytes were separated by liquid chromatography (Agilent 1200; Agilent; San Clara, CA) using Hypersil Gold reversed-phase column (50 × 3 mm, 5 μm particle size; Thermo

Fisher Scientific) following sample injection (50 µL for cell lysate samples and 75 µL for plasma samples). A mobile phase of 0.1% v/v formic acid in water (A) and acetonitrile (B) was used, with an elution gradient of 20% B from 0-1 min, 20-95% B from 1.0-4.5 min, 95% B from 4.50-5.25 min, 95-20% B from 5.25-5.80 min and 20% B from 5.8-6.0 min, for a run time of 6 min and flow rate of 0.5 mL/min. The heated electrospray ionization source of the triple quadrupole mass spectrometer (Thermo TSQ Vantage; Thermo Fisher Scientific) was operated in positive mode (3500 V, 350^{o}C) with collision energy set at 25 V. Additional ionization source conditions used were as follows: 40 arbitrary units for sheath gas pressure, 15 arbitrary units for auxiliary gas pressure and 350°C for capillary temperature. Selected reaction monitoring for fexofenadine and fexofenadine-d6 was performed using mass transitions 502.2 → 466.5 m/z and 508.2 → 472.5 m/z, respectively. Both solutes had chromatographic retention time of 3.2 min. Standard curves were linear over the concentration ranges of 2.5-100 ng/mL for cell lysates and 2-100 ng/mL for plasma samples.

3.6 Flavonoid LC-MS/MS Quantification

3.6.1 Sample Preparation

Concentrated (4x) GFJ and AJ (100 µL) and plasma samples (50 µL) were precipitated 1:3 (v/v) with 50% acetonitrile in methanol (v/v) and centrifuged for 10 min at 13,500 rpm. The resulting supernatant (100 µL from fruit juices and 50 µL from plasma) was diluted 1:2 (v/v) in 0.1% formic acid in water (v/v). Flavonoid standard curves were created for juice and plasma in 0.1% formic acid in water (v/v) and blank control plasma (Bioreclamation IVT), respectively.

3.6.2 LC-MS/MS Conditions

Naringin, narirutin, hesperidin, neohesperidin, phloretin, quercetin and kaempferol concentrations were analyzed by ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), consisting of an Agilent 1290 liquid chromatography system (Agilent) and Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific). Analytes were separated on a Kinetex reversed-phase column (50 × 3 mm, 5 μm particle size; Phenomenex, Torrence, CA) following sample injection (5 μL). A mobile phase of 0.1% v/v formic acid in water (A) and 50% v/v acetonitrile in methanol (B) was used, with an elution gradient of 25% B from 0.0-1.5 min, 25-95% B from 1.5-7.0 min, 95-25% B from 7-9 min and 25% B from 9.0-9.5 min, for a run time of 9.5 min and flow rate of 0.5 mL/min. Mass spectrometer ionization conditions used were as follows: 15 arbitrary units for sheath gas pressure, 5 arbitrary units for auxiliary gas pressure and 300°C for capillary temperature. Naringin, narirutin, hesperidin, neohesperidin, phloretin, quercetin and kaempferol were subjected to collision energies of 17, 17, 19, 19, 17, 28 and 31 V, respectively for detection in positive mode using mass transitions 581.2 → 273.0 m/z, 581.2 → 273.0 m/z, 611.2 → 303.0 m/z, 611.2 → 303.0 m/z, 275.1 → 107.1 m/z, 303.1 → 229.1 m/z and 287.1 → 153.1 m/z, respectively with solute retention times of 1.95, 1.65, 2.10, 2.42, 2.61, 2.42 and 3.31 min, respectively.

Naringenin, hesperetin and phloridzin concentrations were analyzed separately by LC-MS/MS instrumentation and source conditions as described in **Section 3.5.2**. A mobile phase of 0.1% v/v formic acid in water (A) and 50% v/v acetonitrile in methanol (B) was used, with an elution gradient of 25% B from 0.0-1.5 min, 25-95% B from 1.5-6.0 min, 95% B from 6.0-7.0 min, 95-25% B from 7.0-8.0 min and 25% B from 8.0-8.5 min, for a

run time of 8.5 min and flow rate of 0.5 mL/min. Naringenin, hesperetin and phloridzin were subjected to collision energies of 21, 27 and 18 V and detected in negative mode using mass transitions 272.2 → 151.0 m/z, 301.1 → 164.1 m/z and 435.2 → 273.2 m/z with chromatographic retention times of 4.40, 4.53 and 3.64 min, respectively

Standard curves for flavonoids in juice were linear over the ranges of 60-300, 40-200, 10-50, 20-100, 0.02-0.10, 0.6-3.0, 0.02-0.10, 0.01-0.1, 0.2-1.0 and 2-10 µg/mL for naringin, narirutin, hesperidin, neohesperidin, phloretin, quercetin, kaempferol, naringenin, hesperetin and phloridzin, respectively. The lower limits of quantification in plasma were 6, 4, 1, 2, 0.006, 0.06, 0.006, 0.004, 0.02 and 0.2 µg/mL for naringin, narirutin, hesperidin, neohesperidin, phloretin, quercetin, kaempferol, naringenin, hesperetin and phloridzin, respectively.

3.7 Animals

Adult, male mice were used in all experiments to limit age- and sex-dependent variation in intestinal drug transporter expression (Cheng *et al.*, 2005). Oatp2b1$^{-/-}$ mice (*Slco2b1*$^{tm1a(KOMP)Wtsi}$) were generated in collaboration with the Knockout Mouse Project at UC Davis (manuscript in preparation). In this mouse model, a gene trap cassette was inserted into the *Slco2b1* gene in Intron 3 of C57BL/6J mice by homologous recombination in embryonic stem cells to produce global knockdown of Oatp2b1 gene expression. Preliminary investigations indicate that Oatp2b1$^{-/-}$ mice are viable, fertile and produce litters of normal size. Serum biochemistry and histological analysis of Oatp2b1$^{-/-}$ mice are unremarkable. C57BL/6 mice (WT background strain of Oatp2b1$^{-/-}$ mice) were obtained from Jackson Laboratories (Bar Harbor, MA). Mdr1a$^{-/-}$ mice (Crl/CF1-

Abcb1a^{mds}) and its corresponding WT strain, CF-1 mice, were purchased from Charles River Laboratories, Inc. (Wilmington, MA). C57BL/6 and Oatp2b1$^{-/-}$ mice were maintained in a barrier, sterilized, temperature controlled, animal facility, following a 12-hour light/dark cycle. CF-1 and Mdr1a$^{-/-}$ mice were housed in a conventional, temperature controlled, animal facility, following a 12-hour light/dark cycle. Standard chow diet and water were available to all mice *ad libitum*.

3.8 Mouse Pharmacokinetic Study

To determine the *in vivo* effect of fruit juices on fexofenadine exposure, pharmacokinetic studies were conducted in adult, 10-19 week old C57BL/6, Oatp2b1$^{-/-}$, CF-1 and Mdr1a$^{-/-}$ mice. Animals were fasted for 4 hours prior to drug administration. Mice were administered fexofenadine (1 mg/kg) dissolved in 200 µL of PBS (pH equivalent to GFJ and AJ), 4x concentrated GFJ or 4x concentrated AJ, by oral gavage. Blood (30 µL) was sampled from the saphenous vein at 5, 15, 30 min and 1, 2 and 3 hours post drug administration and stored in heparinized microcentrifuge tubes. Following the 1-hour blood sampling, subcutaneous saline (1 mL) was administered as fluid replacement. Mice were euthanized by isoflurane at the 3 hours and a final blood sample (500 µL) was collected by cardiac puncture. Blood samples were centrifuged at 5000g for 10 min, 4^{o}C. Plasma (200 µL from final time point, 10 µL from all other time points) was obtained and stored at -80^{o}C until fexofenadine and flavonoid levels were measured by LC-MS/MS. This animal study protocol (No. 2014-012) was approved by Western University's Animal Use Subcommittee (London, ON).

3.9 Pharmacokinetic Analysis

Plasma fexofenadine concentration-time curves were generated. Area under the plasma concentration-time curve from 0 to 3 hours (AUC_{0-3}) was calculated by the linear trapezoidal method. Peak plasma concentration (C_{max}) and time to reach peak plasma concentration (T_{max}) were determined directly from the data obtained.

3.10 Mouse Intestinal Permeability Study

To explore the possibility of intestinal mucosa damage by GFJ, an intestinal permeability test was performed in adult, 10 week old WT (CF-1) and $Mdr1a^{-/-}$ mice. Following a protocol described by Johnson *et al.* (2015), mice were fasted for 6 hours then administered fluorescein isothiocyanate labelled dextran (FITC-dextran, 4 kDa), at 600 mg/kg dissolved in either 200 μL of PBS or 4x concentrated GFJ by oral gavage. At 1-hour post administration, 130 μL of blood was collected from the saphenous vein and centrifuged at 12,000 g for 3 min. Plasma was collected and diluted 1:2 (v/v) in PBS. FITC-dextran concentration in plasma, an indicator of paracellular absorption, was measured by fluorescence spectroscopy (excitation at 485nm, emission at 535nm) on a Luminoskan Ascent instrument (Thermo Fisher Scientific). FITC-dextran doses, blood and plasma samples were protected from light exposure. Mice were returned to the conventional animal facility for recovery following the experiment.

3.11 Statistics

Unpaired, two-tailed, Student's t-test or one-way or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test were used to determine statistical

differences between groups. Statistical significant was deemed at a *P* value of <0.05. All statistical analysis was conducted using GraphPad Prism Version 6.0f (La Jolla, CA).

4 Results

4.1 Fexofenadine is a Substrate of Multiple Intestinal Transporters and their Mouse Orthologs

Clinical studies have suggested that fruit juices limit intestinal fexofenadine absorption, a process mediated by enterocyte uptake and efflux transporters (Banfield *et al.*, 2002; Dresser *et al.*, 2002; Glaeser *et al.*, 2007). While the antihistamine is a well described *in vitro* substrate of OATP1A2, OATP2B1 and P-gp, (Cvetkovic *et al.*, 1999; Dresser *et al.*, 2002; Nozawa *et al.*, 2004), the entire complement of intestinal transport proteins involved in its absorption has not been fully elucidated. Additionally, there have been no reports demonstrating transport by mouse orthologs of the human fexofenadine transporters. Species differences in drug transporter substrate specificity may impact the use of mouse as a relevant model to understand mechanisms of FJDIs. Therefore, we conducted transport experiments using a HeLa cell expression system to identify additional candidate *in vivo* human and mouse fexofenadine transporters.

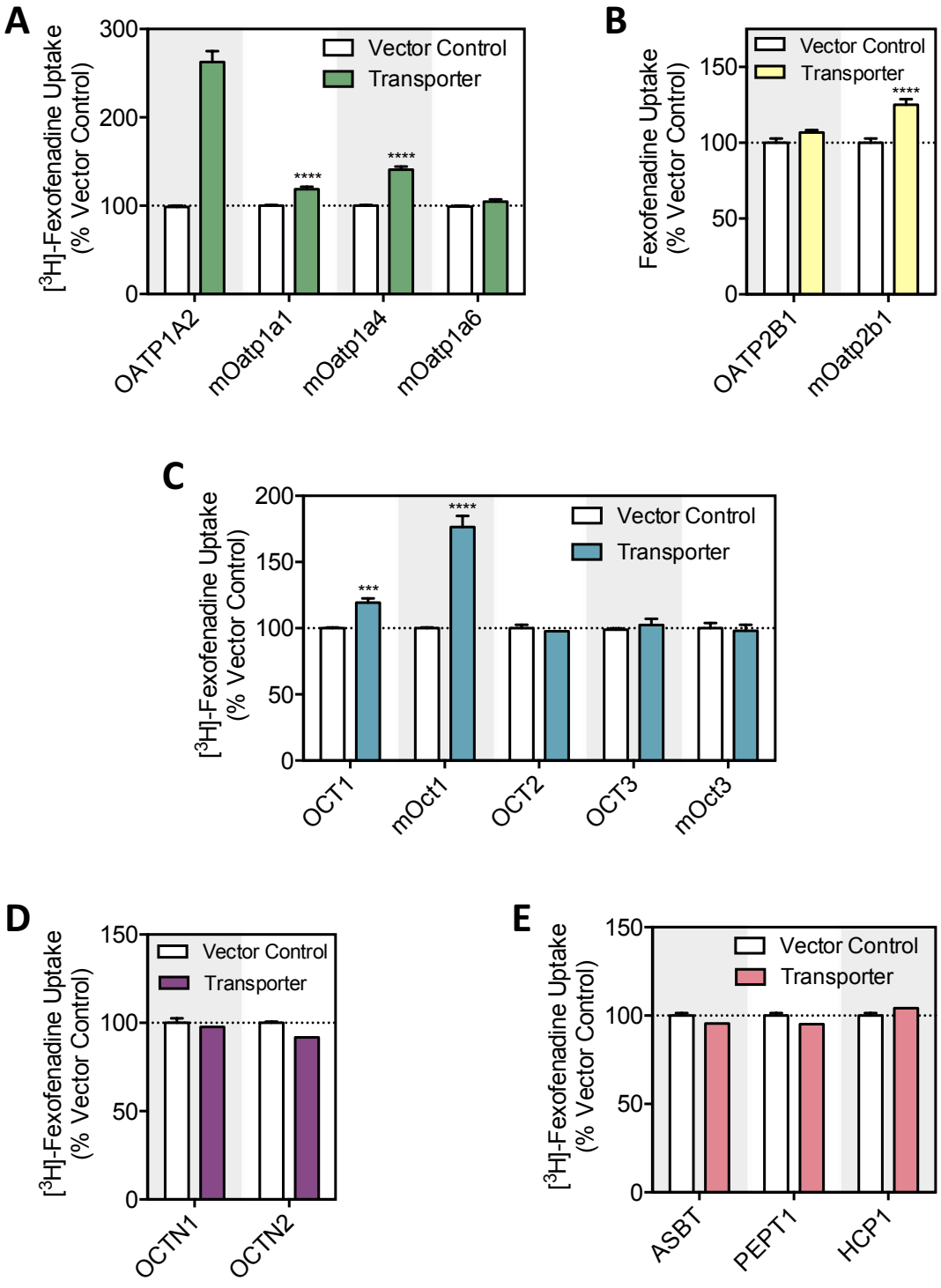
4.1.1 Identification of Fexofenadine Uptake Transporters

We performed transient transfection of a variety of intestinal transporters in cultured HeLa cells to screen for novel fexofenadine transporters. Transfection and functional heterologous expression were validated for mOatp1a1, mOatp1a4, mOatp1a6, OATP2B1, mOatp2b1 and mOct1 by demonstrating transport of known substrates and plasma membrane localization by immunofluorescence microscopy (for mOatp2b1 only) (**Appendix A: Transfection Validation, Supplementary Figures 1 and 2**). For uptake membrane proteins, the degree of intracellular fexofenadine accumulation after 30 min drug exposure was used as a measure of transport activity. Fexofenadine uptake was then reported as a percent of uptake by blank vector control transfected cells. While additional

replicates were required for statistical analysis (n = 2), fexofenadine uptake by cells expressing OATP1A2 was 2.6-fold higher than control cells transfected with blank vector control plasmid, in accordance with previous reports (Dresser *et al.*, 2002). Likewise, the mouse orthologs, mOatp1a1 and mOatp1a4 significantly transported fexofenadine, albeit to a lesser efficiency than OATP1A2 (19% and 41% greater than control, respectively, $P<0.0001$). However, mOatp1a6 exhibited negligible drug uptake, suggesting substrate specificity differences between mouse Oatp1a transporters (**Figure 4.1 A**). Although OATP2B1 has previously been described as a fexofenadine transport protein, in our *in vitro* expression system, the antihistamine did not appear to be a substrate of the human transporter as intracellular drug levels were comparable between OATP2B1 expressing cells and control cells (**Figure 4.1 B**) (Nozawa *et al.*, 2004; Imanaga *et al.*, 2011; Shirasaka *et al.*, 2011; Shirasaka *et al.*, 2013b; Akamine *et al.*, 2014; Akamine, 2015). On the other hand, uptake of the antihistamine by the mouse ortholog, mOatp2b1, was 22% higher than control ($P<0.0001$) (**Figure 4.1 B)**. Interestingly, fexofenadine was a substrate of both OCT1 and mOct1, as they showed significantly greater cellular drug accumulation when compared to their respective controls (19% and 76% higher than control, $P<0.001$ and $P<0.0001$, respectively). Other human OCT and OCTN family of transporters, as well as ASBT, PEPT1 and HCP1 did not appear to transport the antihistamine. While these findings do not definitively rule out the contribution of these transporters in mediating intestinal fexofenadine absorption, it suggests limited roles played by these membrane proteins (**Figure 4.1 C, D and E)**. Overall, the current findings would suggest that if expressed in the intestinal apical membrane, fexofenadine absorption may be mediated by OATP1A2 and OCT1 in humans and mOatp1a1,

mOatp1a4, mOatp2b1 and mOct1 in mice.

Figure 4.1. Fexofenadine transport by HeLa cells expressing human intestinal uptake transporters and their mouse orthologs. HeLa cells transiently transfected with human or human and mouse orthologs of (**A**) OATP1A2, (**B**) OATP2B1, (**C**) OCTs, (**D**) OCTNs and **(E)** other uptake transporters were evaluated for intracellular accumulation of [^{3}H]-fexofenadine (0.1 μM for A, C, D and E) or unlabeled fexofenadine (100 μM for B) at pH 6 (A, B and E) or pH 7.5 (C and D) following a 30-minute incubation period. Data are expressed as percent of vector control, mean ± SEM, n = 1 for OCTN1 OCTN2, OCT2, ASBT, PEPT1 and HCP1, n = 2 for OATP1A2 and mOct3 and n ≥ 3 for controls and all other transporters. Student's t-test was conducted, where ***P<0.001 and ****P<0.0001 represent significant differences from vector control.


A
[3H]-Fexofenadine Uptake
(% Vector Control)
300
200
100
0
Vector Control
Transporter
****
****
OATP1A2
mOatp1a1
mOatp1a4
mOatp1a6
B
Fexofenadine Uptake
(% Vector Control)
150
100
50
0
Vector Control
Transporter
****
OATP2B1
mOatp2b1
C
[3H]-Fexofenadine Uptake
(% Vector Control)
200
150
100
50
0
Vector Control
Transporter
***
****
OCT1
mOct1
OCT2
OCT3
mOct3
D
[3H]-Fexofenadine Uptake
(% Vector Control)
150
100
50
0
Vector Control
Transporter
OCTN1
OCTN2
E
[3H]-Fexofenadine Uptake
(% Vector Control)
150
100
50
0
Vector Control
Transporter
ASBT
PEPT1
HCP1

4.1.2 Identification of a Novel Fexofenadine Basolateral Efflux Transporter

OSTα/β is a bidirectional transporter that facilitates substrate movement down their chemical gradient and is implicated in basolateral bile acid efflux by enterocytes (Ballatori *et al.*, 2005). *In vitro*, exposing OSTα/β expressing cells to extracellular fexofenadine prompts cellular uptake of the drug. Interestingly, intracellular fexofenadine retention was 45% ($P<0.0001$) higher than vector control in OSTα/β transfected cells (**Figure 4.2**). Analogous to its human ortholog, the antihistamine appeared to be a substrate of mOstα/β. However, strong conclusions cannot be drawn from this result and no statistical analysis was performed as mOstα/β transport experiments were conducted on two occasions (n = 2) with technical replicates. Overall, it would appear that fexofenadine is a convincing, novel *in vitro* substrate of OSTα/β and probable, novel *in vitro* substrate of mOstα/β, suggesting that these transporters may play a role in the intestinal absorption of fexofenadine.

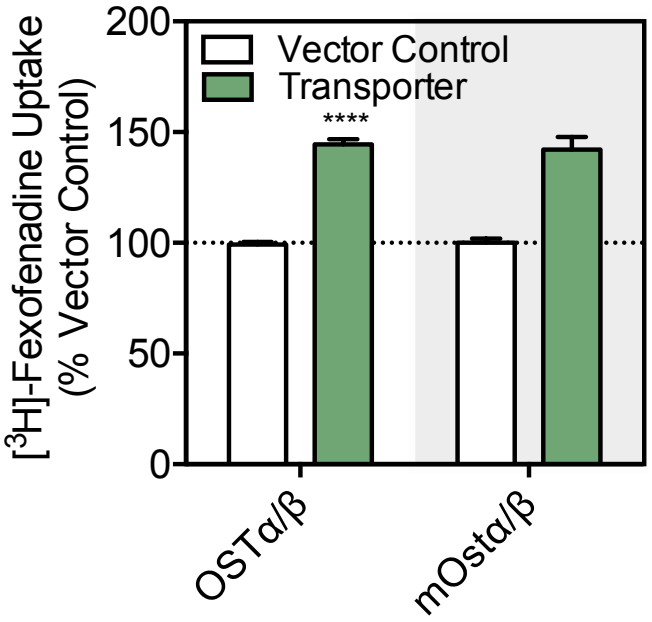


Figure 4.2 Fexofenadine transport by HeLa cells expressing human intestinal basolateral efflux transporter and its mouse ortholog. HeLa cells transiently transfected with basolaterally localized OSTα/β and mOstα/β were evaluated for intracellular accumulation of [^{3}H]-fexofenadine (0.1 μM) at pH 6 following a 30-minute incubation period. Data are expressed as percent of vector control, mean ± SEM (n = 2 for mOstα/β and n ≥ 4 for controls and OSTα/β). Student's t-test was conducted, where ****P<0.0001 represent a significant difference from vector control.

4.2 Flavonoid Profiles of GFJ and AJ

Flavonoids have been proposed as the fruit juice components responsible for causing transporter dependent FJDIs. GFJ and AJ possess different flavonoid compositions that may explain the greater reduction in oral drug bioavailability observed with AJ co-administration when compared to GFJ (Dresser *et al.,* 2002; Tapaninen *et al.,* 2010; Tapaninen *et al.,* 2011). Furthermore, individual flavonoids have been shown to elicit unique *in vivo* effects on fexofenadine exposure and *in vitro* effects on transporter activity (Bailey *et al.*, 2007; Kim *et al.*, 2009; Mandery *et al.*, 2012; Shirasaka *et al.*, 2013b; Mimura *et al.*, 2015). To ensure that the fruit juices used in our experiments contained the expected chemical components and in consideration of fruit juice-specific effects, the concentrations of major flavonoids in 4x concentrated GFJ and AJ were measured by LC-MS/MS.

Similar to what has been reported in the literature, naringin, narirutin, neohesperidin and hesperidin were the most concentrated flavonoids in GFJ while phloridzin was the predominant flavonoid in AJ (Shirasaka *et al.*, 2013a). These results highlight the fact that the major flavonoid constituents differ between GFJ and AJ. As expected, naringenin, the aglycone of both naringin and narirutin was not a significant component in GFJ. Although quercetin was expected to be a major flavonoid in AJ, greater levels of this fruit juice component was detected in GFJ (Yao *et al.*, 2004; Shirasaka *et al.*, 2013a; Mimura *et al.*, 2015). Furthermore, we expected prominent levels of hesperitin in AJ, however, its glycosylated forms hesperidin and neohesperidin were found at significantly greater concentrations (Shirasaka *et al.*, 2013a). In general, flavonoid concentrations in the 4x concentrated GFJ and AJ were higher than those reported in the literature for

normal strength fruit juices (**Table 4.1**). Together, these findings indicated that the GFJ and AJ used in our experiments contained the proposed flavonoid mediators of fruit juice interactions at high concentrations. Furthermore, their unique flavonoid profiles may explain differences in the *in vitro* and *in vivo* fruit juice specific effects on drug transport.

Table 4.1 Flavonoid concentrations in 4x concentrated grapefruit juice (GFJ) and 4x concentrated apple juice (AJ) used in experiments and literature reports.

Flavonoid	Concentration (μM)			
	GFJ (n = 6)	GFJ in Literature	AJ (n = 7)	AJ in Literature
Naringin	2540 ± 80	140 - 1640	Not detectable	0 - 0.001
Narirutin	874 ± 41	45 - 210	Not detectable	0
Naringenin	0.44 ± 0.03	0 - 450	Not detectable	0.001
Hesperidin	99.0 ± 5.0	25 - 50	3.4 ± 0.2	0 - 0.2
Neohesperidin	412 ± 18	5 - 20	2.0 ± 0.5	0
Hesperitin	0.28 ± 0.01	0.6	0.13 ± 0.03	1.5
Quercetin	24.8 ± 1.4	0.005 - 30	0.2 ± 0.06	0.5-3
Kaempferol	5.80 ± 0.24	0.02	0.02 ± 0.003	0-0.7
Phloridzin	1.1 ± 0.2	0.005	106 ± 5.2	17-100
Phloretin	Not detectable	0.001	1.56 ± 0.05	0.2

Data are presented as mean ± SEM. Literature values are presented for 1x concentrated GFJ and AJ and obtained from Gliszczynska-Swiglo and Tyrakowska (2003), Mullen *et al.* (2007), Zhang (2007), Godycki-Cwirko *et al.* (2010) and Shirasaka *et al.* (2013a).

4.3 Effects of GFJ and AJ on Human and Mouse Fexofenadine Transporters

Having identified candidate human intestinal fexofenadine transporters and their mouse orthologs (**Section 4.1**), we next sought to determine the *in vitro* effects of GFJ and AJ on their transport activities. HeLa cells, transiently expressing transporters were exposed to fexofenadine dissolved in control medium, 5% normal strength GFJ or 5% normal strength AJ and intracellular drug accumulation was measured 30 min after drug administration. Fexofenadine uptake was reported as a percent of uptake relative to blank vector control transfected cells, exposed to control medium.

There was strong fruit juice inhibition of fexofenadine uptake by OATP1A2 expressing cells as intracellular drug levels was reduced by 63% (GFJ) and 54% (AJ) upon juice exposure. This finding agreed with the report by Dresser *et al.* (2002). Similarly, GFJ and AJ significantly inhibited mOatp1a1 mediated fexofenadine cellular accumulation by 18% ($P<0.001$) and 12% ($P<0.05$) respectively. For mOatp1a4, there was a juice specific effect on fexofenadine transport evidenced by an effect of GFJ (28% reduction in drug uptake, $P<0.0001$) but not AJ on cellular drug accumulation (**Figure 4.3 A**).

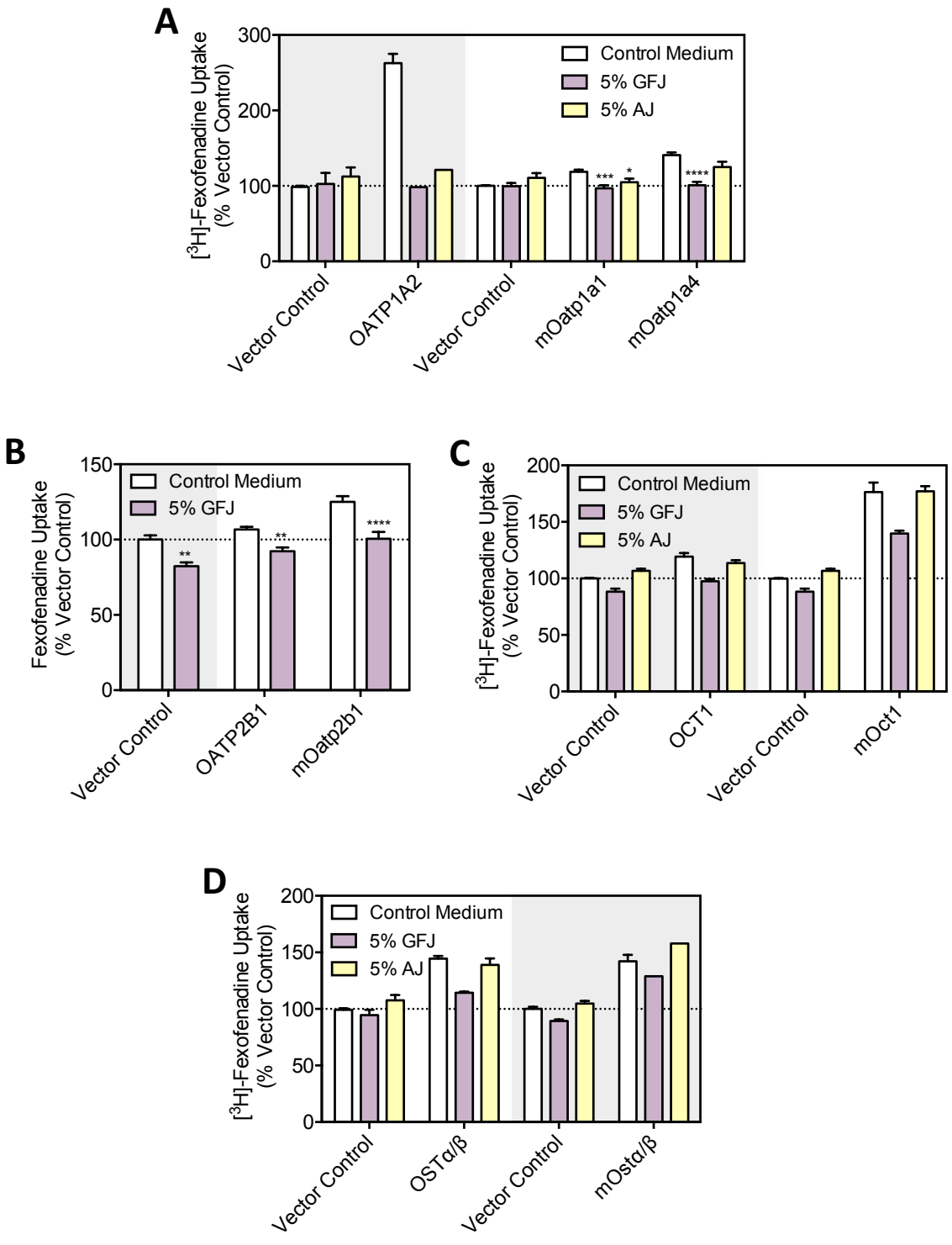
In experiments with OATP2B1, we found that GFJ decreased fexofenadine cellular retention in both vector control and OATP2B1 expressing cells. Again, we did not observe OATP2B1-specific transport of fexofenadine. Uptake difference (difference in the intracellular accumulation of fexofenadine between cells exposed to control medium and GFJ treatment within the same expression system) was not significantly different between vector control and OATP2B1 transfected cells. Together, these findings suggest that the effect of GFJ on the two expression systems is caused by GFJ inhibition of

endogenous, HeLa cell, fexofenadine transporters and not a result of fruit juice inhibition of transfected OATP2B1. In contrast to the human transporter, we found that transfection of mOatp2b1 stimulated fexofenadine cellular accumulation. GFJ also reduced drug uptake into mOatp2b1 expressing cells ($P<0.0001$). Interestingly, the uptake difference calculated from the mOatp2b1 expression system was significantly greater than vector control ($P<0.05$). These findings imply that in addition to endogenous transporters, components of GFJ inhibit fexofenadine uptake by mOatp2b1 (**Figure 4.3 B**).

For organic cation transporters, it would appear that GFJ but not AJ attenuated influx of the antihistamine mediated by OCT1 (18% reduction) and mOct1 (20% reduction) (**Figure 4.3 C**). With OSTα/β, GFJ inhibited fexofenadine transport activity by 21%, but interestingly AJ had no effect on OSTα/β-mediated drug accumulation (**Figure 4.3 D**). Additional experimental replications are required to confirm these results. With mOstα/β, neither fruit juice appeared to inhibit the transporter (**Figure 4.3 D**). However, this experiment was performed once and therefore the (lack of) effect of fruit juices on fexofenadine transport by mOstα/β requires further confirmation.

Taken together, these *in vitro* transporter inhibition results implicate the probable involvement of OATP1A2, OCT1 and OSTα/β in the fruit juice-fexofenadine interaction in humans. Furthermore, while GFJ appeared to inhibit all three transporters, AJ only inhibited OATP1A2, demonstrating juice-type specific effects. With respect to mouse transporters, the current *in vitro* data predicts that mOatp1a1, mOatp1a4, mOatp2b1 and mOct1 could be potential players in a fruit juice-fexofenadine interaction if these proteins are indeed expressed in the mouse enterocytes.

Figure 4.3. Fruit juice inhibition of fexofenadine transport by HeLa cells expressing human intestinal fexofenadine transporters and their mouse orthologs. HeLa cells transiently transfected with human and mouse orthologs of (**A**) OATP1A2, (**B**) OATP2B1, (**C**) OCTs and (**D)** OSTα/β were evaluated for intracellular accumulation of [^{3}H]-fexofenadine (0.1 μM for A, C and D) or cold fexofenadine (100 μM for B) in control medium, 5% grapefruit juice (GFJ) in vehicle or 5% apple juice (AJ) in vehicle, at pH 6 (A, B and D) or pH 7.5 (C) following a 30-minute incubation period. Data are expressed as percent of vector control cells exposed to control medium, mean ± SEM, n = 1 for GFJ and AJ treated OATP1A2 and mOstα/β, n = 2 for KHB treated mOstα/β, GFJ and AJ treated OCT1, OSTα/β and mOct1 and n ≥ 3 for controls and all other transporters/conditions. One-way ANOVA was conducted where *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 represent significant differences from vehicle treated cells within the same expression system.


A
[3H]-Fexofenadine Uptake
(% Vector Control)
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4.4 Effect of GFJ Co-Administration on Fexofenadine Pharmacokinetics in WT and Oatp2b1$^{-/-}$ Mice

In humans, fexofenadine co-ingestion with GFJ dramatically drops plasma drug exposure (by up to 67%) with minimal changes to $t_{1/2}$ and clearance (Dresser *et al.*, 2002). Lately, there has been much attention focused on intestinal OATP2B1 as the primary transporter mediator of this food-drug effect. Unfortunately, we were unable to demonstrate facilitation of fexofenadine uptake by this transporter *in vitro*. However, we had a unique opportunity to study the *in vivo* role of mOatp2b1 with the recent development of a transporter KO mouse in our laboratory. We have found mOatp2b1-mediated fexofenadine transport, which was inhibited by fruit juices *in vitro.* Furthermore, mRNA expression of the transporter in the mouse small intestine is well documented (Cheng *et al.*, 2005; Fu *et al.*, 2016) (**Appendix C: Expression of Fexofenadine Transporters in Mouse Small Intestine, Supplementary Figure 4**). Therefore, we investigated the *in vivo* role of mOatp2b1 in the fruit juice-fexofenadine interaction using WT (C57BL/6) and Oatp2b1$^{-/-}$ mice. A pharmacokinetic study was conducted whereby fexofenadine (1mg/kg) was co-administered with 200 µL (volume equivalent to 500 mL or two glasses of fluid ingestion in humans) of PBS (control) or 4x concentrated GFJ (used to magnify the fruit juice effect) by oral gavage and plasma drug levels were measured at set intervals post drug intake.

Fexofenadine was rapidly absorbed into circulation in both PBS treated WT and Oatp2b1$^{-/-}$ mice, with T_{max} occurring within the first 15-30 min post drug administration, implying early absorption of the drug across the proximal small bowel (**Figure 4.4**). Interestingly, there was a lack of significant differences between T_{max}, C_{max} and AUC_{0-3} between WT

and Oatp2b1$^{-/-}$ mice suggesting a limited role played by mOatp2b1 in fexofenadine disposition (**Table 4.2**). Moreover, in contradiction to clinical findings, GFJ co-administration did not lead to a significant change in fexofenadine disposition in WT mice as T_{max}, C_{max}, and AUC_{0-3} were not statistically different between PBS control and GFJ groups (**Table 4.2**). However, there was a trend toward decreased fexofenadine plasma concentrations at 15 min post drug administration in GFJ treated WT mice in comparison to PBS control treated WT mice ($P = 0.08$) (**Figure 4.4**). In Oatp2b1$^{-/-}$ mice, GFJ co-ingestion did not significantly alter fexofenadine pharmacokinetics (**Table 4.2**). Taken together, these results indicate that mOatp2b1 does not play a significant role in fexofenadine disposition nor is it responsible for GFJ interaction in the mouse *in vivo*. Importantly, the WT (C57BL/6) mouse does not recapitulate the clinical GFJ-fexofenadine pharmacokinetic interaction, indicating that it is an inadequate model of the human condition.

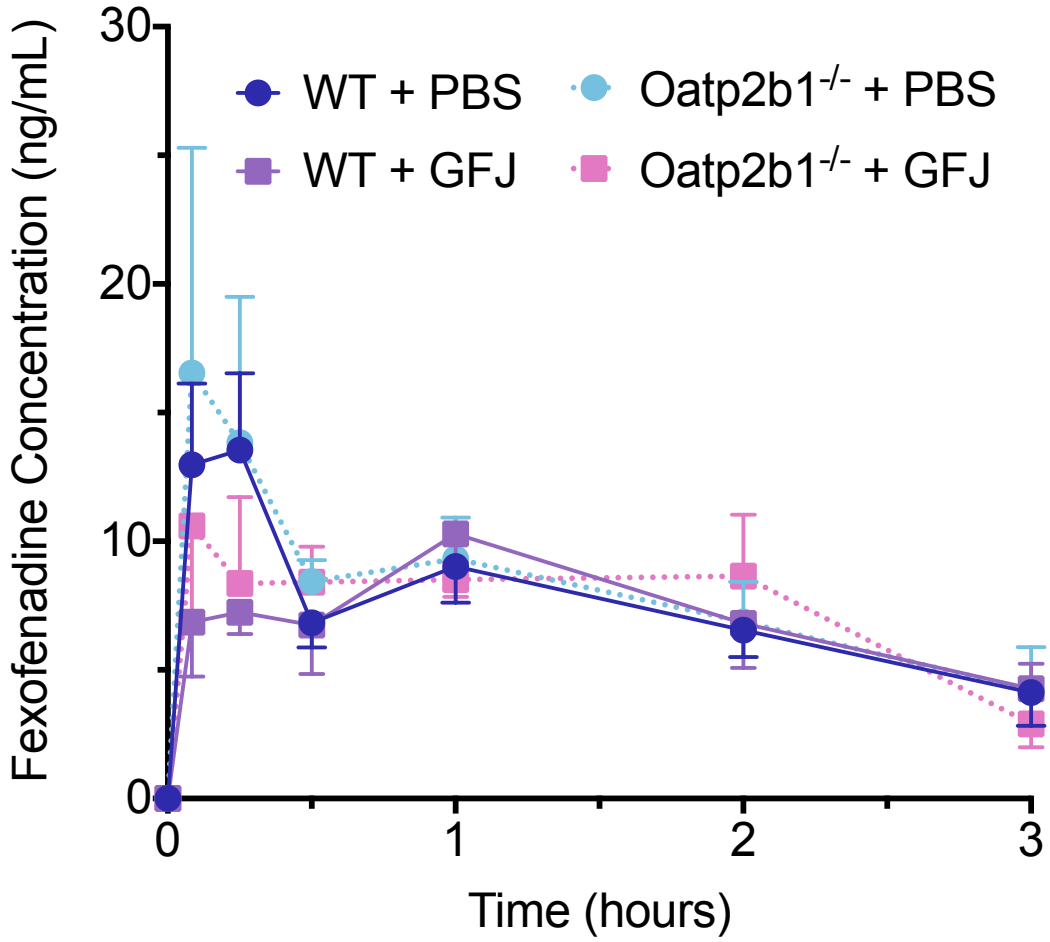

Figure 4.4 Role of mOatp2b1 in fexofenadine disposition following grapefruit juice (GFJ) co-ingestion. Mean ± SEM plasma concentration-time curves of fexofenadine (1 mg/kg) after oral administration with 200 µL phosphate-buffered saline (PBS) or 4x concentrated GFJ in wild-type (WT) (n = 7 for PBS and n = 5 for GFJ) and Oatp2b1$^{-/-}$ (n = 4 for PBS and n = 4 for GFJ) mice.

Table 4.2 Plasma pharmacokinetic parameters of fexofenadine (1 mg/kg) after oral administration with 200 μL phosphate-buffered saline (PBS) or 4x concentrated grapefruit juice (GFJ) in wild-type (WT) and Oatp2b1$^{-/-}$ mice.

Data are presented as mean ± SEM. AUC_{0-3} is area under the plasma drug concentration-time curve from 0 to 3 hours. C_{max} is peak plasma drug concentration. T_{max} is time to reach peak plasma drug concentration.

	WT (C57BL/6)		Oatp2b1$^{-/-}$	
Parameters	PBS (n = 7)	GFJ (n = 5)	PBS (n = 4)	GFJ (n = 4)
AUC_{0-3} (ng · h/mL)	22.4 ± 2.10	21.6 ± 3.52	24.1 ± 4.44	22.7 ± 3.22
C_{max} (ng/mL)	18.0 ± 3.00	11.5 ± 1.00	18.4 ± 8.00	15.3 ± 3.03
T_{max} (h)	0.37 ± 0.16	0.82 ± 0.18	0.23 ± 0.10	0.60 ± 0.47

4.5 Effects of GFJ and AJ Co-Administration on Fexofenadine Pharmacokinetics in WT and Mdr1a$^{-/-}$ mice

High Mdr1a efflux activity may be substantially more limiting to intestinal fexofenadine absorption in mice than in humans (Cvetkovic *et al.,* 1999; Tahara *et al.,* 2005; Smith and Gums 2009). This notion could explain the low oral fexofenadine bioavailability of less than 3% in mice a value much lower than that found in humans (33%) (Cvetkovic *et al.*, 1999; Tahara *et al.*, 2005; Smith and Gums, 2009). Indeed, in the Mdr1a$^{-/-}$ mouse model, plasma fexofenadine levels have been previously reported to be 4-5 times higher than WT mice (Cvetkovic *et al.,* 1999; Tahara *et al.,* 2005). We therefore hypothesized that in normal WT mice, high Mdr1a efflux activity may mask the impact of inhibition of fexofenadine uptake transport by fruit juices. We anticipated that by eliminating significant efflux transport, the effects of fruit juice on oral fexofenadine bioavailability may be exaggerated and thus more evident than that observed in WT mice. To that end, we investigated the effect of GFJ and AJ on fexofenadine pharmacokinetics in WT and Mdr1a$^{-/-}$ mice.

Mdr1a$^{-/-}$ mice exhibited a 2.75-fold increase in plasma fexofenadine exposure compared to WT mice when the drug was administered in PBS control vehicle (P<0.01), a result that is in agreement with previous reports (Cvetkovic *et al.,* 1999) (**Figure 4.5**). This finding confirms the dominant role of P-gp in determining fexofenadine bioavailability in rodents. Concomitant ingestion of GFJ or AJ with fexofenadine did not significantly alter the $AUC_{0\text{-}3}$, C_{max} or T_{max} in WT (CF-1) mice. Similarly, no difference in any pharmacokinetic parameter was observed in Mdr1a$^{-/-}$ mice following either juice intake (**Figure 4.5**) (**Table 4.3**). Overall, it appears that while Mdr1a is a major determinant of

fexofenadine bioavailability *in vivo*, the absence of its expression did not unmask a latent fexofenadine-fruit juice interaction to recapitulate the human situation. Current mouse models remain poorly reflective of the clinical fexofenadine-fruit juice interaction.

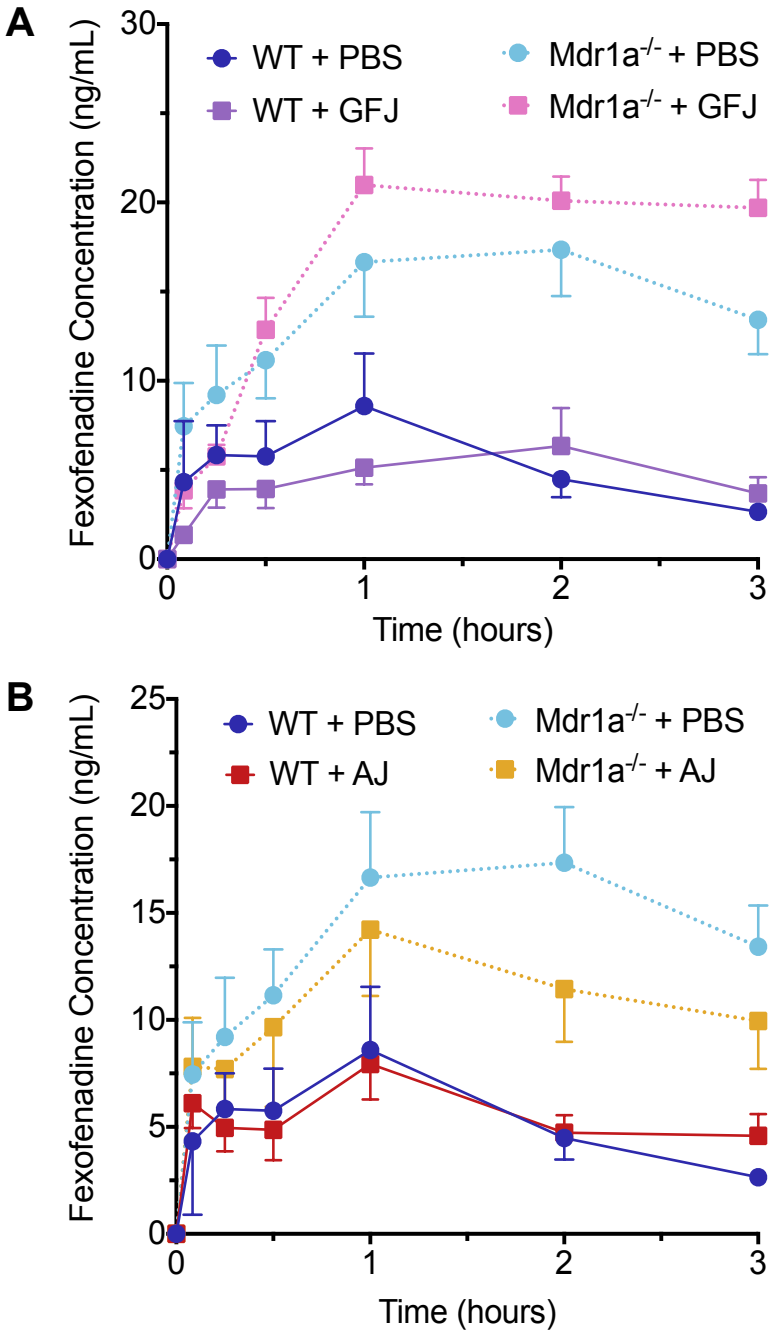

Figure 4.5 Role of Mdr1a in fexofenadine disposition following fruit juice co-ingestion. Mean ± SEM plasma concentration-time curves of fexofenadine (1 mg/kg) after oral administration with 200 µL phosphate-buffered saline (PBS), (**A**) 4x concentrated grapefruit juice (GFJ) or (**B**) 4x concentrated apple juice (AJ) in wild-type (WT) (n = 7 for PBS, n = 7 for GFJ and n = 8 for AJ) and Mdr1a$^{-/-}$ (n = 9 for PBS, n = 7 for GFJ and n = 7 for AJ) mice.

Table 4.3 Plasma pharmacokinetic parameters of fexofenadine (1 mg/kg) after oral administration with 200 µL phosphate-buffered saline (PBS), 4x concentrated grapefruit juice (GFJ) or 4x concentrated apple juice (AJ) in wild-type (WT) and Mdr1a$^{-/-}$ mice.

	WT (CF-1)			Mdr1a$^{-/-}$		
Parameters	PBS (n = 7)	GFJ (n = 7)	AJ (n = 8)	PBS (n = 9)	GFJ (n = 7)	AJ (n = 7)
$AUC_{0\text{-}3}$ (ng · h/mL)	16.2 ± 3.51	14.5 ± 3.39	16.6 ± 2.44	43.6 ± 6.71**	52.2 ± 3.42	33.3 ± 6.84
C_{max} (ng/mL)	12.4 ± 3.46	7.55 ± 1.84	9.68 ± 1.61	19.8 ± 2.78	23.7 ± 1.46	17.5 ± 3.16
T_{max} (h)	0.66 ± 0.16	1.32 ± 0.26	1.08 ± 0.35	1.56 ± 0.33	1.72 ± 0.36	1.23 ± 0.37

Data are presented as mean ± SEM. $AUC_{0\text{-}3}$ is area under the plasma drug concentration-time curve from 0 to 3 hours. C_{max} is peak plasma drug concentration. T_{max} is time to reach peak plasma drug concentration.

**P<0.01 represents a significant difference from PBS treated WT mice.

4.6 GFJ Does Not Compromise Intestinal Mucosa Integrity

The lack of observable changes to fexofenadine plasma concentrations upon fruit juice co-administration in mice, which differs from that observed in humans, may have resulted due to a potential impact of fruit juices on intestinal mucosa integrity. Intestinal epithelial cells form a protective barrier against the external environment through the use of tight junctions (Assimakopoulos *et al.*, 2011). It is considered that drugs such as fexofenadine can only enter the systemic circulation through transcellular uptake via solute carriers in the apical and basolateral enterocyte membranes (Han *et al.*, 2013). However, xenobiotics, dietary factors, enzymes and stress are factors that may disrupt intestinal tight junctions (Assimakopoulos *et al.*, 2011). Damaged mucosal integrity increases intestinal permeability, leading to potential paracellular uptake of fexofenadine, a process independent of transport proteins. As such, a reduction in plasma drug levels due to inhibition of uptake transporters or induction of efflux transporters may be overcome and masked by increased bioavailability due to paracellular absorption.

To explore the possibility that fruit juices caused intestinal mucosa damage, an intestinal permeability test was performed in WT and Mdr1a$^{-/-}$ mice. Here, the cell-impermeable compound, FITC-dextran, was orally administered to mice and differences in plasma concentrations served as an indicator of altered intestinal paracellular transport. No significant difference was observed in plasma FITC-dextran concentrations between PBS control and GFJ treatments in WT mice (**Figure 4.6**). Likewise, GFJ did not significantly affect FITC-dextran plasma levels in Mdr1a$^{-/-}$ mice. Furthermore, plasma FITC-dextran concentrations were not significantly different between WT and Mdr1a$^{-/-}$ mice (**Figure**

4.6). These results suggest that the administered GFJ does not alter intestinal tight junctions. Furthermore, the plasma fexofenadine exposure differences that were observed between WT and Mdr1a$^{-/-}$ mice were not related to differences in intestinal paracellular permeability.

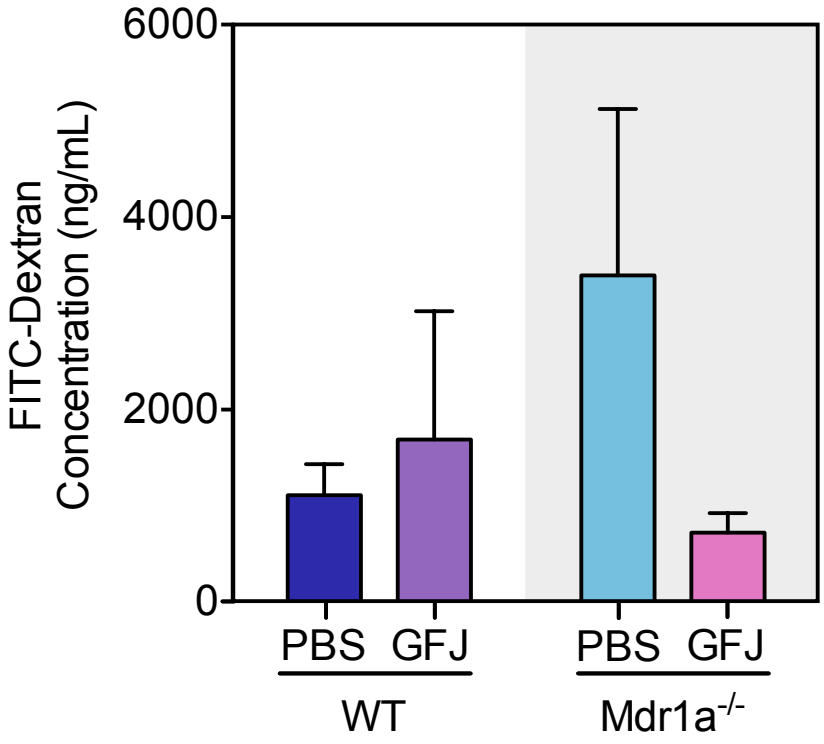

Figure 4.6 Effect of grapefruit juice (GFJ) on intestinal permeability. Mean ± SEM plasma concentration of FITC-dextran (600 mg/kg) after oral administration with 200 µL phosphate-buffered saline (PBS) or 4x concentrated GFJ in wild-type (WT) (n = 4 for PBS and n = 4 for GFJ) and Mdr1a$^{-/-}$ (n = 3 for PBS and n = 4 for GFJ) mice.

4.7 Flavonoids are Minimally Absorbed in Mice

In humans, the site of the fruit juice-fexofenadine interaction has been localized to the intestine, as fruit juices do not change fexofenadine $t_{1/2}$ or clearance. This implies that fruit juice flavonoids do not affect the activity of transport proteins in excretory organs such as the kidney and liver. Furthermore, it suggests that fruit juice flavonoids do not enter the circulation at appreciable levels to influence fexofenadine elimination pathways. To determine if this is in fact the case, we attempted to measure the concentrations of administered flavonoids after GFJ and AJ consumption in the mouse fexofenadine pharmacokinetics studies at the final time point plasma sample (3 hours post juice administration).

We were unable to detect any flavonoids (naringen, narirutin, naringenin, hesperidin, neohesperidin, hersperitin, quercetin, kaempferol, phloridzin and phloretin) in mouse plasma. Therefore, orally administered flavonoids are not likely present at sufficient circulating concentrations to modulate the activity of transport proteins in the kidney and liver. In mice, the fruit juice flavonoids would appear to impact fexofenadine pharmacokinetics only in the gut.

5 Discussion

5.1 Summary of Main Findings

Although several intestinal drug transporters are proposed mediators of FJDIs involving non-metabolized drugs such as fexofenadine, the exact transport proteins involved *in vivo* and the mechanistic basis of the effect remains unclear. Our working hypothesis is that GFJ and AJ limit the absorption of fexofenadine through their interactions with specific intestinal transporters.

In **Aim 1**, we evaluated candidate gut transporters and their mouse orthologs involved in the fruit juice-fexofenadine interaction. Here, we hypothesized that in addition to the previously suspected transporters, OATP1A2 and OATP2B1, other intestinal transport proteins involved in this food-drug effect exist. Using *in vitro* transporter screens, we confirmed that OATP1A2 and OCT1 are intestinal fexofenadine transporters (Cvetkovic *et al.*, 1999; Dresser *et al.*, 2002; Glaeser *et al.*, 2007; Ming *et al.*, 2011). However, we did not find OATP2B1 to be a fexofenadine transporter, contrary to findings by other investigators. Furthermore, we demonstrated that not only was OATP1A2 transport of fexofenadine subject to fruit juice inhibition as previously reported, but also potentially affected was OCT1, suggesting that this transporter may be a new player in FJDIs. One of our most important findings was the identification of OSTα/β as a novel potential contributor to the fruit juice-fexofenadine interaction as we showed for the first time that it transports fexofenadine and is likely inhibited by GFJ *in vitro*. In support of studies aimed to assess the mouse as a model for FJDIs, we have described for the first time that mOatp1a1, mOatp1a4, mOatp2b1, mOct1 and mOstα/β, murine orthologs of human intestinally expressed fexofenadine transporters, are also functional fexofenadine solute carriers that are subject to fruit juice inhibition to varying degrees. Interestingly,

mOatp2b1, mOct1, mOstα and mOstβ, but not mOatp1a1 and mOatp1a4 mRNAs are expressed in the small bowel of mice (Cheng *et al.*, 2005; Fu *et al.*, 2016). Together, these findings predicted that mOatp2b1 and mOct1 could contribute to fexofenadine absorption in mice, while minimizing the potential roles for mOatp1a1 and mOatp1a4. *In vitro*, GFJ inhibited all identified fexofenadine transporters, whereas AJ was only able to elicit inhibitory effects on OATP1A2. This differential fruit juice effect may be explained by the unique flavonoid composition of each juice that we characterized by LC-MS/MS. Overall, these results support the hypothesis that in addition to the current suspected OATPs, other intestinal transport proteins may be involved in this food-drug effect, namely OCT1 and OSTα/β.

In **Aim 2**, we characterized the mouse as an *in vivo* model for the fruit juice-fexofenadine interaction and investigated the *in vivo* role of mOatp2b1 in this food-drug effect. It was hypothesized that mice would faithfully recapitulate the human fruit juice-fexofenadine interaction. Furthermore, the FJDI would be mediated through inhibition of mOatp2b1 by GFJ. However, we showed that mice do not recapitulate the human FJDI as GFJ co-administration resulted in no change to plasma fexofenadine exposure in WT mice. Contradicting *in vitro* and gene expression findings obtained in Aim 1, studies in knockout mice revealed that mOatp2b1 was not a significant contributor to fexofenadine oral absorption and that this transporter was not involved in FJDIs *in vivo*. In an attempt to unmask fruit juice effects on uptake transporters, we eliminated the significant intestinal efflux activity of Mdr1a by conducting fruit juice-fexofenadine pharmacokinetic experiments in Mdr1a$^{-/-}$ mice and its corresponding background strain (CF-1) as control. Indeed, we found that Mdr1a is a substantial contributor to

fexofenadine bioavailability and plasma levels in mice (Cvetkovic *et al.*, 1999; Tahara *et al.*, 2005). However, its dominant activity in the gut did not mask the effect of fruit juices on uptake fexofenadine transporters as GFJ and AJ co-ingestion did not alter fexofenadine pharmacokinetics in Mdr1a$^{-/-}$ mice. Additionally, the lack of fruit juice effect in mice was not due to changes in intestinal paracellular permeability resulting from GFJ administration. Overall, the findings from the *in vivo* studies did not support the hypothesis that the mouse is a good model for FJDIs.

5.2 The Intestine as the Site of FJDIs

The mechanisms of FJDIs involving non-metabolized drugs have been extensively studied and debated over the last decade and a half. In the initial report in 2002, Dresser and colleagues localized this food-drug effect to the site of intestinal drug absorption as GFJ and AJ co-ingestion elicited no effects on fexofenadine $t_{1/2}$ and renal clearance. Since then, this idea that FJDIs is mediated through an intestinal mechanism has become entrenched as subsequent clinical studies by Dresser *et al.,* (2005), Bailey *et al.,* (2007), Glaeser *et al.,* (2007), Won *et al.,* (2013) and Akamine *et al.,* (2014) have all observed this lack of change in pharmacokinetic parameters describing of systemic drug elimination with fruit juice co-intake. Evidence gathered from this thesis adds further support for this notion. We found that in mice, flavonoids, the presumed transporter inhibitors in fruit juices, were not detected in plasma post juice administration. This suggests that these key constituents would not have the potential to act on transporters in the liver and kidneys, organs of drug elimination, as they are not readily absorbed into circulation to reach these distal sites at sufficient concentrations to affect fexofenadine clearance. This finding is consistent with the fact that the major fruit juice flavonoids are

glycosides that may be too hydrophilic to be absorbed by passive diffusion (Oteiza *et al.*, 2005). Therefore, interactions with transporter proteins likely occur prior to systemic absorption, in the intestine. The aglycone-type flavonoids in fruit juices are thought to be absorbed, but they are rapidly metabolized by glucuronidation during first-pass through the intestine and liver (Lee and Reidenberg, 1998). Since we did not measure these flavonoid metabolites in blood, it cannot be entirely ruled-out that some metabolized components of fruit juices are present in circulation to alter drug elimination in liver and kidney. However, it should be noted that should flavonoids or its metabolites be present at sufficient concentrations systemically, their effects on transport proteins would have to be stimulatory as opposed to their well accepted inhibitory effects, in order to cause a decrease in plasma drug exposure.

While the current results have shed some new insights into the molecular basis of this FJDI, no clear and compelling mechanisms have yet emerged. In fact, findings presented by this thesis reduce support that OATP1A2 and OATP2B1 are the key transporters involved. Furthermore, our results have provided support for additional players in the fruit juice-fexofenadine interaction, namely OSTα/β and OCT1. Consequently, it becomes more plausible that fruit juices interact with multiple fexofenadine uptake and efflux transporters resulting in a complex pharmacokinetic interplay.

5.3 Evidence to Support or Contradict a Role for Specific Intestinal Transporters in Fruit Juice-Fexofenadine Interactions

5.3.1 OATP1A2

5.3.1.1 Supportive Evidence

OATP1A2 was the first proposed mediator of the fruit juice-fexofenadine interaction. Repeatedly, *in vitro* studies have shown that fexofenadine is an excellent substrate of the drug transporter (Cvetkovic *et al*., 1999; Dresser *et al*., 2002; Bailey *et al.,* 2007; Glaeser *et al.,* 2007; Won *et al.,* 2013). Furthermore, influx of the antihistamine is inhibited by GFJ, AJ and GFJ flavonoids naringin and hesperidin in cellular OATP1A2 expression systems (Dresser *et al.,* 2002; Bailey *et al.,* 2007; *Won et al.,* 2013). In support of its role in FJDIs, our findings confirmed OATP1A2-mediated fexofenadine transport and fruit juice inhibition in a HeLa cell model. As such, the OATP transporter is a candidate mediator of FJDIs owing to strong *in vitro* support.

5.3.1.2 Contradictory Evidence

In spite of convincing *in vitro* evidence, the *in vivo* role of OATP1A2 remains highly debated. Much of this is due to heterogeneity in reports regarding the mRNA and protein expression of this membrane transporter in the small intestine. Recent studies using highly sensitive and accurate methods such as targeted proteomics favor the absence of OATP1A2 protein in human small bowel (Nishimura and Naito, 2005; Hilgendorf *et al.*, 2007; Meier *et al.*, 2007; Eechoute *et al.*, 2011; Drozdzik *et al.*, 2014). To further disqualify its involvement, a study found that intestinal fexofenadine oral absorption was not altered in Oatp1a/1b KO mice when compared to WT mice, implying minimal roles of mouse OATP1A2 orthologs in facilitating fexofenadine uptake from the gut (van de

Steeg *et al.*, 2010). Finally, while our findings demonstrate that mOatp1a1 and mOatp1a4 are fexofenadine transporters and are functionally inhibited by fruit juices *in vitro*, studies have repeatedly shown that they are not expressed in the mouse small intestine (Cheng *et al.*, 2005; Fu *et al.*, 2016). If these results for mouse transporters translate to humans, an *in vivo* role of OATP1A2 in FJDIs would seem unlikely.

5.3.2 OATP2B1

5.3.2.1 Supportive Evidence

Recent literature reports have focused on OATP2B1 as the primary facilitator of FJDIs. While we were unable to demonstrate fexofenadine uptake by OATP2B1 using a HeLa cell expression system, similar to Glaeser *et al.* (2007), fexofenadine was found to be a transport substrate of this OATP by other research groups using the *Xenopus laevis* oocyte expression system (Nozawa *et al.*, 2004; Imanaga *et al.*, 2011; Shirasaka *et al.*, 2011; Shirasaka *et al.*, 2013b; Akamine *et al.*, 2014; Akamine, 2015). In other expression systems, GFJ and AJ dramatically inhibited fexofenadine influx by the OATP2B1 transporter (Imanaga *et al.*, 2011; Shirasaka *et al.*, 2013b). There is also *in vivo* support for the role of OATP2B1 in the fruit juice-fexofenadine interaction. First, OATP2B1 is unquestionably expressed in the small intestine, where the food-drug effect is localized (Kobayashi *et al.*, 2003; Nishimura and Naito, 2005; Meier *et al.*, 2007; Drozdzik *et al.*, 2014). Second, Imanaga *et al.* (2011) examined the AJ-fexofenadine interaction in a cohort of individuals harboring the reduced function *SLCO2B1* c.1457C>T genetic polymorphism. They found that individuals with the variant allele experienced lower fexofenadine exposure when the drug was taken alone and a less dramatic change to fexofenadine AUC when the drug was taken with AJ in comparison to individuals

carrying WT alleles. Taken together, it appears that fexofenadine is an *in vivo* substrate of OATP2B1 and that the AJ-fexofenadine effect is mediated in part by the inhibitory interaction of AJ with intestinal OATP2B1.

5.3.2.2 Contradictory Evidence

Despite persuasive *in vitro* and clinical support, our novel results reduce support for the mechanistic involvement of OATP2B1 in the fruit juice-fexofenadine interaction. We presented new findings that mOatp2b1 is an *in vitro* fexofenadine transporter and experiences inhibition by GFJ. mOatp2b1 mRNA is detected at appreciable amounts in the mouse small intestine (Cheng *et al.*, 2005; Fu *et al.*, 2016). Therefore, based on the *in vitro* findings and expression reports, we presumed that a fruit juice-drug interaction in the mouse model would be mediated by mOatp2b1 *in vivo*. Unexpectedly, fexofenadine exposure in Oatp2b1$^{-/-}$ mice was not significantly different from WT mice. Furthermore, GFJ co-intake did not alter fexofenadine pharmacokinetics in WT or Oatp2b1$^{-/-}$ mice. Together, we demonstrated that despite *in vitro* transport and inhibition results and reported expression data that all pointed towards the potential involvement of mOatp2b1 *in vivo*, this Oatp transporter does not play a significant role in neither fexofenadine absorption nor the GFJ-fexofenadine interaction. The *in vitro-in vivo* disconnect revealed by our results calls into question the presumed role of OATP2B1 in clinical FJDIs.

5.3.3 OCT1

5.3.3.1 Supportive Evidence

As OCT1 is localized to the apical membrane of intestinal epithelial cells, it has become a contender in the FJDI mechanism (Han *et al.*, 2013). Results from our experiments confirmed that OCT1 mediates fexofenadine transport *in vitro* (Glaeser *et al.*, 2007).

Furthermore, we showed for the first time that transport of the antihistamine by the membrane protein is likely inhibitable by GFJ in a cellular expression model. OCT1 has also been implicated in the atenolol–AJ interaction. Mimura *et al.,* (2015) found that the beta-blocker was an OCT1 substrate and that transport could be inhibited by the AJ flavonoids phloretin and quercetin. In our experiments, we did not observe AJ inhibition of OCT1-mediated fexofenadine cellular uptake. This discrepancy can be attributed to the 5% normal strength AJ used in our experiments which translates to nanomolar concentrations of phloretin and quercetin, rather than the micromolar levels used by Mimura and colleagues. Therefore, there is some *in vitro* support for OCT1 as a candidate mediator of FJDIs.

5.3.3.2 Contradictory Evidence

We found that mOct1 was an *in vitro* fexofenadine transporter that could be inhibited by GFJ. Moreover, the solute carrier is localized to the apical membrane of mouse small bowel epithelial cells (Han *et al.*, 2013). Thus, it was reasonable to presume that there could be a GFJ-fexofenadine interaction in mice that occurs at the level of intestinal mOct1 (Han *et al.*, 2013). However, no pharmacokinetic interaction was observed, suggesting that mOct1 does not facilitate fexofenadine absorption in mice. These results further highlight the many *in vitro-in vivo* disconnects that complicate our ability to establish mechanisms.

5.3.4 OSTα/β

5.3.4.1 Supportive Evidence

We propose OSTα/β as a novel candidate transporter involved in fruit juice-fexofenadine interactions because it transports fexofenadine and is inhibited by GFJ *in vitro*.

Furthermore, its localization to the basolateral membrane of enterocytes implies that it may promote the vectorial movement of fexofenadine for absorption across the enterocyte *in vivo* and can potentially interact with inhibitory fruit juice components (Ballatori *et al.*, 2005).

5.3.4.2 Contradictory Evidence

Since OSTα/β is responsible for bile acid reabsorption in the gut, it follows that this transporter could promote fexofenadine oral absorption (Ballatori *et al.*, 2005). This possibility is somewhat weakened by the fact that OSTα/β is expressed largely in the ileum and therefore it would represent a pathway for the late-in-time absorption of fexofenadine (Ballatori *et al.*, 2005). However, oral fexofenadine is significantly absorbed in the proximal (duodenum) and medial (jejunum) regions of the small intestine (Smith and Gums, 2009). Importantly, plasma pharmacokinetic profiles indicate that the fruit juice-fexofenadine interaction occurs shortly after co-administration, inconsistent with mechanisms involving the distal small intestine. Moreover, because OSTα/β is expressed on the basolateral ileocyte membrane, flavonoids require traversal of the brush border membrane of enterocytes to have access to the transporter. However, most fruit juice components have very poor membrane permeability that likely limits their ability to interact as sufficient levels to inhibit OSTα/β (Oteiza *et al.*, 2005). This poor passive membrane permeability is evidenced by the lack of detectable flavonoids in the plasma of mice administered fruit juices. On the other hand, non-glycoside flavonoids are membrane permeable and in fact, several gut transporters including OATP1A2, OATP2B1 and OCT1 can drive the cellular uptake of quercetin (Glaeser *et al.*, 2014). These types of flavonoids are more likely to interact with OSTα/β.

5.4 Future Studies

5.4.1 *In Vivo* Role for OCT1 and OSTα/β

Our findings have implicated the involvement of two new candidate transporters, OCT1 and OSTα/β, in the fruit juice-fexofenadine interaction. Although we have provided *in vitro* support for their roles, much like current proposed mediators, their *in vivo* contributions remain to be elucidated. Luckily, transporter KO mouse models exist for mOct ($Oct1^{-/-}$) and mOstα ($Ost\alpha^{-/-}$) which may be used to address this knowledge gap (Jonker *et al.*, 2001; Soroka *et al.*, 2010). It is notable that because mOstα/β function as a heteromeric complex, KO of mOstα results in the dramatic reduction of mOstβ protein expression in the gastrointestinal track of mice, sufficiently eliminating mOstα/β function (Rao *et al.*, 2008). Future fexofenadine pharmacokinetic interaction studies with GFJ and AJ should be performed in these transporter KO mice to determine their *in vivo* roles in drug absorption and the FJDI. Additionally, it may be beneficial to generate dual transporter KO mice, combining Mdr1a with a suspected transporter to minimize dominant efflux activity.

5.4.2 Do Fruit Juices Stimulate Apical Efflux Transporter Activity in the Gut?

One of the earliest proposed mechanisms for FJDIs involving intestinal transporters was the possibility that GFJ could "activate" P-gp (Soldner *et al.*, 1999). Such stimulation of P-gp on the apical enterocyte membrane would result in reduction of oral bioavailability of both metabolized and non-metabolized drugs. However, this notion has since been dismissed as fruit juices and their associated flavonoids have mostly been found to inhibit P-gp (Takanaga *et al.*, 1998; de Castro *et al.*, 2007; Shirasaka *et al.*, 2010). Another more

likely possibility is that fruit juices stimulate the activity of apically-expressed MRP2 in the gastrointestinal tract. We found that fexofenadine is a likely transport substrate of MRP2 (**Appendix B: Identification of Fexofenadine Apical Efflux Transporters, Supplementary Figure 3**) and many compounds have been found to stimulate MRP2 activity in a species-specific manner (Lagas *et al.*, 2009). Moreover, flavonoids and their metabolites are well-known substrates of MRP2 (Morris and Zhang, 2006). Therefore future studies should examine whether fruit juices and their individual constituents stimulate the activity of apical fexofenadine efflux transporters in the gut such as MRP2.

5.4.3 Are Unknown Fexofenadine Transporters Involved?

We found that the clinical fruit juice-fexofenadine interaction could not be reproduced in mice. While disappointing, such species differences may however provide some clues to the FJDI mechanism. It is possible that the fruit juice-fexofenadine interaction may be attributed to the existence of unknown transport proteins. For instance, humans may possess a currently unrecognized fexofenadine transporter that is not found in mice through which the FJDI is mediated. It is also plausible that there exists an unknown fexofenadine transporter found in both humans and mice that is subject to human-specific inhibition by fruit juices. In light of the fact that we already find fexofenadine as a substrate of numerous uptake and efflux transporters located on the apical and basolateral membranes of intestinal epithelial cells, the possibility arises that the unknown transporter(s) actually represent the major pathway for oral absorption and modulation by fruit juices. We also showed that while GFJ significantly inhibited the activity of all tested fexofenadine transporters, AJ only elicited inhibitory effects on OATP1A2-mediated drug uptake, signifying a differential effect of fruit juices *in vitro*. However, it

is well established that clinically, both GFJ and AJ are capable of mediating the FJDI, if not to a greater extent by AJ. This disconnect between *in vitro* and *in vivo* findings suggest that AJ may interact with unknown fexofenadine transporters to mediate its effect on drug exposure. Future studies should therefore be conducted to identify these unrecognized membrane proteins. For instance, a functional cloning approach which involves a process of RNA fractionation followed by heterologous expression and evaluation of fexofenadine transport activity may be used to discover novel transporters. Another strategy would be large scale cloning of candidate solute carriers expressed in the intestine based on human genome data and known cDNA sequences, followed by *in vitro* characterization of fexofenadine transport activity. Finally, a genome-wide association study examining individuals with unusually low and high fexofenadine levels is a human genetic approach to identifying unknown fexofenadine transport proteins.

5.4.4 What are the Other Fruit Juice Constituents that Act as *In Vivo* Modulators of Fexofenadine Absorption?

While we have some knowledge of the fruit juice constituents responsible for eliciting transporter mediated-FJDIs, most studies have examined the effects of single flavonoids on individual transport protein activity *in vitro*. This however, does not reflect the collective effect of each flavonoid on the complement of intestinal transporters *in vivo*. For instance, quercetin has been shown to inhibit the activities of OATP2B1 and OCT1 in cellular models. However, a human pharmacokinetic study revealed that quercetin co-administration resulted in an increase in fexofenadine exposure, a result that is opposite to what *in vitro* data would suggest. This demonstrated that although quercetin has an clear effect on *in vitro* drug transport, it does not decisively contribute to the effect *in*

vivo (Kim *et al.*, 2009). Consequently, there is a need to better characterize the essential fruit juice components involved in transporter mediated FJDIs. This may be accomplished by conducting clinical fexofenadine pharmacokinetic interaction studies with individual flavonoids similar to those performed by Bailey *et al.* (2007) for naringin and Kim *et al.* (2009) for quercetin.

5.5 Conclusions

FJDIs involving non-metabolized drugs are a cause of altered drug exposure leading to a potential loss of therapeutic efficacy. Understandably, this food-drug effect has become a growing concern for its impact on optimal patient therapy and safety. While several transporter mediators have been proposed, the underlying mechanistic basis remains unclear. The overall goal of this thesis was to elucidate the specific intestinal transporters involved in this food-drug interaction. We found evidence for a growing complement of intestinal fexofenadine transporters and the effect of fruit juices on their activity *in vitro*. Moreover, we characterized mouse transporter orthologs of human intestinal fexofenadine transport proteins as the groundwork for targeted studies in this rodent model. In our experiments in mice, we found that this species does not recapitulate human FJDIs. Finally, while mOatp2b1 appeared to be a potential mediator of FJDIs based on convincing *in vitro* and gene expression results, we demonstrated using Oatp2b1$^{-/-}$ mice that the transporter does not contribute to the effect *in vivo*. Overall, while our results have not revealed that a single convincing mechanism exists, we have provided evidence to suggest complex interactions among multiple intestinal transporters with fruit juices is responsible for the observed pharmacokinetic interactions. We expect that a better understanding of the precise mechanisms behind transporter mediated-FJDIs

will allow for the provision of safer and more effective pharmacotherapy as well as allow for more rational drug design.

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Appendices

Appendix A: Transfection Validation

Methods

Transport Study

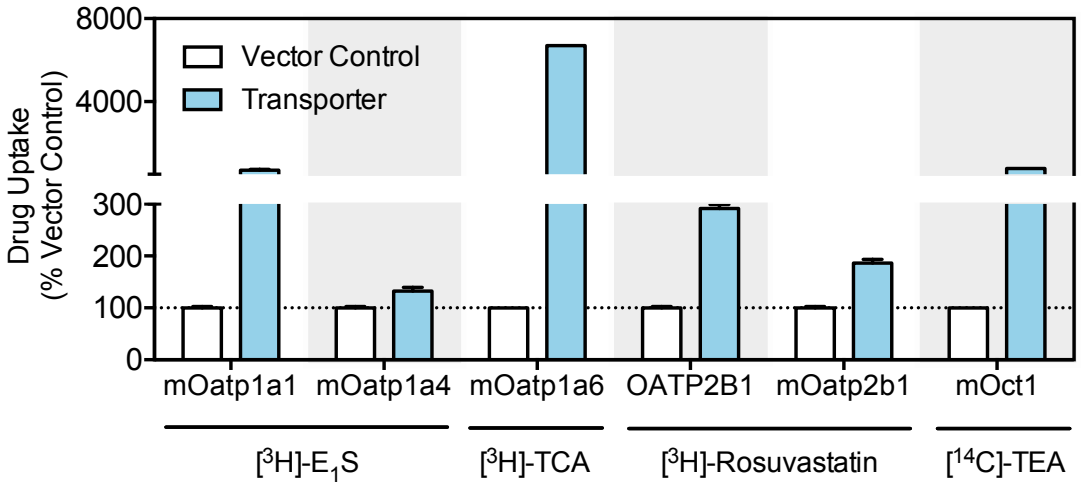
The success of HeLa cell transfection as determined by functional activity were evaluated for mOatp1a1, mOatp1a4, mOatp1a6, OATP2B1, mOatp2b1 and mOct1 using transport studies conducted according to methods outlined in **Section 3.4.2** and by Cvetkovic *et al.,* (1999). Greater uptake of well-established drug substrates, namely [^{3}H]-estrone 3-sulfate (E_1S) for mOatp1a1 and mOatp1a4, [^{3}H]-taurocholic acid (TCA) for mOatp1a6, [^{3}H]-rosuvastatin for OATP2B1 and mOatp2b1 and [^{14}C]-tetraethylammonium (TEA) for mOct1, by cells overexpressing these transporters versus control cells (transfection with blank expression plasmids) validated transfection and served as positive controls for transport function (Jonker *et al.*, 2003; Ho *et al.*, 2006; Gong *et al.*, 2011; Tian *et al.*, 2015). In brief, the drug dose, which consisted of radiolabelled E_1S, TCA, rosuvastatin or TEA and their unlabeled drug (100 μM) dissolved in PBS when evaluating OATP2B1 and mOatp2b1 transport or KHB for all other transporters at pH 6 or pH 7.5 for mOct1, was applied to cultured cells for 30 min at 37°C, 5% CO_2. Subsequently, cells were washed with ice-cold PBS, lysed and intracellular radioactivity was determined by liquid scintillation spectrometry.

Immunofluorescence Microscopy

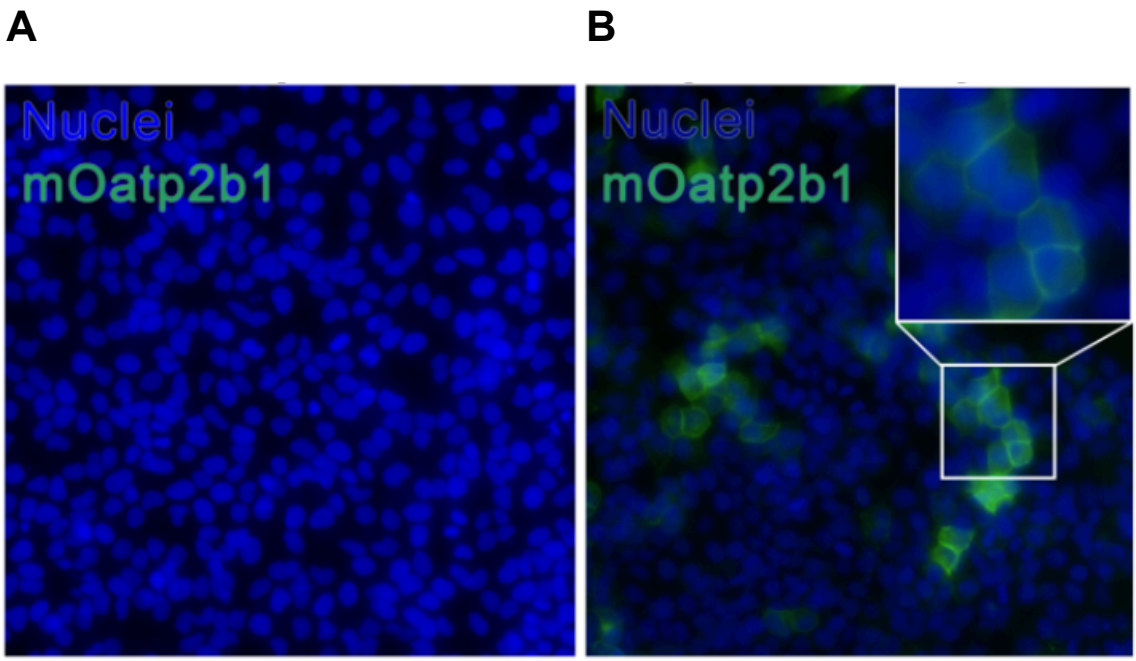
Another indicator of transfection success is the appropriate localization of protein. In the HeLa cell model, mOatp2b1 is expected to be expressed in the plasma membrane in order to facilitate intracellular drug accumulation. To validate localization, HeLa cells were

grown on 4-well culture slides (seeding density of 2.5 x 10^5 cells/well) at 37°C, 5% CO_2 for 48 hours. Cells were transfected with blank expression plasmids (control) or expression plasmids containing mOatp2b1 cDNA (1 μg DNA/well). After 48 hours, cells were fixed with ice-cold 70% methanol in water (v/v) for at -20°C for 10 min. Subsequently, 0.3% Triton X-100 in PBS (v/v) was used to permeabilize cells at RT and blocking was performed using 2% bovine serum albumin in PBS (w/v) at RT for 30 min. Cells were exposed to a custom-made rabbit polyclonal mOatp2b1 antibody (Invitrogen) using a 1:200 dilution in PBS containing 0.05% v/v Tween-20 (PBST) at RT. At 1-hour post incubation, cells were washed using PBST and subjected to Alexa Fluor 488 anti-rabbit secondary antibody (Invitrogen) using a 1:200 dilution in PBST at 37°C for 30 min. Following a final wash using PBST, cells were mounted using VECTASHIELD Medium containing 4', 6-diamidino-2-phenylindole (DAPI) counterstain (Vector Laboratories; Burlington, ON) and imaged by fluorescence microscopy (Nikon Eclipse; Nikon Instruments Inc.; Melville, NY).

Results



Supplementary Figure 1. Transport of well established drug substrates by HeLa cells expressing human and mouse intestinal uptake transporters. HeLa cells transiently transfected with intestinal uptake transporters were evaluated for intracellular accumulation of [^{3}H]-estrone 3-sulfate (E_1S), [^{3}H]-taurocholic acid (TCA), [^{3}H]-rosuvastatin and [^{14}C]-tetraethylammonium (TEA) (100 μM) at pH 6 or pH 7.5 for mOct1 following a 30-minute incubation period. Data are expressed as percent of vector control, mean ± SEM, n = 1 for mOatp1a6 and mOct1 and n = 2 for all other transporters.



Supplementary Figure 2. Plasma membrane localization of mOatp2b1 as demonstrated by immunofluorescence. HeLa cells transiently transfected with blank expression plasmid as control (A) or mOatp2b1 expression plasmid (B) were stained with DAPI for nuclei localization (blue) and Alexa Fluor 488 for mOatp2b1 localization (green).

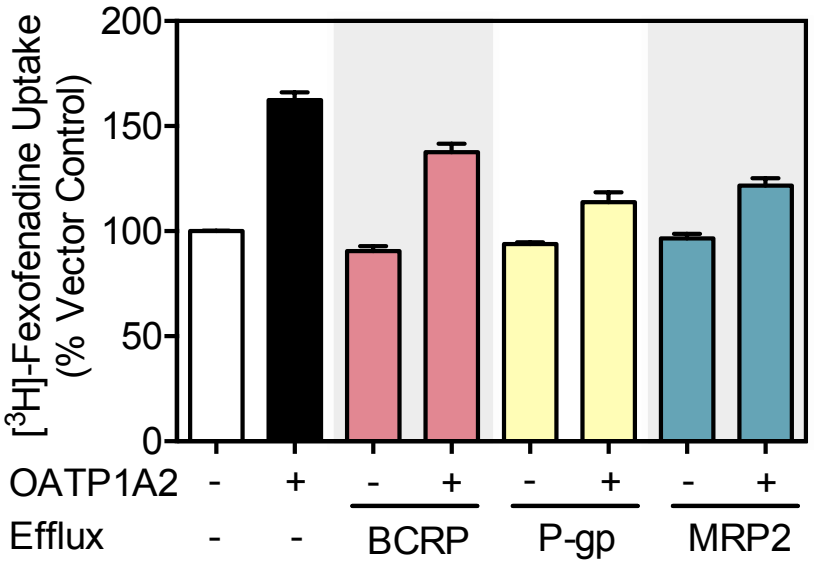
Appendix B: Identification of Fexofenadine Apical Efflux Transporters

Methods

Transient transfection of HeLa cells was performed using Lipofectamine 3000 as described in **Section 3.4.1**. Double transfections were performed where each efflux transporter was expressed with an uptake transporter or blank plasmid at a 1:4 ratio of uptake transporter/empty vector to efflux transporter (1 µg DNA/well total). Fexofenadine transport studies were conduced as outlined by **Section 3.4.2**.

Results

To examine fexofenadine transport activity by human apical efflux transporters, a dual overexpression system was used where each efflux transporter was expressed in the presence and absence of human OATP1A2. OATP1A2 was used to drive fexofenadine into cultured HeLa cells to allow for modulation of drug accumulation by P-gp, BCRP and MRP2 mediated efflux. A reduction in cellular fexofenadine levels in cells that co-expressed both OATP1A2 and an efflux transporter when compared to cells only expressing OATP1A2 would indicate fexofenadine transport by the expressed efflux protein. We found that fexofenadine is a substrate of P-gp and MRP2 and perhaps BCRP, in agreement with the literature (**Supplementary Figure 3**) (Cvetkovic *et al.*, 1999; Akamine *et al.*, 2010; Ming *et al.*, 2011). However, no strong conclusions can be made from these results and no statistical analysis was performed as the efflux transport experiment was conducted on one occasion (n = 1) with technical replicates.



Supplementary Figure 3. Fexofenadine transport by HeLa cells expressing human intestinal apical efflux transporters. HeLa cells transiently transfected with OATP1A2 and/or human apically localized efflux transporters were evaluated for intracellular accumulation of [^{3}H]-fexofenadine (0.1 μM) at pH 6 following a 30-minute incubation period. The presence (+) or absence (-) of transporter expression in HeLa cells is shown below and data are expressed as percent of vector control, mean ± SEM of triplicates (n = 1).

Appendix C: Expression of Fexofenadine Transporters in Mouse Small Intestine

Methods

Liver, kidney and small intestinal mucosa (scraped from the duodenum, jejunum and ileum) were harvested from C57BL/6, Oatp2b1$^{-/-}$, CF-1 and Mdr1a$^{-/-}$ mice, placed in TRIzol Reagent (Thermo Fisher Scientific) and stored at -20^{o}C until RNA extraction. Tissues were homogenized and total RNA was isolated according to manufacturer's instructions (TRIzol). cDNA was synthesized from 2 µg total RNA by MultiScribe Reverse Transcriptase with random hexamer primers (Thermo Fisher Scientific). SYBR green-based (Thermo Fisher Scientific) qPCR (Applied Biosystems ViiA 7) was performed using generated cDNA to determine the relative mRNA expression of mouse uptake and efflux transporters using primers listed in **Supplementary Table 1**. Transporter expression was normalized to 18S ribosomal RNA (TaqMan-based qPCR) (Thermo Fisher Scientific). Analyses were performed in triplicates per transporter per tissue and relative gene expression was calculated by the $\Delta\Delta C_T$ method.

Supplementary Table 1. Primer sequences for qPCR.

Gene	Forward Primer	Reverse Primer
mOatp1a1	5'-ACTCCCATAATGCCCTTGG-3’	5’-TAATCGGGCCAACAATCTTC-3’
mOatp1a4	5’-CCCAGAGCTCTCCAGTTTTG-3’	5’-TCCCATGTTGTTCTTCTGATTG-3’
mOatp2b1	5’-CTTCATCTCAGAACCATACC-3’	5’-ACTGGAACAGCTGCCATTG-3’
mOct1	5’-GGGTGTACGACACTCCCG-3’	5’-GCCCAAGTTCACACAGGACT-3’
Mdr1a	5’-CTCTTTGACTCGGGAGCAGAA-3’	5’-CGGAAACAAGCAGCATAAGAAA-3’
mMrp2	5’-CTGAGTGCTTGGACCAGTGA-3’	5’-CAAAGTCTGGGGGAGTGTGT-3’
mOstα	5’-TACAAGAACACCCTTTGCCC-3’	5’-CGAGGAATCCAGAGACCAAA-3’
mOstβ	5’-GTATTTTCGTGCAGAAGATGCG-3’	5’-TTTCTGTTTGCCAGGATGCTC-3’

Results

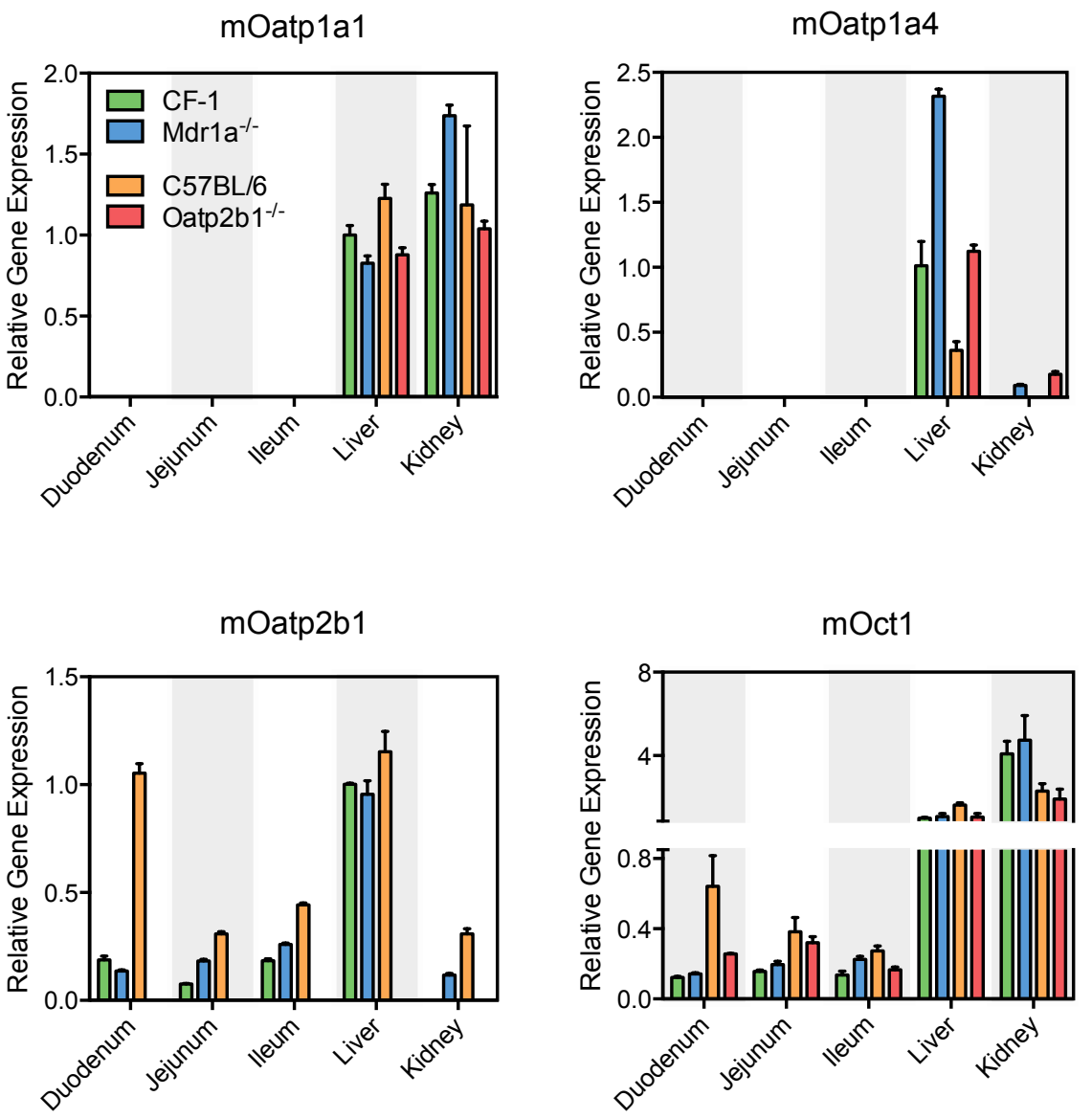
In **Section 4.3**, we demonstrated that several mouse orthologs of human intestinal transporters are functional *in vitro* fexofenadine transport proteins and are subject to inhibition by fruit juices. To understand the potential relevance of these findings to *in vivo* FJDIs, expression of these transporters in the mouse small intestine must be confirmed in the mouse strains used in our experiments. Therefore, by qPCR, we examined the mRNA expression of mouse fexofenadine transporters in mucosal scrapings from various sections of the small bowel of all mouse strains (CF-1, Mdr1a$^{-/-}$ [CF-1 background], C57BL/6 and Oatp2b1$^{-/-}$ [C57BL/6 background]) used in subsequent *in vivo* fruit juice-fexofenadine interaction experiments. Intestinal expression of transporters was compared to liver and kidney. Analyses were conducted in triplicate with one mouse tissue sample per mouse strain.

In accordance with reports by Cheng *et al.* (2005) and Fu *et al.* (2016), the fexofenadine transporters mOatp1a1 and mOatp1a4 were not expressed along the small intestine of all 4 mouse strains. Consequently, these results rule out these mouse Oatp1a transporters as mediators of a fruit juice-fexofenadine effect in this species. Analogous to its human ortholog, there was intestinal expression of mOatp2b1 in CF-1, Mdr1a$^{-/-}$ and C57BL/6 mice. As expected, mOatp2b1 mRNA was not detected in the Oatp2b1$^{-/-}$ mouse. mOct1 mRNA was expressed across all small intestinal sections as was recently reported by Fu *et al.* (2016) (**Supplementary Figure 4**).

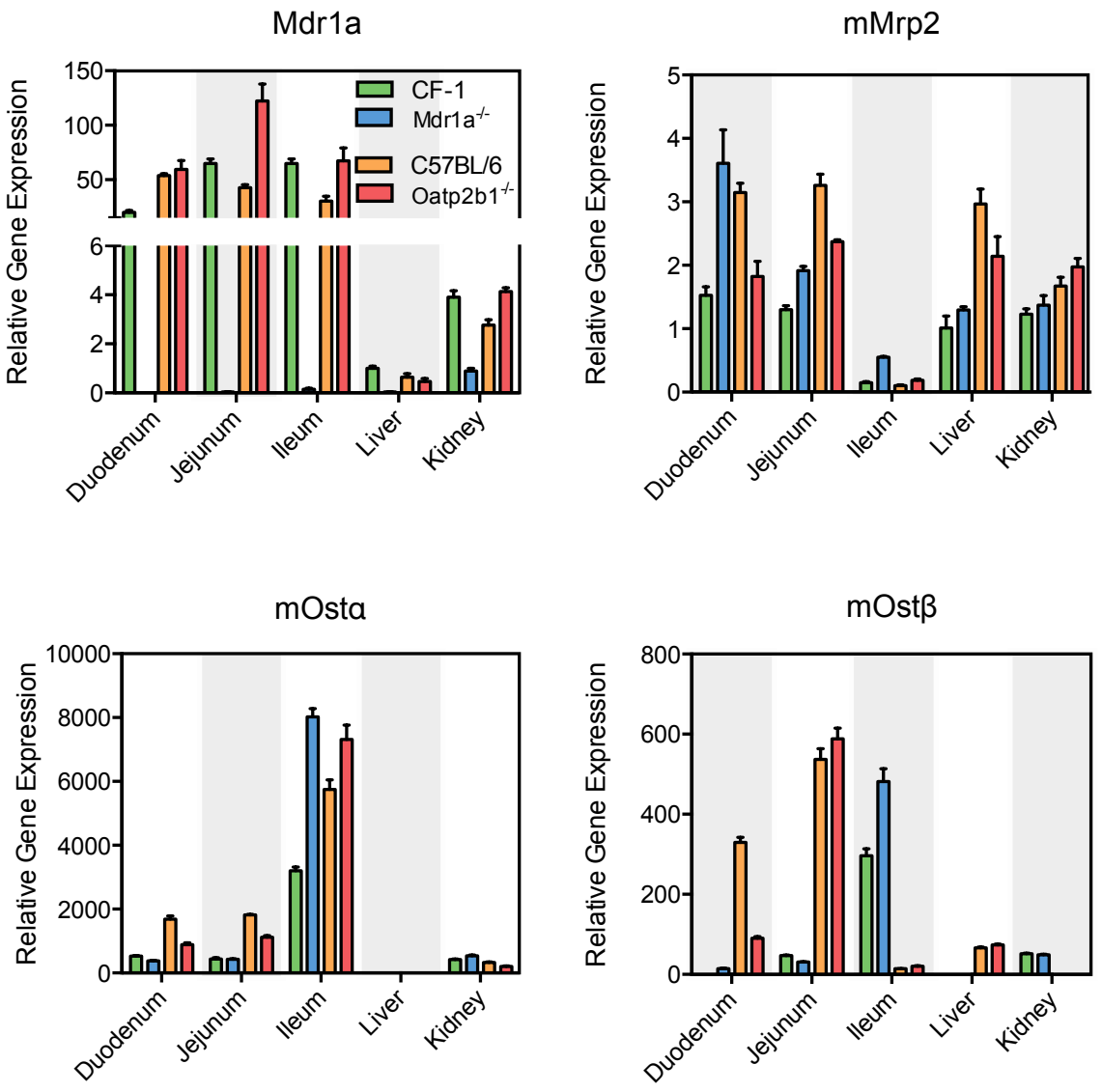
Mdr1a was expressed in all intestinal mucosa sections, with the exception of Mdr1a$^{-/-}$ mice as expected. mMrp2 and mOstα was detected along the small intestine across all

strains. Finally, with the exception of the CF-1 duodenum, mOstβ was expressed in all small intestinal mucosa sections in all strains of mice (**Supplementary Figure 5**).

Overall, our expression results agree with the current literature. The combination of *in vitro* transport activity and inhibitory profiles by fruit juices, together with gene expression findings predict that mOatp2b1 and mOct1 could mediate a fruit juice-fexofenadine interaction in mice.



Supplementary Figure 4. mRNA expression of fexofenadine uptake transporters in mouse small intestine. Gene expression of uptake transporters was measured by real-time PCR in liver, kidney, and mucosal scrapings of the duodenum, jejunum and ileum of CF-1 (wild-type) and its corresponding knockout mouse Mdr1a$^{-/-}$ and C57BL/6 (wild-type) and its corresponding knockout mouse Oatp2b1$^{-/-}$. Values are relative to CF-1 liver and data are presented as mean ± SEM of triplicates, n = 1 per strain.



Supplementary Figure 5. mRNA expression of fexofenadine efflux transporters in mouse small intestine. Gene expression of efflux transporters was measured by real-time PCR in liver, kidney, and mucosal scrapings of the duodenum, jejunum and ileum of CF-1 (wild-type) and its corresponding knockout mouse Mdr1a$^{-/-}$ and C57BL/6 (wild-type) and its corresponding knockout mouse Oatp2b1$^{-/-}$. Values are relative to CF-1 liver and data are presented as mean ± SEM of triplicates, n = 1 per strain.

Appendix D: Animal Use Protocol Approval

Western

2014-012::1:
AUP Number: 2014-012
AUP Title: Interaction of medications and diet in Oatp1b2 KO animals
Yearly Renewal Date: 07/01/2015
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2014-012 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
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Curriculum Vitae

Name: Mandy Meng Jiao Li

Education

M.Sc. Physiology and Pharmacology	2015-2016
University of Western Ontario	London, Ontario, Canada
B.M.Sc. Honours Specialization in Pharmacology	2011-2015
University of Western Ontario	London, Ontario, Canada

Awards and Scholarships

CIHR - Frederick Banting and Charles Best Canada Graduate Scholarships	2015
Ontario Graduate Scholarship	2015
Susan Vitali-Lovell Gold Medal and Award	2015
Western Science Strategic Undergraduate Student Research Award	2013
Western In-Course Scholarship	2012-2013
Laurene Paterson Estate Scholarship	2012
Western's 125th Anniversary Alumni Award	2012
Dean's Honour List	2011-2015
Western Scholarship of Excellence	2011

Teaching Experience

3rd Year Pharmacology Lab	2015-2016
The University of Western Ontario	London, Ontario, Canada

Presentations

Li M, Tirona R. Evaluating the effect of fruit juices on drug disposition in wild-type and P-glycoprotein deficient mice. London Health Research Day, London, Ontario. March 2016. [Top 100 Poster].

Li M, Tirona R. *In vivo* evaluation of the role of organic anion-transporting polypeptide 2B1 in fruit juice-drug interactions. Physiology and Pharmacology Research Day, University of Western Ontario, London, Ontario. November 2015. [Poster].