Title
The role of transporters in nephrotoxicity: an investigation from the bench to populations-based studies

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The role of transporters in nephrotoxicity; an investigation from the bench to population-based studies

by

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DISSERTATION

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ABSTRACT

Renal transporters are primary modulators of drug disposition and elimination. The goals of this dissertation are: 1) to understand the role of renal transporters and genetic variation in renal transporters in disease through epidemiological studies and genetic association studies; 2) to characterize the role of transporters and assess the contribution of genetic variation in drug transport and cytotoxicity through functional experimental studies, 3) to identify and predict selective inhibitors of OAT1 and OAT3 through high throughput screening and computational modeling.

Our epidemiological study of tenofovir associated renal insufficiency demonstrates that tenofovir is associated with mild decline in renal function and that HIV therapy, in general, may improve renal function in women with pre-existing renal impairment. Functional studies demonstrate that SLC22A6 is a primary transporter for tenofovir and adefovir uptake in the renal proximal tubule. We found that rare variants in SLC22A6 may play a role in differential transport and cytotoxicity of tenofovir and adefovir. A genetic association study using DNA from 565 children with pediatric leukemia on methotrexate therapy demonstrated that in 8 candidate transporter genes chosen, 11 single nucleotide polymorphisms (SNPs) out of 44 SNPs in ABCC1, ABCC2, ABCC4, ABCG2, and SLC22A8 were significantly associated with changes in glomerular filtration rate (GFR) post-methotrexate. Haplotype blocks of the ABCG2 were found to be significantly associated with changes in GFR after methotrexate treatment. Findings from our chemo-informatics screening study suggest that SLC22A6 and SLC22A8 molecular interactors vary in structure and that small changes in electrostatics and steric effects can drastically change the inhibition activity of SLC22A6 and SLC22A8. A number of non-selective and selective inhibitors were identified. SAR studies identified several chemical descriptors unique to OAT1 and OAT3 interacting molecules.

This dissertation provides a unique look at drug-induced nephrotoxicity from the vantage of pharmacogenetic variation in the SLC22A6 and SLC22A8 transporters. It sets the stage for investigations where other genes involved in the nephrotoxicity pathways of these drugs may be investigated. It is our hope that insights from this dissertation can be further extended to other transporters in the SLC22 family and may inform clinical decisions in the future.

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Chapter 1

The Role of Transporters in Nephrotoxicity: An Investigation from the Bench to Population-based Studies

1.1 Introduction

The kidney is responsible for concentrating and eliminating many environmental agents including naturally occurring toxins and drugs and as such is a frequent site of drug toxicity. Nephrotoxicity represents a substantial problem in drug therapy as well as in the development of new drugs. Some reports demonstrate that up to 20% of acute renal failure cases are drug induced[2]. One study estimated that 14% of acute renal failure in the ICU was caused by drugs[3]. Nephrotoxicity may be defined in the following ways: 1) as a clinical measure of renal function that is medically relevant, or 2) as a comparison of the clinical measure of kidney function with a reference (relative change in renal function from baseline)[2].

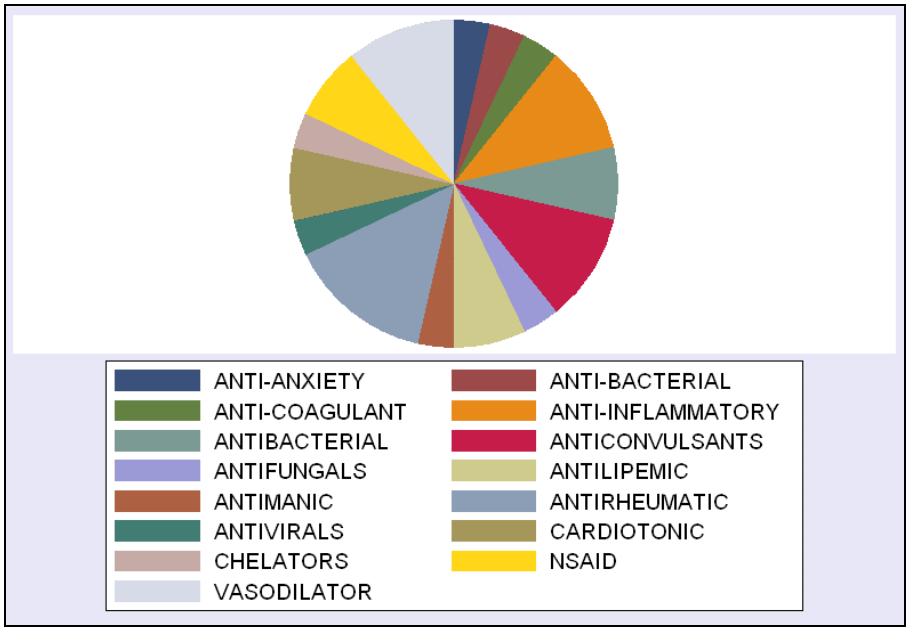

Figure 1.1 The distribution of drugs that cause drug-induced nephrotoxicity by therapeutic class compiled from an analysis of FDA approved drugs with known adverse drug reactions and Rxlist's top 300 prescription drugs.

The renal ultra structure and function makes the kidney susceptible to xenobiotic toxicity. The kidney is particularly vulnerable because of its high vascularity (25% of resting cardiac output is shunted through the kidney), the large surface area for nephrotoxin binding and transport across the renal epithelium of the renal proximal tubule[1]and its ability to concentrate solutes to be excreted from the body.[4]

The mechanisms of drug-induced nephrotoxicity vary and are dependent on the drug or class of drugs. Drugs from a wide variety of therapeutic classes are associated with nephrotoxicity (See Figure 1.1 and Table 1.1). Many risk factors associated with drug-induced nephrotoxicity have been identified. These factors may be patient (host) - associated or drug-associated. Patient associated risk factors include the individual's renal status, gender, age, diabetes, intravascular volume depletion, sepsis, concomitant use of other nephrotoxic agents and underlying pathological state[5].

Table 1.1 Drugs associated with nephrotoxicity in the top 300 prescribed drugs from Rxlist and their therapeutic class. Generic drugs were not included in the top 300 prescribed drugs.

Kidney	Therapeutics
Lisinopril	cardiotonic
Warfarin	anti-coagulant
Naproxen	anti-inflammatory
Lisinopril	cardiotonic
Lovastatin	anti-lipemic
Allopurinol	enzyme inhibitors
Metronidazole	radiation sensitizing
Gemfibrozil	anti-lipemic
Diclofenac	anti-inflammatory
Minocycline	anti-bacterial
Acyclovir	anti-viral
Methotrexate	anti-rheumatic
Phenytoin	anti-convulsant
Lithium	anti-manic
Ketoconazole	anti-fungal
Doxepin	anti-anxiety
Naproxen	anti-inflammatory
Amiodarone	anti-arrhythmic

Here we review the mechanisms of drug-induced nephrotoxicity and describe the major pathophysiologies. We review the current challenges in biomarker identification for renal toxicity and lastly, we describe the few studies that examined the role of polymorphisms in drug-induced nephrotoxicity.

1.2 Mechanisms of drug induced nephrotoxicity

Drug associated risk factors are dependent on the mechanism of nephrotoxicity of the agent and include factors that can be modulated such as concomitant drug use, degree of hydration, and urine pH. Many drug-induced toxicities are drug level dependent and require monitoring of drug levels to ensure their safe use, e.g., aminoglycoside antibiotics. Although there is great variation in susceptibility to kidney injury following exposure to such agents, this variation is due in large part to variation in pharmacokinetic factors including factors that control the exposure of the kidney to the drug. Thus, genetic variation in proteins that play a role in setting systemic as well as renal drug levels are important determinants of nephrotoxicity. These proteins include enzymes and transporters in the intestine and liver, and in the kidney. In some cases, the onset of nephrotoxicity is not clearly related to drug level e.g., non-steroidal anti-inflammatory agents. For these idiosyncratic drug reactions, variation in pharmacokinetic factors may not contribute or may contribute only a small part to the variation in nephrotoxicity. Table 1.2 lists some cohort studies that have drawn associations between genetic polymorphisms and drug-induced nephrotoxicity. Most of these studies included a small number of cases and have not been replicated.

Drug associated risk factors for nephrotoxicity are related to the mechanism by which the drug produces its injury to the kidney. The spectrum of mechanisms is wide (Figure 1.2). It includes hemodynamic renal failure and

vasoconstriction; tubular cell toxicity; obstructive nephropathy; osmotic nephrosis; and interstitial nephritis.

Table 1.2 Examples of cohort studies that have drawn associations between polymorphisms and drug-induced nephrotoxicity.

Drug	Drug Class	Form of Nephrotoxicity	Gene	Polymorphism	Reference
Tenofovir	Nucleotide reverse transcriptase inhibitor	Tubulopathy	ABCC2	G1249A	6
Ifosamide	Anti-cancer alkylating agent	Tubulopathy, Glomerular dysfunction	GSTP1 GSTT1	Deletion GSTT1 A313G GSTP1	7
Lisinopril	ACE inhibitor	End stage renal disease (ESRD)	ACE	I/I (insertion/insertion)	8
Ramipril	ACE inhibitor	Proteinuria/ESRD	ACE	D/D (deletion/deletion)	9
Cyclosporine	Immunosuppressive	Impaired renal function and chronic allograft nephropathy	ABCB1	T3435T	10

Thus, depending on the mechanism, different structures in the kidney may be affected:the glomerulus, renal tubules, interstitium or the blood vessels. Further, multiple

mechanisms are often induced because of pathophysiology of the disease[4, 11].

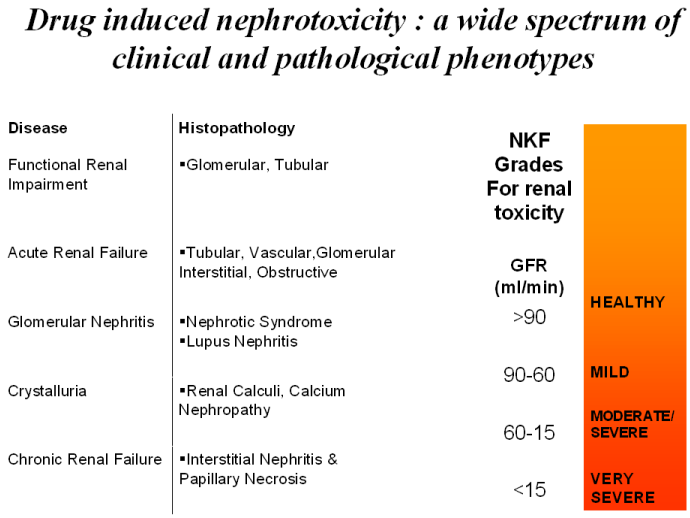

Figure 1.2 The spectrum of drug-induced nephrotoxicity is very broad incorporating many different pathophysiologies within renal dysfunction and injury. The National Kidney Foundation (NKF) has defined clinical measures using glomerular filtration rate (GFR) to estimate the degree of renal insufficiency

1.3 Forms of drug induced nephrotoxicity

1.3.1 Pre-renal effects

Drugs that decrease renal perfusion such as those decreasing cardiac output or increasing peripheral vasoconstriction can have an indirect effect on renal function by acutely decreasing glomerular filtration rate (GFR). Several forms of pre-renal drug induced damage include volume depletion, vascular occlusion, increased catabolism and altered hemodynamics[12].

Volume depletion can result from the excessive use of diuretics and laxatives. This can result in enhanced water and electrolyte loss. A chronic nephropathy can result from the hypokalemia from diuretic and laxative overuse. Lithium, a drug used for psychiatric disorders also presents with similar renal effects[12].

Alterations in renal hemodynamics may result in prerenal azotemia (high levels of nitrogen wastes in the blood) and subsequent acute renal failure. The kidney has

elaborate mechanisms for preserving its blood flow and one of the major mechanisms is through the cyclooxygenase enzymes, COX-1 and COX-2. Prostaglandins produced by arachidonic acid metabolism catalyzed by COX enzymes function in the preservation of renal function and blood flow[13]. Thus, inhibitors of COX enzymes such as Non Steroidal Anti-Inflammatory Drugs (NSAIDs) may produce vasoconstriction and renal ischemia resulting in acute renal failure[11]. COX-2 specific inhibitors appear to be as nephrotoxic as nonselective NSAIDs. For example, aspirin is least likely to cause renal failure whereas indomethacin has the greatest likelihood. Ibuprofen, diclofenac, and naproxen are intermediate in risk[3]. Patients with compromised renal blood flow as a result of pre-existing renal disease, sepsis and heart failure have greater risk for hemodynamic renal failure[3]. The risks for NSAID induced nephrotoxicity vary depending on the drug. Other agents that disrupt renal hemodynamics and thus cause a hemodynamic renal failure include agents that modify the renin-angiotensin systems such as ACE Inhibitors[14] and angiotensin II receptor blockers. ACE inhibitors cause vasodilatory effects on the efferent glomerular arterioles. Notably, polymorphisms in ACE the target for ACE inhibitors have been associated with nephrotoxicity. Calcineurin inhibitors (CNIs) such as cyclosporine cause a direct vasoconstriction to renal arterioles resulting in reduced renal perfusion, a fall in GFR, hypertension and ultimately nephrotoxicity. Vasopressors such as low-dose dopamine and epinephrine are commonly used in adult and pediatric ICU settings[3] and in conditions of chronic use are associated with renal hypoxia and acute tubular necrosis in patients with decreased renal perfusion (patients at severe risk for acute renal failure in hypovolemic, septic, cardiogenic shock)[3].

1.3.2 Obstructive nephropathy

Obstructive nephrotoxicity occurs when crystals are deposited in the kidney[11, 15]. Several poorly soluble drugs have been shown to precipitate in the distal tubule where urine is concentrated. These precipitates cause a nephropathy called crystal

nephropathy and lead to the development of tubular obstruction. Drugs associated with crystal nephropathy include the anti-viral drug, acyclovir, antibacterial sulfonamides, the anti-cancer drug, methotrexate, and the anti-viral protease inhibitor, indinavir[13]. Crystal formation is associated with high doses and is greater in the presence of low urine output. Weakly acidic drugs such as methotrexate may precipitate out at low urine pHs whereas bases such as indinavir may precipitate out at higher pHs[15]. Thus, risk factors for crystal nephropathy include pharmacokinetic risk factors, which produce elevated drug levels in the renal tubule, and factors that affect intravascular volume and concentrate the urine such as aquaporins. For example, heart failure and ascites will reduce effective intravascular volume, predisposing patients to crystal formation from drugs with low solubility. Thus increasing fluid volume by hydration or changing urine pH can reduce risk to crystal nephropathy. Other agents that cause obstructive nephropathy are anticoagulants, which cause bleeding and then clotting that can lead to uretric obstruction. Methyldopa, beta-blockers, ergot derivatives and methysergide have been shown to induce retroperitoneal fibrosis- or an overgrowth of fibrous tissue that obstructs the ureters[14].

1.3.3 Osmotic nephrosis

Renal tubular injury also occurs in the presence of hyperosmolar agents[15]. Such non-metabolizable agents are taken up into proximal tubule cells through pinocytosis and followed by cellular water accumulation due to an osmotic gradient. This results in swelling and vacuolization of the proximal tubule cells. The resultant swollen cells can produce an obstructive nephropathy, which results in acute renal failure. Compounds associated with this type of nephrotoxicity include contrast agents, immune globulin and hydroethyl starch. With the exception of underlying kidney disease and high doses of hyperosmolar agents, no risk factors for osmotic nephrosis have been identified[15].

1.3.4 Tubular toxicity

Direct toxicity to renal epithelia is associated with the use of many drugs including the anti-cancer drug cisplatin, the anti-fungal agent amphotericin B, aminoglycoside antibiotics, methoxyflurane and various anti-viral agents (e.g., adefovir, tenofovir)[15]. Direct nephrotoxicity gives rise to acute tubular or interstitial damage and renal papillary necrosis[14]. More specifically, these drugs may result in acute tubular necrosis, although a myriad of toxicities have been reported ranging from proximal tubule defects (Fanconi syndrome) to severe necrosis. The pathology of tubular toxicity is distinct from interstitial nephritis with loss of brush border, nuclear enlargement and cellular necrosis being the predominant histological features[11]. Unlike interstitial nephritis, there is little evidence of inflammation. In general, agents that cause acute tubular toxicity accumulate in renal tubule cells. Within the tubule cell, distinct mechanisms may lead to tubule toxicity depending on the agent. For example, aminoglycosides result in disrupted protein synthesis, mitochondrial function and protein reabsorption. Direct cell death may also occur from the lysis of lysosomal membranes that result in toxins concentrating in the cytoplasm[3]. Factors that enhance tubular exposure to these agents are potential risk factors for tubular toxicity. Risk factors for tubular toxicity include factors that control exposure of the tubule cells to toxins as well as factors that may be associated with the mechanism of the toxicity such as complex I and II in the electron transport chain.

1.3.5 Allergic or immunological renal damage

Some drugs can induce allergic or hypersensitivity reactions that can lead to vasculitis, interstitial nephritis and glomerulonephritis. The presentation of acute interstitial nephritis is a hypersensitivity reaction where there is a fall in GFR with

accompanying proteinuria, haematuria, and abnormal liver function. Drugs that have been associated with hypersensitivity leading to kidney toxicity include allopurinol, cimetidine, minocycline, penicillins, phenytoin, cephalosporins, clofibrate, isoniazid, thiazides, rifampicin, among others[14]. Another allergic nephropathy is chronic interstitial nephritis, where papillary necrosis is the major presentation and one of the most common forms of drug induced renal failure. This often occurs after prolonged use of analgesics. Lastly, drug-induced glomerulonephritis occurs from antibody complex accumulation in the glomerulus. The deposition of complement and immunoglobulins build up along the glomerular basement membrane and blood vessels and this leads to an inflammatory response. Examples of drug associated with this are NSAIDs, penicillins, probenecid, sulphonamides, thiazide diuretics, halothane and allopurinol[14].

1.3.6 Interstitial nephritis

Drugs that induce interstitial nephritis include: antibiotics (penicillins, cephalosporins, sulfonamides, ciprofloxacin, erythromycin), non-steroidal anti-inflammatory drugs and diuretics (furosemide and thiazides) along with the anti-coagulant, phenindione[4]. In the case of acute interstitial nephritis, if the reaction is severe, acute renal failure can occur. Acute interstitial nephritis is an idiosyncratic reaction primarily characterized by classical signs of hypersensitivity reactions such as fever, rash, and eosinophilia. Kidney manifestations include acute renal failure, pyuria, proteinuria, granulomas and eosinophiluria accompanied by interstitial inflammation. The mechanism of acute interstitial nephritis is thought to involve a hypersensitivity reaction to the drug as manifested by T cell infiltrates in the tubular interstitium[3]. Chronic interstitial nephritis is the most serious complication. Heavy metals (mercurial diuretics in particular), lead, and analgesics may induce chronic interstitial nephritis[4].

1.4 Current challenges in biomarkers for nephrotoxicity

Several challenges for the assessment of drug-induced nephrotoxicity exist. Major challenges are related to the use of biomarkers to diagnose drug-induced nephrotoxicity and to the agreement as to what defines "clinically relevant" changes in renal function. Although biomarkers for kidney function have been in existence for decades (e.g. serum levels of blood urea nitrogen (BUN) and creatinine), the consensus is that the non-linearity with glomerular filtration rate (GFR) may be a common cause of drug overdose; small changes in serum creatinine levels may reflect large changes in GFR. Serum creatinine is a late marker of nephrotoxicity that does not reflect real time, rapid changes in renal function. Early onset of renal injury is more subtle and may be reflected in minor changes in electrolyte excretion or excretion of microproteins[5]. Additionally, many factors beyond creatinine clearance affect the serum creatinine concentration[16]. Established relationships between GFR and creatinine as calculated by the Cockroft-Gault (CG)[17] and the Modification in Diet and Renal Disease equation (MDRD)[16] are mostly validated in individuals with abnormal kidney function. Consequently, these biomarkers have been used primarily to detect and diagnose severe changes in kidney function and severe loss of kidney function.

Pharmacogenomic studies may focus on changes in individuals with normal kidney function in which a change in renal function may not be in the range of severe renal insufficiency. Biomarkers obtained from urinary excretion of enzymes such as alanine aminopeptidase and N-acetyl-β-glucosaminidase have been used in recent studies[18]. Genomic markers such as kidney injury molecule (Kim-1) may also be used as markers of renal injury[5]. Cystatin C, a relatively new biomarker of nephrotoxicity, may be used although it is even less well established as a biomarker of nephrotoxicity. Other indicators such as BUN and glucose are associated with a host of other confounders[18].

1.4.1 Currently used biomarkers for renal toxicity

Controversy remains about appropriate indicators of "renal insufficiency." Parameters used for identifying nephrotoxicity are altered renal tubular function (urinary enzymes, casts), declines in glomerular filtration (such as decreased creatinine clearance and increases in serum creatinine levels). However, biomarkers for nephrotoxicity may not exhibit changes in renal injury. Recently the increase in serum creatinine concentration equal to or greater than 0.4 mg/dl has been superseded by use of estimated creatinine clearance values calculated from algorithms such as Crockoft-Gault and the Modification in diet and renal disease (MDRD) equations that benchmark severe nephrotoxicity as a fall in GFR of equal or greater than 50%[16, 17]. The National Kidney Foundation's classifications of renal insufficiency have recently gained popularity as indices for degree of renal impairment (Grade I: GFR $\geq$ 90, Grade II: 60 $\leq$ GFR < 90, Grade: III 30 $\leq$ GFR < 60, Grade IV: 15 $\leq$ GFR < 30, Grade V: GFR < 15 [19]. Currently, definitive diagnosis of nephrotoxicity is in most cases supported by several other pieces of clinical evidence such as renal biopsy and urine-analysis. For example, evidence implicating tenofovir-associated renal insufficiency is a large fall in estimated GFR, proteinuria, phosphaturia, glucosuria and elevated potassium concentrations. Evidence of improvement in renal function or subsequent fall in renal function through the removal or rechallenge of the offending drug is a third method for assessing association of drug-induced nephrotoxicity.

1.5 Transporters and drug-induced renal toxicity

Membrane transporters play a crucial role in controlling drug levels and therefore may be determinants of intracellular accumulation of toxins in the renal epithelium. Examples of molecules that are nephrotoxic only after being transported into renal tubular cells are cephalosporin antibiotics, which have been shown to produce proximal

tubular necrosis after transport into those cells. Citrinin, a secondary product of fungal metabolism, also produces proximal tubular necrosis, but only after transport into proximal tubular cells [20]. Cephalosporins and citrinin are both substrates of OATs[20]. Various glutathione conjugates (e.g., S-(1,2-dichlorovinyl) glutathione [DCVG]) also are transported into proximal tubular cells resulting in nephrotoxicity. Finally, certain heavy metals also are transported into renal tubular cells[20].

In the human kidney specifically, some common transporters known to transport nephrotoxic agents are the organic anion transporters, OAT1 (SLC22A6), OAT2 (SLC22A7), OAT3 (SLC22A8), OAT4 (SLC22A11), URAT1 (SLC22A12), OATP1A2 (SLCO1A2), BCRP (ABCG2), MRP2 (ABCC2) , MRP4 (ABCC4), P-gp (ABCB1), and the organic cation transporters OCT2 (SLC22A2), OCT3 (SLC22A3) and OCTN2 (SLC22A5) . The following summarizes the three major classes of drugs that are known to be involved in nephrotoxicity and their interactions with transporters.

1.5.1 Transporters and antiviral-induced nephrotoxicity

With respect to antiviral induced nephrotoxicity, nucleoside analogues have been shown to be associated with tubular toxicities. These analogues enter the cell through influx transporters, human organic anion transporters 1 and 3 (OAT1 and OAT3) or human organic cation transporters (OCTs). Efflux transporters, multi-drug resistance transporters and P-glycoprotein (MRP2, MRP4, P-gp) actively remove the drugs. Adefovir, tenofovir and cidofovir accumulation in the cell has been linked to proximal tubular dysfunction[21, 22]. These nucleotide reverse transcriptase inhibitors are known substrates for OAT1 and OAT3[23]. Zidovudine, stavudine, didanosine, zalcitabine, and lamivudine, also are substrates of OAT1, and acyclovir and ganciclovir have been shown to be substrates for OCT1. The efflux transporter MRP4 has recently been shown to

interact with adefovir and tenofovir, while peptide transporters PEPT1 and PEPT2 mediate valacyclovir transport (Figure 1.3).

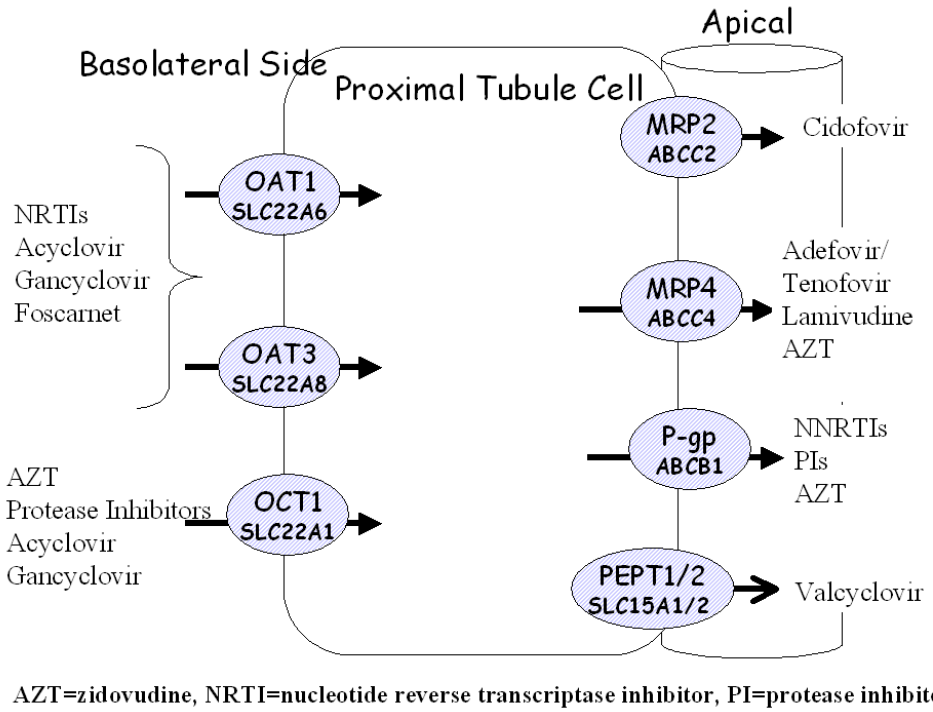

Figure 1.3 Transporter mediated pathways for antiviral accumulation in the proximal tubule adapted from Izzedine and colleagues[1]. Several of these transporters have overlapping substrate specificity.

1.5.2 Transporters in antibiotic induced nephrotoxicity

Tetracycline is active against a broad spectrum of bacteria. Susceptible strains include a wide range of Gram-positive and Gram-negative bacteria, Mycoplasma, Rickettia and chlamydia. Tetracyclines are classified as short-acting (tetracycline and oxytetracycline), intermediate- acting (demeclocycline and methacycline) or long-acting (doxycycline and minocycline) based on their serum half-lives. Studies have suggested that the OATs mediate the urinary excretion and reabsorption of tetracyclines[24].

Tetracyclines have been shown to interact with OAT1 and OAT3 for influx into the renal tubular cell and OAT4 for reabsorption[24].

1.5.3 Transporters in NSAID induced nephrotoxicity

Despite the widespread use of non-steroidal inflammatory drugs (NSAIDs), renal toxicity is an adverse drug reaction that has been reported with their use. The inhibition of penicillin by NSAIDs[25] and the observation that probenecid inhibits NSAID secretion[26] suggested that the organic anion transporters (OATs) could possibly transport NSAIDs. Ibuprofen (OAT1, OAT3), ketoprofen (OAT1, OAT3, OAT4), indomethacin (OAT1, OAT3), salicylate (OAT1, OAT2, OAT3 and OAT4) have been shown to be OAT substrates. Moreover, inhibition studies have shown that NSAIDs in general can interfere with OAT mediated transport[27-30]. Proximal tubular nephropathy and renal papillary necrosis have been linked with acetylsalicylate, salicylate, ibuprofen and mefenamic acid[31-33]. The interaction of NSAIDs with rat isoforms of the organic anion transporters, rOat1, rOat3 and human OAT4 implicates these transporters in playing a large role in drug-induced nephrotoxicities. NSAID induced suppression of prostaglandin synthesis that can result in acute renal failure due to decreased renal blood flow may be modulated by OAT mediated renal transport of NSAIDs.

1.5.4 Transporters in anticancer agent-induced nephrotoxicity

The clinical application of a variety of platinum anti-cancer agents has been limited due to their severe nephrotoxicity. Cisplatin (*Cis*-diamminedichloroplatinum II), is an anti-cancer treatment for solid tumors of the prostate gland, bladder, colon, lung, testis, and brain. Twenty percent of patients receiving high-dose cisplatin have severe renal dysfunction as cisplatin's chief dose-limiting side effect is nephrotoxicity. Cisplatin-DNA crosslinks are associated with cytotoxic lesions in tumors and other dividing cells. Quiescent proximal tubule cells are selectively damaged by cisplatin after its conversion

to reactive thiol through glutathione conjugation, metabolization and cysteine conjugation[34]. Recent studies suggest that inflammation, oxidative stress injury, and apoptosis probably explain the mechanism of cisplatin toxicity.

The organic cation transporters OCT1 (*SLC22A1*), OCT2 (*SLC22A2*), and OCT3 (*SLC22A3*) belong to the SLC22A family[35-37] of transporters. Previous studies of the interaction of cisplatin with the OCTs suggest that cisplatin is not a substrate of human OCT1 but it is for OCT2[38, 39]. Ishida and colleagues demonstrated that the human copper influx transporter Ctr1 is a primary driver in the cellular uptake of cisplatin[40]. The human copper efflux transporters ATP7B and ATP7A[41, 42] also recognize these platinum compounds, and their elevated expression has been associated with cisplatin resistance.

Another agent known to induce renal insufficiency is methotrexate (MTX). MTX therapy is used for the treatment of cancer as well as rheumatoid arthritis. The drug is also associated with crystalluria, which is thought to be due to the concentration of the 7-OH metabolite of MTX in the urine. Co-administration of probenecid with MTX leads to delayed elimination and increased serum half-life for MTX that increases systemic toxicity[20]. Co-administration of NSAIDs, such as indomethacin, caused acute renal failure. Influx transporters OAT1[43], OATP1A2 [44], OAT2[45], OATP-H and OAT3[46] and several rat and mouse isoforms have been demonstrated to transport methotrexate[30, 47-49]. MRP1[50], MRP2[51, 52], MRP3[53], MRP4[52] , MRP5[54], and ABCG2[55] are efflux transporters that have been shown to efflux methotrexate. Figure 1.4 illustrates the transporters involved in the uptake and efflux of MTX.

Several other drugs that are known to interact with the OATs are the chlorinated phenoxyacetates (herbicides), mercury, cadmium and ochratoxin A. All of these compounds are associated with nephrotoxicity.

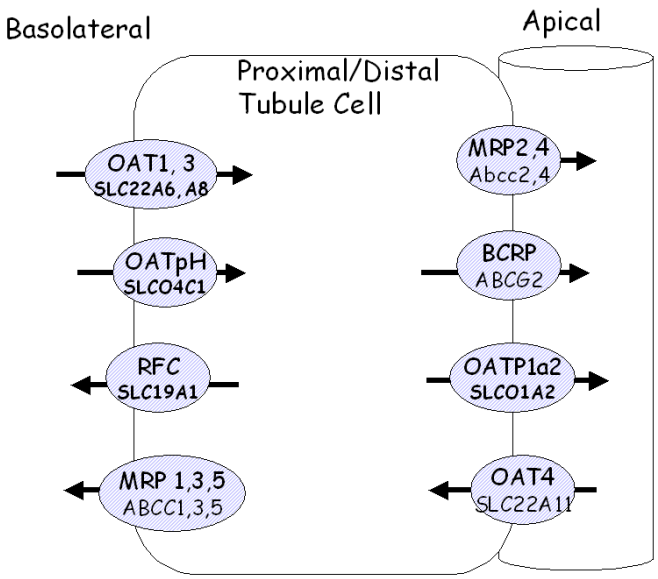

Figure 1.4 Transporters in the proximal tubule and/or the distal tubule that are known to interact with methotrexate. Influx transporters OAT1, OAT3 and OATP-H and efflux transporters MRP1, MRP3, MRP5 and the reduced folate carrier transporter (RFC) are localized on the blood side of the tubule while efflux transporters MRP2, MRP4, BCRP and OATP1A2 along with the reabsorptive transporter OAT4 are localized on the apical lumen.

1.6.The role of genetic variation in membrane transporters in drug-induced renal toxicity

There is limited evidence of the role of genetic variation in membrane transporters in drug-induced renal toxicity. Table 1.2 lists the studies that have looked at the role of genetic variation in drug-induced nephrotoxicity in cohorts. Cyclosporine (CsA) nephrotoxicity is comprised of two different forms of renal impairment-acute or functional nephrotoxicity caused by pre-renal hemodynamic alterations of CsA and chronic nephrotoxicity through long-term CsA exposure. Pre-renal CsA toxicity is modulated by factors in the renin-angiotensin-aldosterone system, the endothelin and nitric-oxide pathway and vasoconstriction through CNS activation. The factors that govern CsA induced chronic nephrotoxicity are angiotensin II, TGF-β and apoptotic genes. Interestingly, the increased expression of multi-drug resistance transporter P-gp

(ABCB1) in proximal tubular cells has also been correlated with decreased CsA deposits and renal tissue and decreased interstitial fibrosis. The C3435T allele was associated with a 2-fold decrease in P-gp expression in homozygous individuals. In a case-control study, 18 of 97 transplant recipients showed complete recovery of renal function when taken off of cyclosporine and switched on to a calcineurin inhibitor[10]. The study found that the donor's ABCB1 3435TT genotype was 13 fold more likely to be associated with CsA nephrotoxicity suggesting that P-gp may play a significant role in CsA nephrotoxicity.[10]

In the area of HIV, polymorphisms in transporters that are thought to modulate the accumulation of tenofovir in the proximal tubules are likely candidates for candidate gene studies for transporter variants that may predispose individuals to tenofovir induced nephrotoxicity. The efflux transporter, multi-drug resistance transporter 2 MRP2 (ABCC2) haplotype CATC was significantly associated with the onset of TDF-induced renal proximal tubulopathy, while CGAC was found to be a protective haplotype[6]. In another study, another efflux transporter multi-drug-resistance transporter (ABCC4) 3463G variant was associated with 35% higher tenofovir concentrations than patients with the wild type ABCC4 [56]. Both studies included few cases and it is not known whether these studies can be replicated.

While ACE inhibitors can slow the progression of diabetic renal disease, at comparable levels of blood pressure control, ACE inhibitors limit proteinuria and end stage renal disease progression in both diabetic and non-diabetic nephropathies. The favorable effect of this blockade is variable and possibly due to genetic factors. Candidate genes that predispose to renal disease progression include the genes that encode the RAS (renin-angiotensin system) proteins and the ACE gene. Recently, the insertion/deletion(I/D) polymorphism in the ACE gene was found to address the reno-protective effect of ACE inhibitor therapy. Further, the I/D polymorphism was found to be

a strong predictor of ACE inhibition-associated reno-protection whereby change in GFR, proteinuria and progression to end stage renal disease were reduced in patients with the DD genotype compared to those with the II or ID genotype[9]. Moreover, a study to determine whether gender or the I/D polymorphism affected patients' disease outcome and patient response to treatment found that proteinuria and change in GFR decreased in women with the DD or II or ID genotype and in men with the DD genotype, but did not change in men with the II and ID genotypes. Thus, in general, men with chronic proteinuric nephropathies are at increased risk of progression due to their lower response to ACE inhibitor treatment.

Decreased GFR and proximal and distal renal tubular dysfunction are diagnosed in 30-70% of children treated with the cytostatic alklyating drug ifosfamide, while 5-8% experience serious to irreversible renal insufficiency with rickets and Fanconi syndrome. Ifosfamide metabolites are formed through oxidation. These metabolites are thought to deplete intracellular glutathioine and thus be the cause of the toxicity. Due to studies in adults that demonstrated interindividual differences in urinary excretion of ifosfamide metabolites, the glutathione S transferases (GSTs) are good candidates for pharmacogenetic study. The GSTs are enzymes responsible for activation or detoxification of electrophilic compounds including ifosfamide metabolites. The mu, pi and theta classes of the GSTs are the GSTM1, GSTP1, and GSTT1 genes. In a study of 76 children, a deletion of the GSTT1 gene was correlated with accumulation of un-metabolized ifosfamide.[7] Concentrations of metabolites were higher in children with a polymorphism in GSTP1 and were thought to correlate with frequency of ifosfamide nephrotoxicity [7]. The high frequency of these polymorphisms in Caucasians (50% have the deletion of GSTT1 and the GSTM1 0/0 genotype) increases the relevance of investigating these genetic polymorphisms.

In the area of anti-cancer therapy, Hulot and colleagues investigated the impact of one heterozygous mutation in the efflux transporter multi-drug resistance protein 2 (ABCC2)[51] on one individual who suffered from impaired MTX elimination and who experienced acute renal failure from severe MTX overdosing. This patient had an A to G mutation in position 412 of MRP2. When the effect of this mutation was studied in-vitro, a significantly lower MTX efflux was observed in cells expressing the variant compared to reference MRP2.

1.7 Dissertation objectives

The objective of this dissertation is to define the role of transporters in nephrotoxicity. Studies in this dissertation highlight the importance of transporters as primary modulators of drug disposition and elimination, as genetic targets for pharmacogenetic interventions, and drug targets for influencing drug safety and efficacy. This dissertation focuses on understanding the role of renal transporters in disease by studying the epidemiology of antiviral-induced and anticancer-induced nephrotoxicities and by assessing the contribution of genetic variation in transporters in these diseases. In experimental studies, the role of transporters in the influx of antiviral drugs is characterized and how genetic variation can play a role in changing drug transport capabilities is determined. Lastly, using a chemocentric and informatics perspective together with screening methods, selective inhibitors of OAT1 and OAT3 are identified and chemical signatures that predict inhibitor activity are computationally determined.

Chapter 2. Epidemiology of Tenofovir-associated renal insufficiency

The prevalence and risk factors for tenofovir-associated nephrotoxicity were determined in a cohort of women living with chronic HIV infection. We compared the prevalence and degree of renal impairment associated with tenofovir to those of other anti-retrovirals (ARVs) in HIV-infected women and identified characteristics associated with the greatest decline in creatinine clearance. Our analysis suggests that the prevalence of tenofovir-associated renal insufficiency in women infected with HIV appears to be in the range of that reported in mostly male cohorts. We demonstrate that tenofovir use is associated with mild decline in renal function. In contrast, a proportion of women also show an improvement in GFR, perhaps due to treatment of HIV.

Chapter 3. Role of organic anion transporters and their genetic variants in the transport and cytotoxicity of Tenofovir and Adefovir

The transport and cytotoxicity of tenofovir and adefovir, two antiviral drugs, by human organic anion transporter 1 (OAT1) was investigated. We confirmed that OAT1 transports tenofovir. Studies in stably transfected cell lines demonstrated that the potency of tenofovir-induced cytotoxicity in OAT1 transfected cells was over 28 fold greater than in empty vector transfected cells (EC_{50} 6.7 μM vs. 200 μM). Cells expressing six OAT1 variants exhibited similar rates of transport and cytotoxicity profiles when treated with tenofovir and adefovir. However, one variant, the R454Q OAT1 variant showed reduced function. Transport and cytotoxicity were markedly reduced in this transfected cell line compared to reference OAT1. Collectively, these data suggest that tenofovir is transported by OAT1 and that cells expressing these transporters, such as human renal proximal tubule cells, are more susceptible to tenofovir-induced cytotoxicity. We established that probenecid, an inhibitor of organic anion transport, could protect cells from the cytotoxic effects of tenofovir.

Chapter 4. A candidate gene study of renal transporters in methotrexate-induced nephrotoxicity

To investigate whether renal membrane transporters are significantly associated with MTX-induced nephrotoxicity, we conducted a pharmacogenetic analysis of 44 SNPs in 8 transporter genes in a cohort of 556 pediatric acute lymphoblastoid leukemia (ALL) patients. We determined whether MTX is associated with a decline in glomerular filtration rate in this cohort. We demonstrated that multiple SNPs in several influx and efflux (OAT1, OAT3, MRP4, ABCG2) renal membrane transporters may play a key role in determining an individual's predisposition towards MTX-induced nephrotoxicity. We determined the influence of ABCG2 haplotypes on methotrexate renal toxicity. We also identified haplotype blocks within ABCG2 that may be the primary drivers of significant GFR change post MTX treatment.

Chapter 5. Predicting and identifying selective inhibitors of organic anion renal transporters

Novel selective inhibitors of renal organic anion transporters can be extremely important as therapeutic agents as well as drugs to prevent off-target effects. We screened over 4000 compounds in two random drug libraries to identify selective inhibitors for organic anion transporter 1 and 3 (OAT1 and OAT3). This study is in progress and on-going. In studies not shown in this dissertation, we identified several compounds in our screen that demonstrated aggregation. It is important to note that these aggregates may potentially confound our current findings. In this chapter we proceed to report our initial findings. In a preliminary analysis, we identified several selective inhibitors for OAT1 and OAT3. Using our screening data, we used a ligand-based algorithm (Similarity Ensemble Approach SEA) to predict novel inhibitors. Preliminary observations suggest that there are several predominant structural features

in the ligands that are selective for OAT1 and OAT3. Our SAR study identifies pharmacophores that may be used to distinguish OAT1 inhibitors from OAT3 inhibitors.

Chapter 6. Discussion and summary

This chapter summarizes the findings of the dissertation and provides suggestions for future directions.

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Chapter 2

HIV and Epidemiology of Tenofovir Associated Renal Insufficiency

2.1 Introduction

The antiretroviral medication, tenofovir, has become one of the most widely used drugs in highly active antiretroviral therapy (HAART) regimens since its accelerated FDA approval in 2001[57]. Tenofovir is a nucleotide reverse transcriptase inhibitor (NtRTI) for the treatment of human immunodeficiency virus (HIV). Tenofovir's long half-life, once a day dosing, co-formulation with other agents, utility in treating hepatitis B-HIV co-infected patients[58], and therapeutic efficacy have contributed to its widespread prescription use. A fixed dose combination of tenofovir, emtricitabine, and efavirenz (*Atripla™*) has recently been approved as the only once-a-day combination tablet for the treatment of HIV infection in adults[59]. The availability of this combination product will likely further increase the prevalence of tenofovir use in the domestic and international setting. Clinical trials of tenofovir as a single agent in pre-exposure prophylaxis for HIV are underway[60] and may lead to increased use of the drug in HIV-uninfected populations. Consequently, any significant adverse events associated with tenofovir use will have widespread implications.

Although pre-clinical studies and phase III trials demonstrated that tenofovir-associated renal impairment was rare, recent case reports and post-market surveillance in large observational cohorts show that tenofovir-associated renal toxicity may be more common than previously reported[21, 61-68]. In well-controlled randomized trials, similar renal safety of tenofovir (TFV) and other nucleoside reverse transcriptase inhibitors (NRTIs) have been reported[69, 70]. However, in an overview of ten non-randomized studies a prevalence of tenofovir (TFV)-associated renal insufficiency that ranged from

1-4%[71] was found. These rates of renal insufficiency on TFV in the nonrandomized setting may reflect factors in "real-world" drug therapy that potentiate TFV-associated toxicities. The mechanism of TFV associated renal toxicity is not known, but may be related to mitochondrial toxicity [72] .

2.2 Rationale for studying the natural history of tenofovir induced nephrotoxicity

By the beginning of 2004, approximately 1 million people in the US living with HIV/AIDS, twenty-seven percent were women. Infection rates in women are rising faster than in men in many regions[73]. By the end of 2003, over 75% of the women living with HIV were women of African American or Latino ancestry, demonstrating the marked racial and ethnic diversity of HIV-infected women in the U.S[73]. AIDS remains the top cause of death for African American women between the ages of 25 and 34 in this country. The increasing diversity of the epidemic in the U.S. and worldwide mandates the study of adverse effects on antiretroviral therapy in women and non-Caucasian populations. Randomized and prospective studies on the incidence of renal failure on TFV have been performed in predominantly male cohorts[63, 73-75]. There have been several studies on the impact of estrogen on the kidney suggesting that men may be more susceptible to kidney dysfunction, irrespective of etiology than women, due to the effect of estrogen on the renin-angiotensin system and other cellular repair machinery[76]. Identifying rates of renal impairment on TFV-based therapies and concomitant factors that may contribute to TFV- associated renal insufficiency in women may reveal modifiable risk factors for toxicities in women that are distinct from men.

2.3 Objective

The goal of this study was to ascertain the prevalence and to identify risk factors of TFV-associated renal impairment in women living with chronic HIV infection. We

compared the prevalence and degree of renal impairment associated with TFV to those of other anti-retrovirals (ARVs) in HIV-infected women and identified characteristics associated with the greatest decline in creatinine clearance. The setting of our study is the Women's Interagency HIV Study (WIHS), a large US multicenter prospective cohort study of HIV-infected women established in 1994[77]. This cohort is representative of the racial and ethnic diversity among HIV-infected women in this country and serves as a "real-world" population in which to study adverse effects of anti-retroviral agents.

2.4 Methods

Participants in the WIHS cohort are seen semi-annually for questionnaire administration, including current antiretroviral use, physical examination and various laboratory measurements, including serum creatinine. The present analysis uses patient data from 1995 to 2005. Creatinine clearance was calculated using the Modification of Diet in Renal Disease equation (MDRD) that estimates creatinine clearance (as a measure of GFR) based on sex, age and African American race of the subject [16]. Only subjects with creatinine measurements at one or more visits prior to tenofovir use were included in the dataset. Baseline GFR, prior to any tenofovir use was calculated by averaging the preceding two creatinine clearance measurements prior to tenofovir treatment to reduce measurement error and minimize regression to the mean in subgroups defined by baseline GFR values. If only one GFR measurement was available, that value was used as the baseline GFR. For random effects repeated measures regression analyses, to ensure that data used were adequately reflective of duration of tenofovir use, only GFR measurements during the first episode of tenofovir treatment were used in the analysis. No data were used after tenofovir was discontinued. The outcome was the log transformed GFR measurement. To determine the estimated associations of GFR with predictors in the model, the exponentiated

regression coefficients from the regression model were back transformed to give the percent effects.

The three main approaches used in the analysis were paired t-tests to model the level of GFR change from baseline to various narrow time intervals during tenofovir, multivariate random intercept random effect model on the duration of tenofovir utilizing GFR as a repeated numeric outcome variable, and survival analysis using a greater than 25% decrease in GFR from baseline to a GFR below 60 ml/min as the clinically relevant endpoint variable. The repeated numeric outcome variable assesses the level of ongoing change in GFR associated with tenofovir use. For comparison purposes, similar analyses were performed for abacavir, a nucleoside reverse transcriptase inhibitor. The analyses of the association between abacavir and change in GFR were limited to the follow up period after 2001 (the time when tenofovir was on the market).

2.5 Results

2.5.1. Prevalence of tenofovir associated renal insufficiency

There were 394 women who were treated with tenofovir with the duration of tenofovir use ranging from 3 to 42 months. Their demographic characteristics and risk factors are presented in Table 2.1. Among the 394 women treated, 190 or 48% experienced a decline in GFR below 90mls/min, 21 (5%) experienced a drop below 60mls/min, and 3 (1%) dropped below 30 mls/min. Among the 255 women on tenofovir who had baseline renal function above 90 mls/min, 70 (18%) experienced a drop below 90mls/min, 3 (1%) experienced a drop below 60 mls/min and none had GFRs that fell below 30mls/min. There were 5 women who experienced a greater than 50% change in GFR from baseline (see Table 2.2). These women ranged from 42-56 years of age at the time of visit. Their baseline GFRs varied from healthy renal function to severely nephrotoxic at baseline. Among the 5 women, three had normal to moderately healthy

renal function at baseline. BMI ranged from 15-33. Most of these women were African American with one being of Hispanic and one being Caucasian.

Table 2.1 Patient clinical and demographic characteristics of women taking tenofovir in WIHS

Variable	N	Outcome
Total women	394	
Ethnicity n (%)		
White	51	13%
Black	204	52%
Hispanic	132	34%
Asian	2	1%
Other	5	1%
Concomitant Antiretroviral Therapy		
PI	198	50%
NRTI	155	39%
NNRTI	374	95%
Ritonavir	322	82%
Didanosine	327	83%
Other nephrotoxic agent	84	21%
Renal Insufficiency Risk Factor		
GFR (mls/min, median and range)		96.4 (37.0-189.9)
Injection drug users at baseline	104	26%
Hepatitis C	104	26%
Diabetes	96	24%
Women with Grade 1 or worse renal abnormalities at baseline	255	65%
Women with Grade 2 or worse renal abnormalities at baseline	155	39%
Women with Grade 3-5 renal abnormalities at baseline	14	4%
Median Age (range)		34 (19-61)
Individuals with Systolic Blood Pressure above 140	35	9%
Individuals with Diastolic Blood Pressure above 90	45	9%
Body mass Index (median)		26.8 (17.2-61.3)
HIV-related Risk Factors		
Median Viral Load		6500 (80-6mil)
Median CD4 count		323 (2-1505)

Because one of the women experienced a very large decline in GFR (a 94.3% drop) after tenofovir treatment, we considered excluding her from the random effects random intercept analysis. A sensitivity analysis conducted excluding this individual yielded similar results as an analysis with the individual included. Thus, we decided to include this individual in the final analysis.

Table 2.2 Profile of individuals who had large changes in their creatinine clearance after tenofovir treatment

Age (yrs)	Avg.GFR post tenofovir (mls/min)	Baseline GFR (mls/min)	Percent Effect	Months on tenofovir	BMI	Race
44	14.5	35.9	-59.6	5.0	15.4	Hispanic
56	6.86	16.3	-57.9	9.4	33.8	Af..Am.
42	63.3	141.2	-55.2	10.7	20.9	Af..Am.
49	43.8	98.3	-55.4	20.2	17	A..Am.
49	4.3	76.1	-94.3	24.4	28.6	Caucasian

2.5.2 Renal function after tenofovir use over time.

Figure 2.1 illustrates the proportion of women who were in each NKF grade over the course of 6 time periods on tenofovir. Approximately 50% of women had healthy or grade 1 renal function, and the majority of the remainder resided in grade 2 (mild renal impairment). A few women fell into grade 3, 4 and 5.

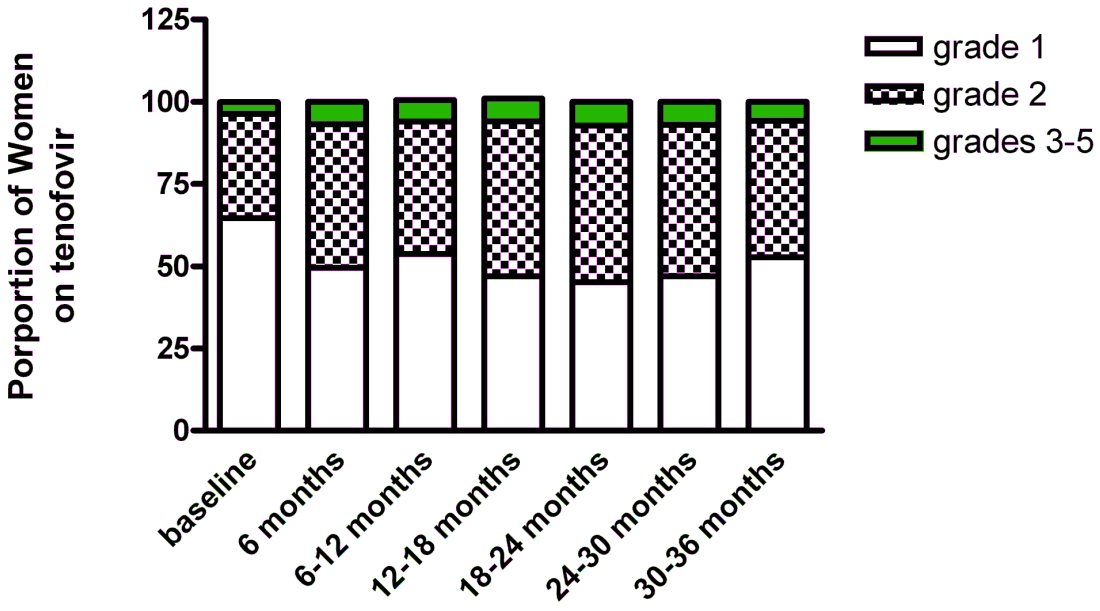

Figure 2.1 Percent of women on tenofovir with grade 1-5 renal function at 6 month intervals on tenofovir

Paired t-tests were used to compare pre-tenofovir GFR with post-tenofovir GFR at six month intervals. Average GFR declined from 3.6 to 7.38% at six month intervals up to 3 years on tenofovir. After 0 to 6 months, 6 to 12 months, 12 to 18 months, 18 to 24 months, 24 to 30 months and 30 to 36 months on tenofovir, the difference in means was 3.57 mls/min, 4.54 mls/min, 4.93 mls/min, 5.06 mls/min, 7.38 mls/min and 4.29 mls/min from baseline, respectively (Table 2.3).

Table 2.3 Mean changes and paired t-tests to compare calculated GFR at baseline to calculated GFR on tenofovir at 6 month intervals

Month intervals	Number of women	Decrease in GFR (mls/min) from baseline	P value	95% CI	
0-6	206	3.57	0.005	1.08	6.06
6-12	289	4.54	0.62	-11.22	6.69
6-18	190	4.93	0.0005	2.20	7.67
18-24	119	5.06	0.0057	1.5	8.63
24-30	81	7.38	0.0014	2.93	11.81
30-36	51	4.29	0.18	-2.11	10.69

2.5.3 Renal function after tenofovir treatment in women with health kidneys and in women with pre-existing renal insufficiency

In a random intercept random slope repeated measures analysis of renal decline on tenofovir where the outcome was a change in GFR level (Table 2.4), the covariate that was statistically significantly associated with change in GFR level was age, -1% per year (P<0.001). Concomitant ritonavir use was not statistically significantly associated with change in renal function; GFR increased 4.1% from baseline (p=0.51) (CI: -7.6%-17.2%). After controlling for a number of covariates, tenofovir use was associated with a 2.4% decrease in GFR from baseline.

Table 2.4 Repeated measures random intercept random effects analysis of women on tenofovir for 3-42 months

Variable	Percent change in GFR from baseline	P value	95% CI	
Age at visit	-1.0%	0.0001	-1.4%	-0.7%
Tenofovir use	-2.4%	0.104	-5.2%	0.5%
African American	15.8%	0.001	6%	23%
Hispanic American	5.4%	0.271	-4%	15%
Diabetes	1.1%	0.424	-2%	4%
Injection drug user	-6.2%	0.309	-19%	6%
Hepatitis C	-2.1%	0.563	-9%	5%
High Systolic blood pressure	0.9%	0.73	-4%	6%
High Diastolic blood pressure	4.0%	0.101	-1%	9%
CD4 count (per 100 cells/ml)	0.3%	0.277	0%	1%
Log viral load (centered at 5)	0.2%	0.56	0%	1%
Concomitant PI use	-2.6%	0.249	-7%	2%
Concomitant NNRTI use	0.2%	0.918	-4%	5%
Concomitant NRTI use	-1.7%	0.509	-7%	3%
Body mass index (centered at 27)	-0.2%	0.265	-1%	0%
Other nephrotoxic drug	-1.4%	0.54	-6%	3%

When baseline renal function and tenofovir use (Table 2.5) was taken into account, women with healthy renal function at baseline experienced an average decline in GFR of 5.6% (p =0.001) whereas women with baseline renal function grade 2 experienced an average improvement in renal function of 9.3% (p < 0.001) after tenofovir exposure. Women with baseline renal function grade 3 experienced approximately 1% average decreases, although this value was not statistically significant (p=0.89). Controlling for all of the same covariates, there was a slight trend towards significance (an 11.1% decrease in estimated effect of tenofovir on GFR) (p =0.073) (CI: -1% to -24.7%) in the interaction effect of tenofovir on GFR between African Americans and Caucasian women.

Table 2.5 Effect of tenofovir on GFR in 394 women on tenofovir accounting for baseline renal function

Variable	N	Percent change in GFR from baseline	P value	95% CI	
Tenofovir use (regardless of baseline renal function)	394	-2.4%	0.104	-5.2%	0.5%
Tenofovir use in women with healthy renal function at baseline	256	-5.6%	0.001	-8.9%	-2.5%
Tenofovir use (baseline renal function Grade 2)	125	9.3%	<0.001	4.0%	13.7%
Tenofovir use (baseline renal function Grade 3)	12	-0.9%	0.89	-13.8%	12.0%

The model used in the table above was adjusted for the same covariates as in Table 2.4

Repeated measures analysis of women on abacavir was also conducted (Table 2.6). The percent effect on GFR was +1.6% and not statistically significant after adjusting for all of the same covariates in Table 2.4. Use of abacavir in women with healthy renal function was associated with a 10% decrease in renal function. While women with baseline grade 2 and baseline grade 3 renal function experienced a significant improvement in renal function of 15% and 62%, respectively (Table 2.6).

Table 2.6 Percent effect on GFR level in 423 women on abacavir accounting for baseline renal function

Variable	N	Percent change in GFR from baseline	P value	95% CI	
Abacavir Use	423	1.6%	0.85	-5.7%	9.4%
Abacavir use in women with healthy renal function at baseline	243	-9.6%	0.010	-16.3%	-2.4%
Abacavir use (baseline renal function Grade 2)	145	15.3%	0.003	4.9%	26.8%
Abacavir use (baseline renal function Grade 3)	25	62.0%	<0.001	34.5%	95.2%

The model used in the table above adjusted for the same covariates as in Table 2.4

2.5.4 Incidence rate of renal insufficiency after tenofovir treatment among tenofovir users and abacavir users

We included a total of 825 women in our survival analysis from both the subset of tenofovir and abacavir treated women. There were 61 events in 17360 person years in both groups. There were 19 events in the tenofovir group and 42 events in the abacavir group. The incidence rate among tenofovir users of experiencing a 25% decrease in

GFR from baseline to a GFR below 60mls/min was 0.0035 per person year. The rate among abacavir users was 0.0038 per person year.

2.6 Discussion

2.6.1 Summary

In this analysis, we report the incidence, degree and risk factors of tenofovir associated renal impairment in WIHS, a multi-center prospective cohort of US women infected with HIV. We found that the prevalence of severe renal impairment among tenofovir users was similar to that reported in other cohorts, approximately 1-2%. The incidence of moderate to severe renal impairment was 1% in women who started with healthy renal function and 5% in all women. Of the 394 women on tenofovir for durations ranging from 3 to 42 months, tenofovir use and age were predictors of lower GFR, whereas African American race and concomitant NRTI use were predictors of higher GFR. Concomitant ritonavir use was not a statistically significant predictor of decline in GFR.

Baseline renal function appears to be an important risk factor in modulating the effect of tenofovir on renal function. Among women with pre-existing renal impairment at baseline, tenofovir was associated with an improvement in renal function. Yet, the improvement in renal function was not limited to tenofovir. An analysis of abacavir also demonstrated improvement in renal function among those women who started with renal impairment. This suggests that this improvement is not tenofovir specific but possibly due to the beneficial effects of HAART.

2.6.2 Among women with healthy baseline kidney function, the prevalence of tenofovir-associated, severe renal impairment was similar to that reported in the literature.

For many drugs that are potentially nephrotoxic, it is recommended that particular caution be exercised when treating patients with impaired baseline kidney function. Patients with normal renal function are considered to be at reduced risk for drug-induced nephrotoxicity. Paradoxically, we observed a GFR decline in women with normal baseline kidney function (GFR $\geq$ 90 ml/min) in comparison to women who had pre-existing renal dysfunction. Among women in WIHS with GFR $\geq$ 90 ml/min at baseline, the prevalence of moderate to severe renal impairment was 1%. This prevalence and the prevalence among women regardless of their baseline renal function is similar to that observed in cohorts that primarily consist of men with HIV, where the reported range is between less than 1% and 4.4% over 12 to 26 months of follow up[63, 74, 75, 78].

2.6.3 The prevalence of tenofovir-associated moderate renal impairment in women was approximately 18%.

Depending on baseline renal status, approximately 1-5% of women on tenofovir experienced a moderate to severe form of renal impairment (greater than 25% drop in GFR and below 60 mls per min estimated GFR). While 18% of women on tenofovir experienced a 25% drop in GFR to below 90mls/min, this latter proportion was similar to that reported by Gallant and colleagues who observed that 17.8% of men experienced a 25% or greater decline in GFR while on tenofovir[63] for up to 1 year.

2.6.4 The degree of renal decline in tenofovir treated women was approximately 3-7% from baseline on average.

Although both improvement and worsening of kidney function were associated with tenofovir use in this cohort, in general, a significantly greater proportion of women experienced a decline rather than an improvement in GFR. Furthermore, most women

did not experience a severe decline in renal function (Figure 2.1). We found that on average, adjusted GFR declined by 5.6% from baseline in women with healthy renal function after initial treatment with tenofovir for over 3 to 42 months. This level of decline was in the range of the average decline in GFR after tenofovir use for 12 to 26 months reported by other cohorts consisting mostly of men [63]. A similar analysis with abacavir demonstrated that on average, GFR declined by 9.6% from baseline in women with healthy renal function at baseline after initial treatment with abacavir over 42 months. Thus, although our analysis suggests that GFR declines with tenofovir exposure and that this decline is greater in women with healthy kidney function at baseline, greater decline in GFR among abacavir users with healthy kidney function suggests that tenofovir exposure may not be the sole contributor towards decline in GFR in women with HIV who are on antiviral medication.

2.6.5 Renal function declines acutely after tenofovir and remains decreased over time

The effect of tenofovir appeared to be an acute drop in renal function which remained constant over time. The level of decline agrees with those of Julg and colleagues who observed that GFR declined steadily by 5 ml/min over a one year period of exposure[79]. Using the paired t-tests similar to our method, Gallant et al. observed an acute initial decline in GFR followed by a stable renal function after exposure to tenofovir[63].

A 50% or greater decline in renal function is considered clinically significant and requires medical intervention. Our analysis suggests that while 1.3% women treated with tenofovir experienced a 50% or greater decline in renal function, 17% of the women experienced >25% decline in GFR after tenofovir use. These declines were shown in women who were on tenofovir for up to 42 months. Overall, our results suggest that use

of tenofovir among women does warrant attention; however, on average, tenofovir is associated with an acute and mild to moderate effect on renal function.

2.6.6. Risk factors for tenofovir associated renal insufficiency.

We examined which factors might affect GFR in women on tenofovir therapy. Previous studies suggested that concomitant use of ritonavir could be a significant risk factor in tenofovir associated renal impairment because most patients who were found to experience renal impairment were also treated with concomitant ritonavir. Ritonavir is also known to increase tenofovir levels in the body [80]. Concomitant ritonavir was used in over 40% of the subjects who were treated with tenofovir. Concomitant ritonavir did not appear to be a strong predictor of tenofovir associated renal insufficiency.

Importantly, while our analysis demonstrates that the average decline in renal impairment agrees with previously published data, a closer look at the association of tenofovir and GFR in women with pre-existing renal impairment demonstrates some interesting results. Women with pre-existing renal impairment who were exposed to either tenofovir or abacavir therapy demonstrated improved renal function. The increase in GFR observed among women who started on tenofovir with pre-existing renal insufficiency may be explained in several ways. Regression to the mean effects may contribute to the frequency of women with Grade 1 renal function who experienced a decline in GFR to Grade 2 or worse after tenofovir, since we defined Grade 1 as the best category. This effect could have also contributed to the frequency of women in Grades 2-5 of renal insufficiency who improved after tenofovir use. However, we minimized regression to the mean by taking the average of the last two GFRs prior to tenofovir administration. Another possible explanation is that the kidney has a great ability to recover on its own from injury. It is also possible that the improved renal function in subjects in the Grade 2 and 3 categories is due to the beneficial effects of tenofovir

against HIV associated nephropathy. We found abacavir therapy was also strongly associated with improvement in renal function in women with pre-existing renal impairment (Table 2.6). This is consistent with the idea that treatment of HIV, in general, improves renal function.

This analysis does not purport to assign a definitive diagnosis of renal disease to subjects treated with tenofovir. It takes a large-scale view of the trends and the associated effects of tenofovir-associated renal dysfunction in a cohort of HIV infected women. Only detailed chart review, urinalysis and in some cases tissue histology can establish whether these women have Fanconi's syndrome or renal disease directly caused by tenofovir. A limitation of this study is that patients who drop out of the cohort or switch from tenofovir to another anti-retroviral medication may be switching or dropping out for reasons due to nephrotoxicity. Furthermore, patients who physicians suspect may be predisposed to nephrotoxicity may not be given tenofovir. This differential loss to follow-up and pre-selection can result in underestimation of the degree and prevalence of nephrotoxicity. It appears to be unlikely that subjects who had moderate renal impairment prior to tenofovir use were screened and not treated with tenofovir as a result. Clinical intervention such as administration of medication to improve renal function could have also been administered to patients with moderate or severe renal dysfunction.

Clinical markers of renal abnormalities in our analysis were based solely on GFR using the MDRD formula. We acknowledge the highly variable nature of creatinine and that one creatinine measurement may not accurately reflect kidney status. We minimized regression to the mean effects by averaging the last two GFRs to generate each individual's baseline GFR value. We chose to define moderate to severe renal impairment as a greater than 25% decline in GFR to below 90 ml/min. Under this definition, 18% of the 255 women previously tenofovir naïve women with healthy renal

function (GFR ≥90ml/min) at baseline experienced renal insufficiency associated with use of tenofovir. This rate is similar to most of those reported in the literature with less restrictive clinical endpoints[75, 79]. Trends in the data and the renal status distribution of women on tenofovir suggest that on average GFR does decrease after the initial administration of tenofovir and remains constant over time on tenofovir.

2.6.7 Conclusion

This is the first analysis of tenofovir-associated renal insufficiency in a cohort of women with HIV. Our analysis suggests that the prevalence of tenofovir-associated renal insufficiency in women infected with HIV appears to be in the range of that reported in mostly male cohorts. We demonstrate that tenofovir use is associated with mild decline in renal function. In contrast, a proportion of women also show an improvement in GFR, perhaps due to treatment of HIV.

2.7 References

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Chapter 3
Role of Organic Anion Transporters in Transport and Cytotoxicity of Tenofovir And Adefovir

3.1 Introduction

Three nucleotide reverse transcriptase inhibitors (NtRTIs) (adefovir, cidofovir and tenofovir) are used in the treatment of various viral infections. Nephrotoxicity is the major dose limiting adverse effect for both cidofovir and adefovir ([9-(2-phosphonylmethoxyethyl) adenine] or PMEA). In one study, 22 to 32% of individuals taking adefovir at high doses developed serious kidney toxicity[1, 81], ranging from Fanconi syndrome to acute tubular necrosis. Histological studies demonstrate that the kidney damage due to acyclic nucleoside monophosphonates occur primarily in the proximal tubule[11]. To exert their nephrotoxic effects, these anionic drugs must gain access to and accumulate in the proximal tubule. Thus, it is important to understand the mechanisms responsible for their accumulation in the proximal tubule.

Three major organic anion transporters (OATs, SLC22) are expressed in abundance in the proximal tubule. OAT1 (SLC22A6) and OAT3 (SLC22A8) are localized to the basolateral membrane of the proximal tubule cell [82]; whereas OAT4 (SLC22A11) is an apical membrane transporter [83, 84]. It is reasonable to propose that one or more of these transporters may mediate uptake and toxicity of acyclic nucleoside monophosphonates. Cihlar and colleagues have determined that OAT1 and to a lesser extent OAT3 mediate uptake of adefovir whereas cidofovir only interacts with OAT1. Further studies by this group suggest that OAT1 facilitates adefovir and cidofovir induced cytotoxicity[23].

Tenofovir, (9-(2-phosphonomethoxypropyl)adenine) or PMPA, was developed for HIV treatment because of its high antiretroviral activity and low potential for nephrotoxicity in comparison to its two predecessors adefovir and cidofovir[85]. Tenofovir is increasingly used as part of highly active antiretroviral therapy (HAART) for chronic management of HIV and is being tested for prevention of HIV infection in individuals with

high-risk behavior. Because of its widespread use, there are increasing case reports suggesting that tenofovir can also induce nephrotoxicity in the form of acute renal failure and Fanconi's syndrome [86, 87]. However, direct studies in isolated human proximal tubules have failed to show that tenofovir can produce cytotoxicity at clinically relevant concentrations. Further, the prevalence of tenofovir associated Fanconi's Syndrome in patients on the drug is 1-2% nationwide[74]. Because of the potentially significant role of organic anion transporters in controlling the levels of adefovir and tenofovir and thus their toxicity, genetic variation in the major organic anion transporters for NRTIs may contribute to tenofovir induced Fanconi's.

Studies from the laboratory of Bleasby and colleagues suggest that genetic variants of OAT1 may transport tenofovir and adefovir at altered rates in comparison to the common form of the transporter. Collectively, these data suggest that cells expressing OAT1 such as human renal proximal tubule cells are more susceptible to tenofovir-induced cytotoxicity and that genetic variants of this transporter may modulate its cytotoxicity[88].

The goal of our study was two-fold: 1) to determine whether or not tenofovir and adefovir transport and cytotoxicity is enhanced in OAT1 and OAT3 transfected cells; 2) to determine the role of genetic variation in the primary organic anion transporter that modulates tenofovir and adefovir transport and cytotoxicity. In particular, we ascertained that transport and cytotoxicity were increased by the organic anion transporter, OAT1. Through cellular uptake studies, we characterized the interaction of adefovir and tenofovir with OAT1 We established that probenecid, an inhibitor of organic anion transport, can protect cells from the cytotoxic effects of these NRTIs. We then demonstrated that a genetic variant in OAT1 shows differential transport and cytotoxicity of adefovir and tenofovir.

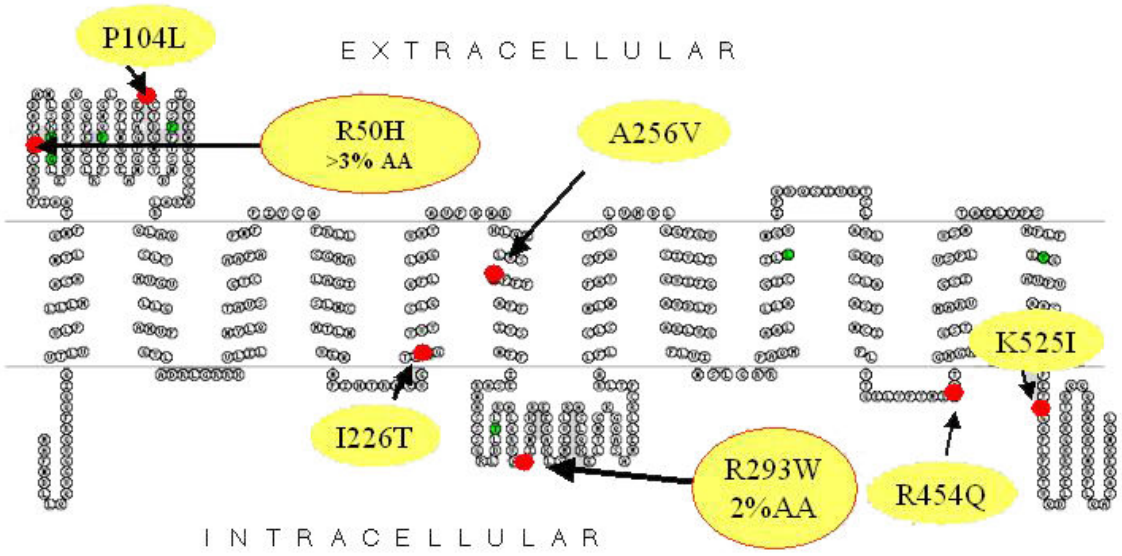


Figure 3.1 Topoimage of genetic variation in OAT1 in a cohort of 256 ethnically diverse healthy individuals

3.2 Methods

3.2.1 Materials

[H^3] labeled tenofovir (3.4 Ci/mmol), [H^3] labeled adefovir (10 Ci/mmol), [H^3] para-aminohippuric acid (PAH) (15 Ci/mmol), and [H^3] estrone sulfate (57.3 Ci/mmol) were purchased from Moravek Biochemicals.

3.2.2 Cells

Human Embryonic Kidney Cells (HEK-293) were purchased from the UCSF Cell Culture Facility. HEK-293 stably transfected with OAT1 (HEK^{OAT1}) was obtained through an interaction with Pfizer. OAT3 (HEK^{OAT3}) and empty vector (HEK^{EV}) were

generated in our laboratory as previously described[89]. OAT4 (HEK^{OAT4}) was generated in our laboratory as described below.

3.2.3 Identification of SLC22A6 variants

Genomic DNA samples were collected from a cohort of unrelated healthy individuals in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. The cohort was comprised of 270 individuals, including 80 African-Americans, 80 European-Americans, 60 Asian-Americans (50 Chinese-Americans and 10 Japanese-Americans), and 50 Mexican-Americans. Variants of SLC22A6 were identified by direct sequencing of individual DNA samples, as has been previously described elsewhere[43]. Briefly, the reference cDNA sequence was obtained from GenBank (http://www.ncbi.nlm.nih.gov, accession number AF097490). Sequencing primers were designed manually, in order to span the exonic as well as 50-200 bp of flanking intronic, regions of SLC22A6. The primer sequences are listed at http://www.pharmgkb.org.

3.2.4 Generation of stably transfected genetic variants of human OAT1

Human OAT1 cDNA was obtained by homology cloning and then subcloned into the mammalian expression vector pcDNA5/FRT (Invitrogen, Carlsbad, CA, USA) to obtain an hOAT1-reference-containing plasmid. The sequence of OAT1-reference corresponds to the OAT1coding region that had the highest frequency in all ethnic groups.

Site-directed mutagenesis of the OAT1-reference-containing plasmid was used to construct the 5 non-synonymous OAT1 variants identified in the population using the QuickChange mutagenesis protocol (Stratagene, La Jolla, CA, USA). The sequence of each variant was verified by complete DNA sequencing, to ensure that the appropriate

nucleotide change occurred, and to confirm that no other alterations had been introduced. Following confirmation, each OAT1 variant in plasmid was transformed into Subcloning Efficiency DH5α™ *E.coli* cells (Invitrogen). Using the manufacturer's protocol, these bacterial cells were grown in selective media overnight, and the OAT1-containing plasmid DNA was then isolated using the QIAprep® Miniprep Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol.

3.2.5 Expression of OAT1 genetic variants and OAT4 in mammalian cells

The transfection process was performed as follows. Human embryonic kidney cells (Flp-In™-293; Invitrogen) were obtained and propagated per the manufacturer's recommendations. Cells were plated at 25% confluence on Biocoat® poly-d-lysine-coated 24-well plates (Becton Dickenson, Bedford, MA, USA) 18-24 hr prior to transfection. After cells reached 70-90% confluency, cells were transfected with OAT1-reference-containing plasmid using a lipid vehicle, Lipofectamine™ 2000 (Invitrogen), and Opti-MEM I Reduced Serum Media (UCSF Cell Culture Facility), and following the Lipofectamine™ 2000 manufacturer's protocol, optimized to a total DNA content of 1 µg/well and a Lipofectamine content of 5 µL/well. Cells were incubated overnight in the transfection mixture. After replacement of media, the cells were again incubated overnight after the media change.

3.2.6 Uptake assays

10^6 HEK cells were plated onto 96 well poly-D-Lysine coated plates (Becton Dickinson Labware, Bedford MA) and grown to 95% confluence. Cell monolayers were cultured for 3 days in MEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 75 ug/ml hygromycin in a humidified incubator at 37°C and 5% CO_2. Prior to transport measurements, cells were incubated with 100 µl 5mM glutaric acid in

phosphate-buffered saline (PBS; UCSF Cell Culture Facility) at pH of 7.4 for 20 minutes at 37°C. This solution was then removed and replaced with a buffered Na^+ - containing solution (128 mM NaCl, 4.73 mM KCL, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM Hepes or Tris, in H_20, pH 7.4), and the cells were incubated for 5 minutes. For the uptake study, for functional screens, cells were incubated with [^{3}H] tenofovir or [^{3}H] adefovir for 2 minutes. Cells were then washed three times with ice cold cell choline buffer and lysed with in 100 µl 0.1N NaOH/0.1% sodium dodecyl sulfate (SDS). Aliquots were removed for protein assay using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin used as a standard and for liquid scintillation counting using 15 ml of Ecolite (ICN Biomedical, Cleveland, OH). [H^3] PAH uptake was normalized to cellular protein. Cellular lysates (75µl) were then placed into scintillation vials for determination of radioactivity using a scintillation counter. For transport rates, the resultant uptake values were normalized for both protein content and uptake incubation time. Protein assays were conducted on a spectrophotometric plate reader using the BCA™ Protein Assay Kit (Pierce, Rockford IL, USA) and following the manufacturer's recommended protocol. Studies were conducted in triplicate and the mean +/- SEM uptake was calculated. Kinetic constants were obtained by fitting to the following equation: V= Vmax [S]/(Km + [S]), where V refers to the rate of substrate transport, Vmax refers to the maximum rate of substrate transport, [S] refers to the substrate concentration. The parameters were estimated using GraphPad Prism version 4 (Graphpad Software, Inc.). P values were calculated according to the Wilcoxon Rank Sum Test. A $p< 0.05$ was considered significant.

3.2.7 Cytotoxicity assays

3000 HEK cells per well were plated into 96 well plates. After 24 hours, various concentrations of tenofovir or adefovir were added to the cells. The cells were then

incubated with drug for 72 hours. MTT assays were conducted by adding 20 µl of bromethyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to each well and incubating for 2-4 hours[90] . After removing the remaining liquid from each well, 100 µl of 0.1 N NaOH in isopropyl alcohol was used to dissolve the blue formazan crystals. Absorbance was measured at 580nm and 670nm using a 96 well microplate reader (Versamax). Mean +/- SEM absorbance values were obtained after subtracting absorbance at 580 nm from that at 670 nm for each quadruplicate. Using the following equation: $Y= ([Bottom] + [Top-Bottom]) / (1+X/EC_{50})^{HillSlope}$, (where Y is the observed value, Bottom is the lowest observed value, Top is the highest observed value, and the Hill slope gives the largest absolute value of the slope of the curve). The resulting EC_{50} values were calculated using GraphPad Prism version 4 (Graphpad Software, Inc.). Experimental data from at least 3 other assays were used to confirm the results.

3.2.8 mRNA and protein expression

Cells were seeded onto 12-well plates and transfected, after 24 hours. RNA or proteins were extracted from the cells. RNA was extracted using RNeasy Kit, (Qiagen). OAT1 mRNA was quantified in each sample by Fast- real-time RT-PCR (7900 HT, Applied Biosystems).

For protein extraction, the cells were lysed, solubilized and pelleted by centrifugation. Protein concentrations were quantified by the BCATM assay, and these samples were used for Western blot analysis. A monoclonal antibody to OAT1 (Cosmo Bio Ltd.) and a polyclonal antibody to rabbit IgG (Molecular Probes) were used to visualize OAT1 using the ImmobilonTM Western detection reagent (Millipore corporation, Billerica, CA).

3.2.9 Immunohistochemistry

Cells were grown onto 24-well poly-D-lysine coated coverslips (BD Biosciences Discovery Labware). Immunohistochemical analysis was conducted the next day. The same antibody to OAT1 that was used for Western blotting was used for immunocytochemistry. A fluorescent goat anti-rat secondary antibody (Invitrogen) was used for the detection of OAT1. Staining was visualized under a Zeiss Axioskop epifluorescence microscope.

3.2.10 Construction of GFP-tagged proteins

Site directed mutagenesis was conducted to create the GFP-labeled reference and R454Q OAT1 variant to attach a DNA segment coding for GFP to the 5' end of the OAT1 gene - the beginning corresponding to the N-terminus of the resultant protein. The GFP-labeled OAT1 plasmids were then transfected into HEK cells grown on 24-well plates using the same transfection protocol as detailed above. The negative control was an empty pcDNA/FRT vector containing the GFP label, but no OAT1 gene. After transfection (24-48 hr later), the cells were visualized under a confocal microscope (Leica), using the manufacturer's suggested protocols for optimal imaging of the GFP label.

3.3 Results

3.3.1 Uptake studies in hOAT1, OAT3 and OAT4 expressing cells

In hOAT1 transfected HEK cells, adefovir and tenofovir demonstrated uptake of over 4 fold over empty-vector transfected cells (negative control) as shown in Figure 3.2. Uptake in OAT3 transfected HEK cells was approximately 2 fold over empty-vector

transfected cells and OAT4 did not appear to exhibit transport of tenofovir above that of mock (empty vector) transfected cells.

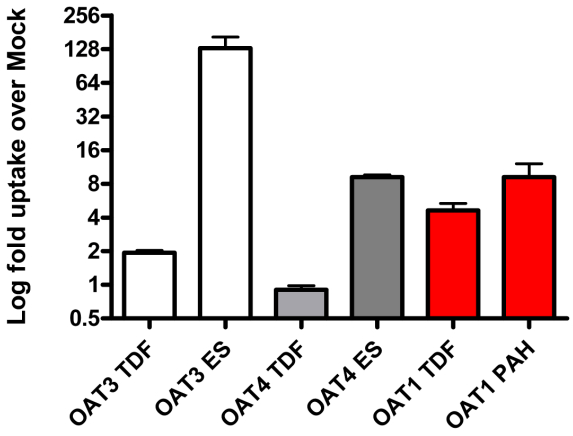
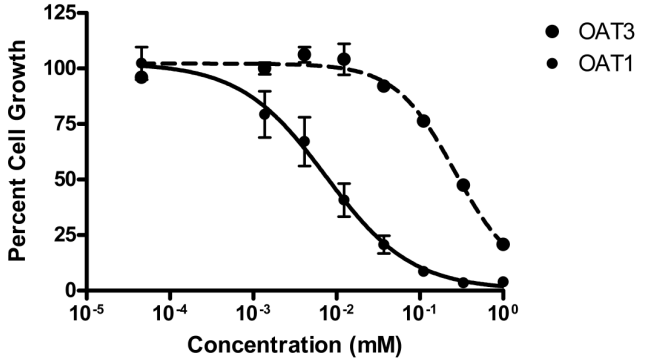


Figure 3.2 Uptake of tenofovir in human organic anion transporter expressing HEK cells. Uptake values were assessed at 2 minutes after start of the uptake reaction. Cells were incubated with each radio-labeled compound (1 µM). Uptake of estrone sulfate (ES) and PAH, typical substrates of OAT4 (ES),OAT3 and OAT1 (PAH) are shown for comparison. Each value represents the mean uptake via OAT3 divided by the mean respective uptake in empty vector transfected cells. Data are from triplicate independent experiments. The uptake of each compound was tested in triplicate samples in each independent experiment.

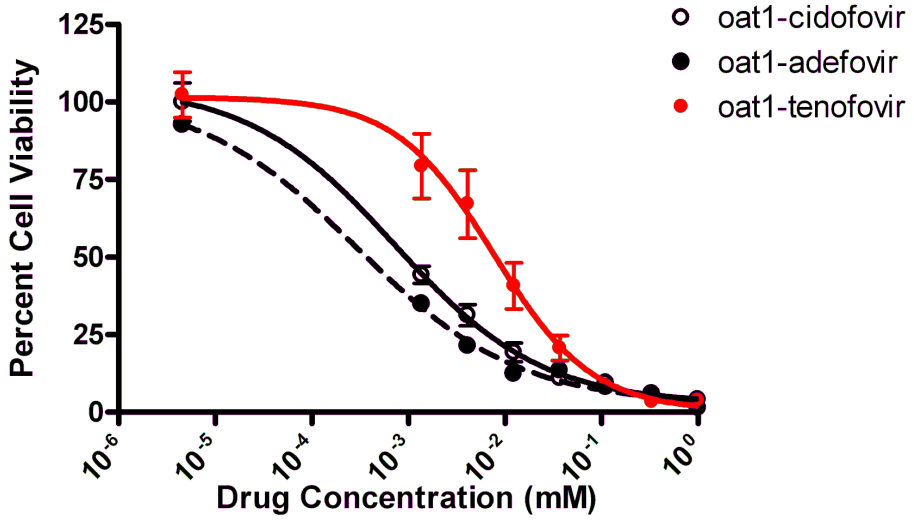


Transfected HEK Cells	TDF EC_{50}(µM)
OAT1	4.78 (2.98-7.66)
OAT3	12.39 (6.15-24.7)

Figure 3.3a.Cytotoxicity of tenofovir in OAT1 and OAT3 expressing HEK cells
$EC_{50\ of}$ OAT3 vs. OAT1, $p<0.01$
(Data shown here are representative of 3 independent experiments)

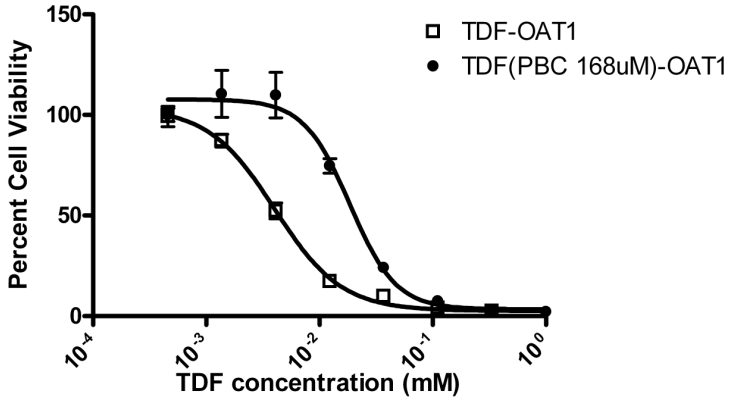
3.3.2 Cytotoxicity studies in OAT1 and OAT3 expressing cells

Figure 3.3a illustrates that the EC_{50} of tenofovir in OAT1 transfected cells in cytotoxicity assays was much lower than that in OAT3 transfected cells (6.7 μM versus 262 μM). In OAT1 transfected cells, adefovir exhibited the greatest toxicity EC_{50} 0.3 +/- 0.2 μM (Figure 3.3b). The EC_{50} of cidofovir was 0.72 +/- 0.4 μM. Tenofovir was the least toxic EC_{50} (10.5 +/- 5.0 μM) however, it was 28 times more toxic in OAT1 transfected cells compared to empty-vector transfected cells (Figure 3.3b). Cytotoxicity from tenofovir was significantly inhibited by probenecid. Figure 3.4 illustrates that the EC_{50} of tenofovir was significantly increased by approximately 100-fold in the presence of 168 μM probenecid from 3.90 +/- 1.06 μM to 336 +/- 200 μM .



	TDF EC_{50}(μM)	ADF EC_{50}(μM)	CDF EC_{50}(μM)
OAT1-Transfected HEK Cells	**10.5 (7.1-15.4)**	**0.30 (0.08 to 1.1)**	**0.72 (0.35-1.48)**
Mock Transfected Cells	**280.3 (97.8-299)**	**263.7 (65.5-1062)**	**>1000**

Figure 3.3b OAT1 facilitates the cytotoxicity of adefovir (ADF), tenofovir (TDF), and cidofovir (CDF). Cells were exposed to various concentrations of each drug and then cell death determined by an MTT assay (see Methods). Data in the table are the mean and +/- standard error (SE) in triplicate samples in a representative experiment.



$EC_{50}(\mu M)$	OAT1	MOCK
TDF	3.90 (2.9-7.7)	198 (61.5-642)
TDF+Probenecid	336 (108-1045)	>1000

Figure 3.4 Inhibition of tenofovir cytotoxicity via OAT1 with 168 μM probenecid. MOCK represents empty vector transfected cells. Cytotoxicity was assessed by MTT assays (see Methods) vs. OAT1 cells incubated with tenofovir alone. $p<0.01$ in Mock vs. OAT1 EC_{50}(tenofovir)

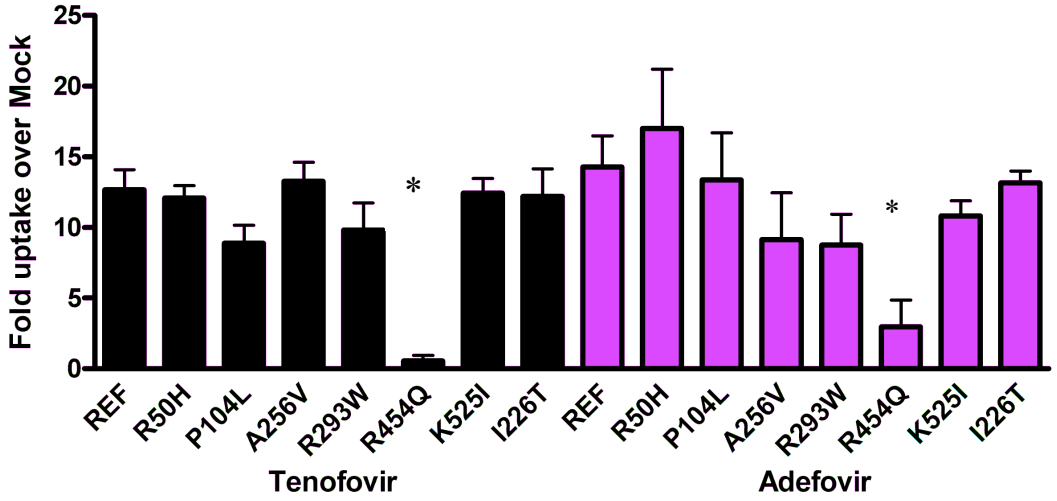


Figure 3.5 Functional activity of all non-synonymous variants with tenofovir and adefovir was assessed. Cells were incubated with each radio-labeled compound (1 μM). Mock transfected cells served as the negative control. Results are expressed as the fold uptake

over mock after correcting for protein per well content. Values represent mean +/- SE in a representative experiment. * p<0.05 vs. OAT1 reference.

3.3.3 Functional screen of tenofovir and adefovir in OAT1 variants

The non-synonymous variants of OAT1 were screened for activity with tenofovir and adefovir. These HEK-293 stably transfected variants were constructed by site-directed mutagenesis of the reference (wild type) cDNA and assayed for function by incubation of the radio-labeled substrate (1 μM) for 2 minutes. The majority of variants demonstrated no statistically significant differences in functional activity from reference except for one variant. Variant R454Q appeared to demonstrate loss of transport activity compared to reference OAT1 ($p<0.05$) (See Figure 3.5).

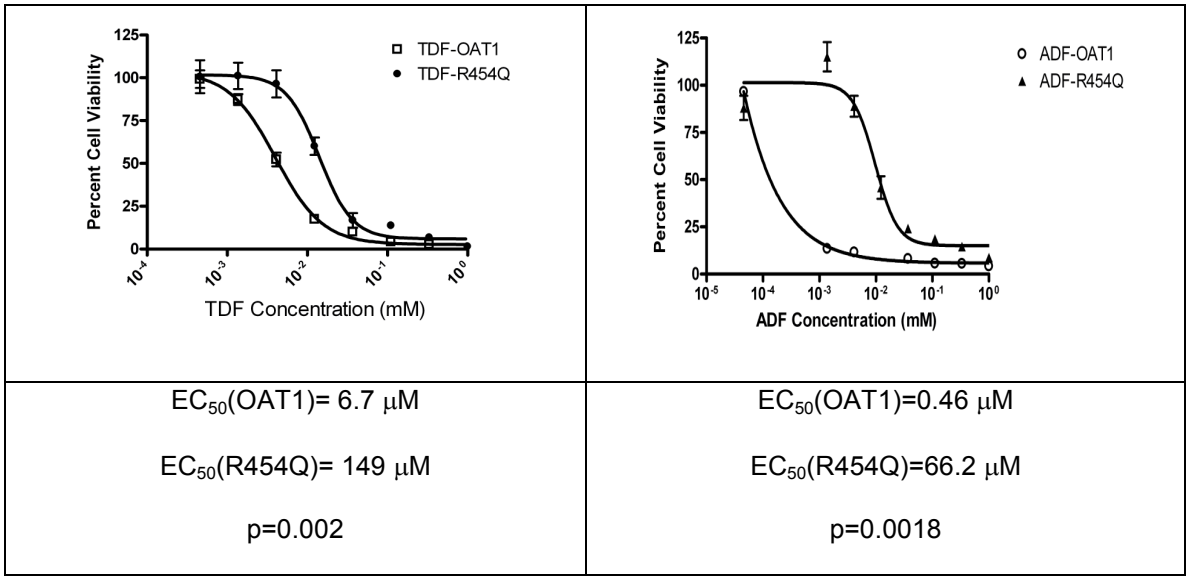
3.3.4 Kinetics and cytotoxicity studies OAT1 variants

In uptake studies using tenofovir and adefovir as well as PAH, of the 7 OAT1 non-synonymous variants, one (R454Q) had significantly reduced function ($p=0.05$) by unpaired Student's *t*-test) relative to OAT1 reference. The other 6 variants demonstrated comparable uptake to OAT1reference (Table 3.1). There were no statistically significant differences in Km and Vmax values across the variants compared to OAT1 reference. Cytotoxicity experiments using both tenofovir and adefovir similarly revealed that all 6 variants except for variant R454Q exhibited similar EC_{50}s compared to OAT1reference (Figure 3.6) (Table 3.2).

Table 3.1 PAH, tenofovir and adefovir kinetics in transfected cells expressing reference OAT1 and its non-synonymous variants.

	PAH			Tenofovir			Adefovir		
		Mean	Std. Error		Mean	Std. Error		Mean	Std. Error
OAT1	Vmax	177.9	45	Vmax	238.9	93.6	Vmax	106.4	16.5
	Km	84.4	22.4	Km	93.8	17.5	Km	50.8	0.5
K525I	Vmax	352.2	14.9	Vmax	127.9	27.2	Vmax	82.9	14.7
	Km	55.3	2.115	Km	128.2	30.2	Km	55.7	9.4
P104L	Vmax	215.0	89.32	Vmax	183.2	77.4	Vmax	102.3	42.7
	Km	62.0	10.67	Km	99.8	14.5	Km	50.8	5.5
A256V	Vmax	405.5	157.6	Vmax	154.9	43.7	Vmax	162.3	17.3
	Km	79.4	17.41	Km	100.8	20.2	Km	64.4	10.8
R293W	Vmax	173.4	91.91	Vmax	84.97	21.6	Vmax	106.3	36.7
	Km	74.9	35.6	Km	102.2	14.8	Km	64.4	8.8
R50H	Vmax	199.3	89.65	Vmax	475.4	292.4	Vmax	224.4	51.7
	Km	127.9	63.14	Km	208.8	88.9	Km	58.4	2.8
I226T	Vmax	205.3	79.93	Vmax	249	40.2	Vmax	69.8	36.9
	Km	49.9	13.76	Km	70.13	8.9	Km	60.6	9.9

No differences in Km and Vmax compared to OAT1 reference using the Wilcoxon Rank Sum Test



EC_{50}(OAT1)= 6.7 μM EC_{50}(R454Q)= 149 μM p=0.002	EC_{50}(OAT1)=0.46 μM EC_{50}(R454Q)=66.2 μM p=0.0018

Figure 3.6 Cytotoxicity of tenofovir and adefovir in R454Q variant transfected HEK cell lines as compared to OAT1 transfected HEK cells. (See Methods) p<0.01 EC_{50}(R454Q vs. OAT1) reference.

Table 3.2 Drug sensitivity of OAT1 variants to tenofovir and adefovir

	Tenofovir				Adefovir			
	Mean EC_{50}	**Std. Error**	**Resistance***			**Mean EC_{50}**	**Std. Error**	**Resistance***
OAT1	6.79	0.97	1		OAT1	0.46	0.08	1
R50H	10.04	2.48	1		R50H	0.349	0.03	1
P104L	20.6	4.19	3		P104L	1.21	0.15	3
I226T	5.97	1.16	1		I226T	0.17	0.01	0.4
A256V	22.4	5.95	3		A256V	0.67	0.02	1
R293W	9.38	1.52	1		R293W	0.51	0.07	1
****R454Q**	**149**	**97.3**	**22**		****R454Q**	**66.2**	**13.7**	**144**
K525I	9.63	1.41	1		K525I	0.54	0.01	1
****MOCK**	**200**	**63.5**	**29**		****MOCK**	**67.8**	**16.3**	**147**

*** resistance was defined as IC_{50} (variant) / IC_{50} (OAT1)**
**** statistically significant p<0.05**

3.3.5 Western blot analysis, quantitative RT-PCR, immunohistochemistry and GFP-tagged constructs

Western blot analysis showed that all OAT1 variants exhibited similar protein expression in the stably transfected cell lines (Figure 3.7). To determine whether any OAT1 protein was found in the cytoplasmic fraction, protein was isolated from both the cytoplasmic and membrane bound fractions. Protein was not found in the cytoplasmic fractions but was found in the membrane fractions (data not shown). Quantitative RT-PCR demonstrated that the R454Q variant did not demonstrate differential mRNAs expression as compared to reference, suggesting that there were no significant transcriptional differences due to the genetic variation (Figure 3.8). Immunohistochemical analysis suggests that the R454Q variant exhibits slightly reduced localization to the plasma membrane compared to reference OAT1 (Figure 3.9). GFP tagged constructs of the variant R454Q and reference OAT1 suggested that OAT1 reference was localized to the membrane of the cell but that the variant showed some intracellular localization as well as plasma membrane localization. The empty vector, negative control demonstrated complete intracellular localization (Figure 3.10).

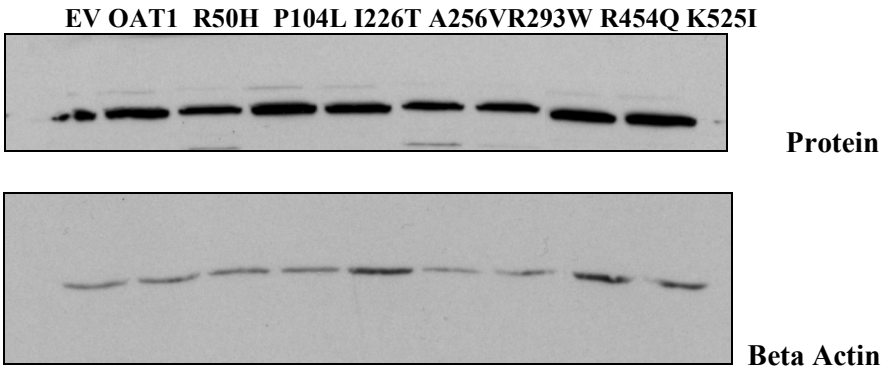


Figure 3.7 Western blot of OAT1 and OAT1 variants.

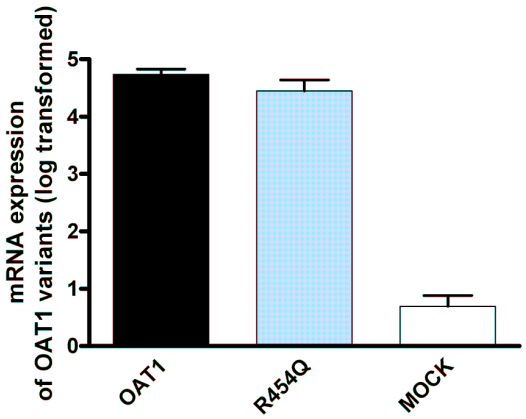


Figure 3.8 mRNA expression of OAT1 in OAT1 transfected, R454Q transfected and empty-vector transfected HEK cell lines.

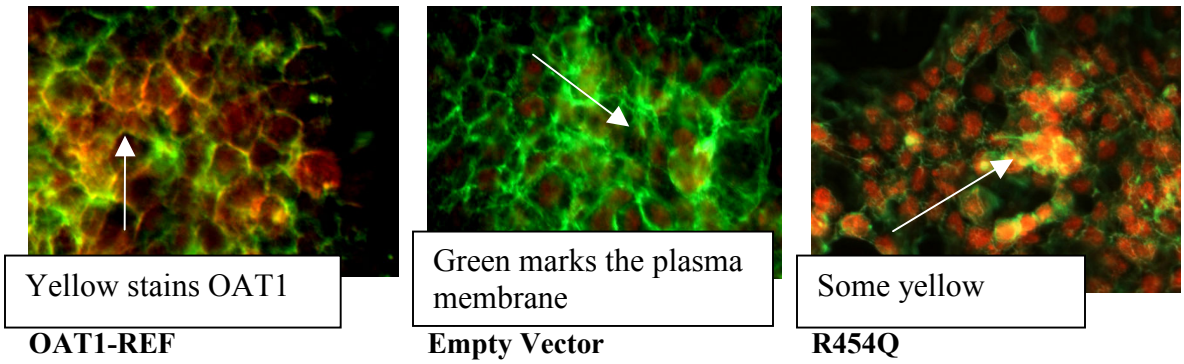


Figure 3.9 Immuohistochemistry of Reference, R454Q and empty vector transfected variants. Figure a illustrates immunostaining of OAT1 transfected cells with rhodamine tagged OAT1 antibody. Plasma membrane of cells was stained in green. Empty Vector or Mock cells demonstrated no OAT1 localization (no yellow) (b). Positive control OAT1 cells showed yellow staining at the plasma membrane where OAT1 was expressed (a). The R454Q variant showed reduced yellow staining correlating with lost of transport activity (c).

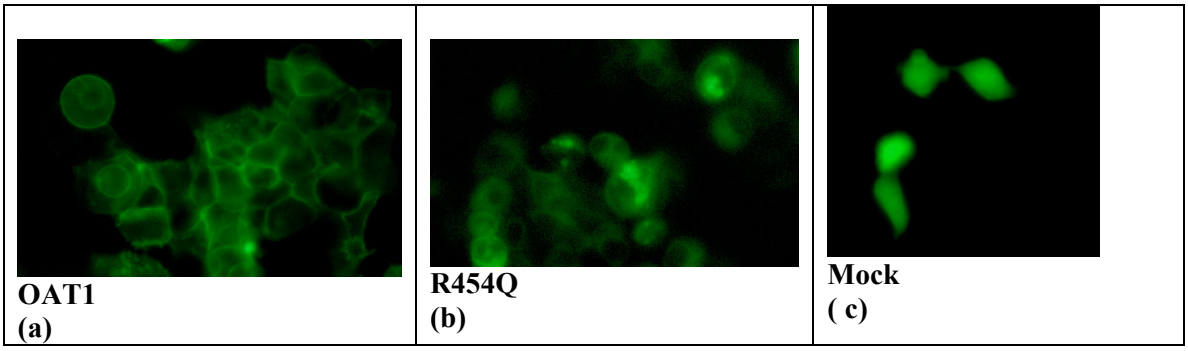


Figure 3.10 GFP tagged OAT1 reference, OAT1-R454Q variant and Mock transfected HEK293 Cells. GFP tagged OAT1 cells show a green signal on the plasma membrane where OAT1 is localized (Figure a). The variant shows some plasma membrane staining as well as intracellular staining (Figure b). Mock transfected cells show no membrane staining but does demonstrate intracellular staining (Figure c).

3.4. Conclusion/discussion

OAT1 is a major transporter for the nucleotide reverse transcriptase inhibitors adefovir and tenofovir. In the field of HIV, OAT1, because of its high expression level in the kidney [91]has been implicated as a candidate for genetic association studies for adefovir and tenofovir associated nephrotoxicity[23, 92]. We functionally characterized the transport and cytotoxicity of tenofovir and adefovir in OAT1 and OAT1 variants.

We show that adefovir and tenofovir are cytotoxic in OAT1 transfected HEK cells. Similar to other studies, tenofovir and adefovir exhibit much greater (4-5 fold) transport in OAT1 transfected cells compared to empty vector transfected cells (Figure 3.3b) suggesting that OAT1 may play a large role in both the cytotoxicity and transport of tenofovir and adefovir.

Fujita and colleagues in our laboratory have previously reported and functionally characterized 7 non-synonymous (amino acid altering) SNPs in the OAT1 protein in an ethnically diverse population[43]. In this study, we determined how these variants interact with the NNRTIs, adefovir and tenofovir and whether the variants could protect against or facilitate cytotoxicity. We found little functional heterogeneity in both transport and cytotoxicity studies among the OAT1 variants (Table 3.1, Table 3.2). Only one variant, found on one chromosome out of 540 chromosomes analyzed, R454Q, exhibited reduced transport (See Figure 3.5) and cytotoxicity of tenofovir compared to reference (Figure 3.6).

The kinetic studies performed on the reduced-function variant (R454Q) suggest that the reduced transport of adefovir, tenofovir and PAH may be due to a combination of factors including differences in the transporter's affinity for these substrates (represented by changes in K_m) and in its maximum substrate transport capacity (represented by changes in V_{max}).

There may be slight differences in the localization of the transporters of the R454Q variant to the cellular membrane. Our immunolocalization and GFP studies suggest that the R454Q variant demonstrates impaired membrane trafficking because of its partial intracellular localization and reduced membrane localization (Figures 3.9 and 3.10) In two previous studies, genetic variants of OAT1 were determined[43, 88]. In oocyte studies Fujita and colleagues determined that one variant, R454Q exhibited nonfunctional activity compared to reference OAT1 in uptake studies with methotrexate,

ochratoxin and para-amminohippuric acid (PAH). Bleasby and colleagues found that the R50H variant exhibited hyperfunctional transport of adefovir, tenofovir and cidofovir as compared to PAH in oocytes.[88] Our results were similar to Fujita's findings; however, we found no significant differences between the R50H variant and reference OAT1. Variation in the experimental system, stably transfected HEK cells versus oocytes may be cause of the inconsistency in results.

OAT1 is not the only known transporter that mediates adefovir and tenofovir influx. Human organic anion transporter 3 (OAT3) has also been found to take up these compounds but at lower affinity[92]. Uwai and colleagues found that OAT3 mRNA expression was at least 3 fold greater than OAT1 expression in the kidney[92]. On the side of efflux, Multiresistance transporter 4 (MRP4) is to date, the known transporter responsible for efflux of these compounds [93, 94]. Therefore, genetic variation in OAT3 and MRP4 may contribute to differences in nucleotide reverse transcriptase inhibitor (NtRTI) transport and cytotoxicity as well. Recently, two groups have characterized the effect of genetic variation in MRP4 on the efflux of adefovir and tenofovir[95]. Since genetic variation in OAT3 may play a role adefovir and tenofovir uptake and cytotoxicity due to its substantial expression in the kidney, further studies are needed to determine whether variants inOAT3 could also play a role in mediating adefovir and tenofovir transport and cytotoxicity.

3.4.1 Implications

This is the first report of adefovir, cidofovir and tenofovir cytotoxicity in OAT1 transfected HEK cells. We also establish the functional implications of genetic variation in OAT1 on tenofovir, adefovir associated cytotoxicity.

3.5 References

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Chapter 4

Candidate gene association study of methotrexate induced renal insufficiency

4.1 Introduction

Methotrexate (MTX) is a folic acid antagonist used against over 60% of cancer malignancies in children[1]. In pediatric oncology, it is used to treat acute lymphoblastoid leukemia (ALL), Non-Hodgkins lymphoma, osteosarcoma and various brain tumors[1]. While highly efficacious, MTX produces several toxicities including liver, bone marrow, kidney and mucosal toxicity. Of these, the most common toxicity is nephrotoxicity. Although the mechanism for MTX-induced renal insufficiency is unknown, tubular obstruction by crystal deposits of MTX and its primary metabolite 7-OH-MTX is thought to be the major driver[1]. Urine alkalinization with sufficient hydration has been observed to significantly reduce the frequency of MTX renal dysfunction suggesting that crystalluria is the main cause of MTX nephrotoxicity[1].

Since MTX is excreted primarily unchanged in the urine, transporters that are highly expressed in the renal proximal and distal tubule may play a role in its renal elimination and concentration in tubular fluid. Several groups have demonstrated that probenecid, an organic anion transport inhibitor totally inhibits renal tubular transport of MTX[2, 3], suggesting that tubular transport plays a large role in MTX excretion. Additionally, several transporters, which are highly expressed in the kidney proximal and distal tubule, transport MTX in vitro[4-8].

ALL is associated with renal dysfunction, which appears to be caused by several treatment related factors. Leukemic infiltration, tumor lysis syndrome can be secondary to chemotherapy. Concomitant use of other cytostatics that are indirectly nephrotoxic, or

nephrotoxins such as aminoglycoside antibiotics, vancomycin and amphotericin B may also provoke and exacerbate ALL associated renal impairment[9]. Further, concomitant diseases can be a contributing factor.

Exploration of the role of genetic variants (i.e. MTHFR, RFC) has been conducted to understand MTX treatment efficacy[10-13]. Fewer studies have looked at the impact of genetics on MTX-induced toxicities[14], and no group to our knowledge has investigated how genetic variation in renal transporters may modulate MTX-induced renal dysfunction.

To investigate whether renal membrane transporters are significantly associated with MTX-induced renal dysfunction, we conducted a pharmacogenetic analysis of 44 SNPs in 8 transporter genes (see Figure 4.1) in a cohort of 414 pediatric ALL patients. Our results suggest that SNPs in several influx and efflux renal membrane transporters play a key role in determining an individual's predisposition towards MTX-induced renal dysfunction.

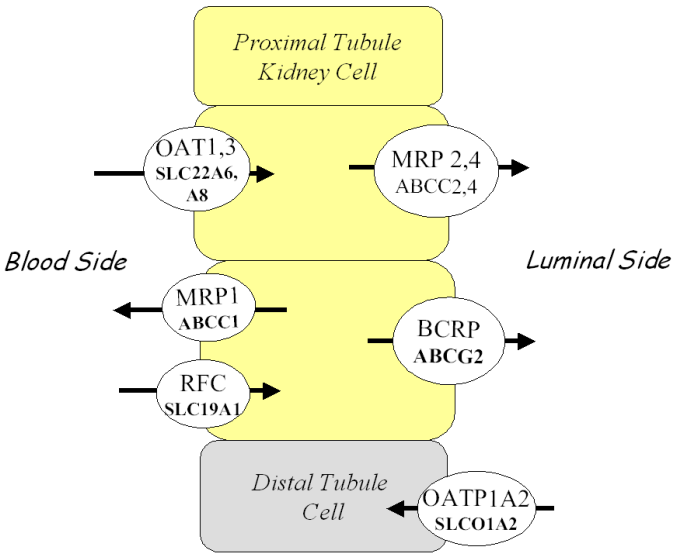


Figure 4.1. Renal transporters that play a role in the tubular transport of MTX. Illustrated is the localization of renal transporters chosen for the candidate gene study

4.2 Methods

4.2.1 Patient population

565 patients from St. Jude Children's Research Hospital with newly diagnosed ALL were enrolled in Therapy studies 13 (N=265), 14 and 15 (N=300) between 1991 and 1998. Dr. Mary Relling, Dr. Cheng Cheng and Dr. Ching-Hong Pui determined cohort inclusion and exclusion criteria, subject selection and data collection. Genotype and information from 414 patients were used in this study. The following describes the details of each protocol: protocol 13 included 10 courses of 2 g/m^2 of intravenous MTX over 2 hours. In protocol 14 and 15, patients received 2.5 (low-risk patients) or 5 g/m^2 (standard or high-risk patients) of MTX given intravenously over 24 hours during the consolidation phase. Remission induction therapy prior to the consolidation phase included prednisone, vincristine, daunorubicin, asparaginase, etoposide, cytarabine and triple intrathecal regimen of hydrocortisone, and cytarabine.[15] All protocols included hydration with 5% dextrose and 40 mEq/L of $NaHCO_3$ in 0.25% normal saline at a rate of 200 ml /m^2/h given over 8 hours and increased leucovorin dosing as needed in patients with greater than 0.8 μM MTX plasma concentration 42 hours after initiation of MTX therapy.[15]

4.2.2 Selection of candidate transporter genes

Forty-four single nucleotide polymorphisms (SNPs) in eight kidney transporter genes, (OAT1 (SLC22A6), OAT3 (SLC22A8), OATP1A2 (SLCO1A2), MRP1 (ABCC1), MRP2 (ABCC2), MRP4 (ABCC4), RFC (SLC19A1), and MXR, (ABCG2)) were selected for genotyping in this cohort. Genes were chosen based on the following criteria: 1) genes must be expressed in the kidney; 2) transporters encoded by the genes must have been shown to be involved in the transport of MTX in cellular studies or in vivo in knockout mice.

Genotyping was conducted using 384 well Sequenom iPlex system and Uniplex by the genome core at Children's Oakland Hospital Research Center. The iPLEX is a multiplexed assay, with up to 36 SNPs combined in a single mutiplexed assay. While the uniplex assay system uses amplifluor allele-specific amplification for SNPs that failed or were unable to be run on the iPLEX system. DNA samples and data including age, weight, cancer risk group (cancer progression status), MTX dose, treatment protocol and creatinine levels were provided by Mary Relling and colleagues at St. Jude's Children Hospital.

4.2.3 Identification of genetic variants in eight transporter genes

We selected SNPs in the eight transporter genes that had a minor allele frequency of equal to or greater than 5% in the Caucasian population at an r^2 value of > 0.8. To identify these SNPs, we used data obtained as part of our large Pharmacogenetics of Membrane Transporters project. In this project, the exonic and adjoining intronic regions in 270 healthy individuals from four ethnic groups (African Americans, Caucasian Americans, Asian Americans and Mexican Americans), were resequenced[16] . The sequencing information was fed into Haploview to determine tag SNPs that captured genetic variants with a minor allele frequency of equal to or greater than 5% in the Caucasian population at an r^2 value of $\geq$ 0.8. These 44 tag SNPs were chosen for genotyping patients in the MTX cohort. (A list of SNPs genotyped is available in the Appendix)

4.2.4 Haplotype construction and analysis

Because the single SNP analysis demonstrated associations with several ABCG2 SNPs, haplotypes from ABCG2 were constructed. Haplotypes were statistically inferred in the Caucasian group using the PHASE[17] method for variants with a minor allele frequency greater than 5%. Haplotype block boundaries were determined using

the LD Spline algorithm in Haploview. Diplotypes were constructed using haplotype information. For information regarding SNP and haplotype frequencies access www.pharmgkb.org.

4.2.5 Association Analysis

4.2.5.1 Outcome measures

Association studies were conducted using STATA™. Two main outcome measures were used in the study. The estimated creatinine clearance (eGFR) and change in eGFR from baseline was calculated by using the following equation: change in eGFR = POST MTX (eGFR) – PRE MTX (eGFR). A negative value indicates impaired renal function while a positive value indicates improvement in renal function. The change in eGFR was used as the outcome in repeated measures random intercept analysis. Secondly, mean pre and post MTX eGFR was used for paired t-tests. More specifically, pre MTX eGFR was compared to post MTX eGFR to determine whether the average differences were statistically significantly different from no change. eGFR was estimated from serum creatinine levels using the pediatric Schwartz equation (see below).

Pediatric Schwartz Equation ml/min/1.73m2

eGFR (ml/min/1.73m2/1.73m^2) =k X Height / [serum creatinine(mg/dl)][18, 19]

k= 0.45 if age < 1 yr
k=0.55 if Male and age ≥ 1 yr and <13 yr or if females age ≥ 1 yr
k= 0.7 if age ≥ 13 yrs and age ≤ 21 and Male

4.2.5.2 Risk factors used in analyses

All analyses were conducted for the subset of Caucasians (n=296) included in the cohort to prevent confounding. Analyses were further extended to all subjects (n=414) for confirmation purposes. Covariates used in the study were age, cancer progression risk, MTX dose, and race. In this cohort, the covariate for cancer risk was

defined as follows: patients were assigned to a higher- or lower-cancer risk group on the basis of their presenting clinical features. These features included the biologic features of their leukemic cells, and their early response to remission-induction treatment). Lower-risk ALL patients were patients 1 to 9 years old with a presenting leukocyte count less than 50,109/L or had a DNA index of 1.16 or more. Patients in this category could not have a CNS-3 status; testicular leukemia (documented by ultrasonographic examination); a T-cell immunophenotype, cytogenetic aberrations such as the t(9;22), t(4;11), t(1;19) associated with a pre–B immunophenotype, an *MLL* gene rearrangement, or near-haploidy (hypo-diploidy); nor could their bone marrow contain 5% or more leukemic blasts on day 15 of remission induction (day 19 after the start of up-front therapy). All other patients were classified as high risk.

4.2.5.3 Statistical models used

Repeated measures random intercept models were used that included data from multiple treatment visits per individual. Several preliminary analyses were run. They included additional stratification by cancer progression and treatment protocol on all patients.

To assess the degree of renal dysfunction due to MTX in this cohort, paired t-tests were conducted to determine whether there were statistically significant differences in eGFR pre and post MTX treatment on all patients. T-tests were also conducted to determine whether there were statistically significant changes in eGFR post MTX among children on different cancer treatments and different cancer progression profiles.

To reinforce our observations, three major types of genetic association analyses were conducted: SNPs were first individually associated with improvements or decreases in average changes in eGFR using a multivariate repeated measures random intercept model. First, a co-dominant model was performed to determine which was the

best genetic model to be used. Then the dominant and recessive genetic models were conducted for the final analyses in all patients.

The same analysis was performed for individuals in standard and high-risk treatment protocols because these children are thought to be the most at risk for MTX-induced renal insufficiency. For those genes where there are a number of SNPs significantly associated, haplotype and stratified haplotype (diplotype) association studies were conducted only for those genes where there were several SNPs significantly associated with outcome in the initial analyses.

4.3 Results

4.3.1 MTX is associated with decreases in creatinine clearance

Table 4.1.a Mean pre MTX eGFR levels stratified by age in 414 pediatric ALL children

Age ranges	N	Mean pre MTX eGFR (ml/min/1.73m^2)	Min eGFR (ml/min/1.73m^2)	Max eGFR (ml/min/1.73m^2)
age < 2yrs	20	116	50.0	196
age 2-5 yrs	147	142	64.9	259
age 5-11 yrs	145	132	53.9	211
age 11-16 yrs	75	130	69.7	252
age > 16 yrs	27	110	66.8	167

194 (46%) of the cohort were female and 220 (53%) of the cohort were male. The cohort was ethnically diverse. Caucasians (n= 296, 71%) and African Americans (n=74, 18%) represented the majority of the population (See Figure 4.2). An analysis stratifying pre MTX eGFR levels by age in this cohort demonstrated that pre MTX eGFR levels in general, appeared to be in the range of those found in other cohorts (Table 4.1.a)[20].

Preliminary analysis of the effect of MTX treatment demonstrated that MTX is harmful to the kidney (Figure 4.3). 9 patients experienced greater than 50% increases

in serum creatinine (mg/dl) (Figure 4.3a). 28 patients experienced greater than 25% drops in eGFR after MTX treatment (Figure 4.3b).

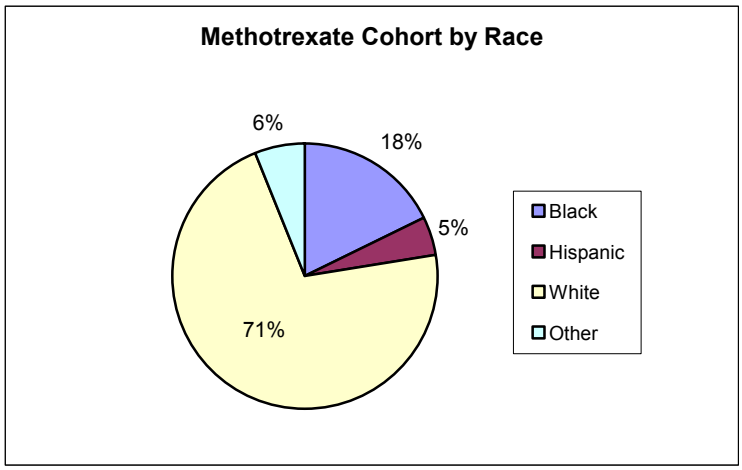
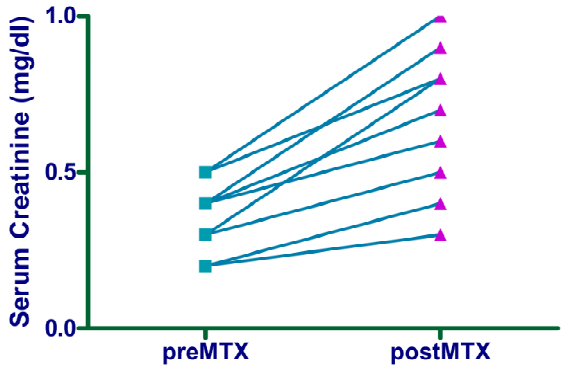
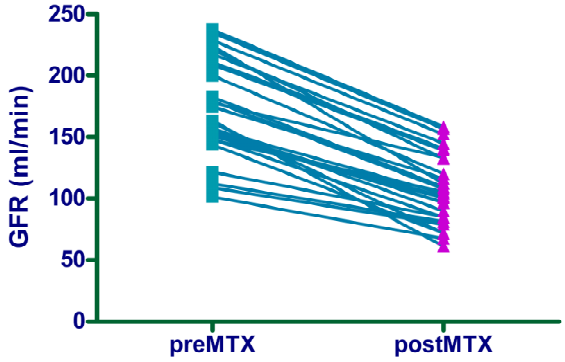


Figure 4.2 MTX patient population table by race. Pie chart shows the racial distribution of the 414 ALL patients. Race was determined through identification by parents.



a)



b)

Figure 4.3. a) Serum creatinine levels pre- and post- MTX treatment in Caucasian patients who experienced a greater than 50% increase in serum creatinine levels after their first MTX treatment regimen. b) Estimated eGFR pre and post MTX treatment in patients experiencing a greater than 25% reduction in estimated eGFR below baseline eGFR

On average, eGFR pre MTX treatment was approximately 133 ml/min/1.73m^2 and similar among genders. Post-treatment eGFR was 125 ml/min/1.73m^2 among females, 121 ml/min/1.73m^2 among males and 123 ml/min/1.73m^2 among all subjects. Differences between pre and post treatment eGFR were 10.0 ml/min/1.73m^2 in females and 9.03 ml/min in males (both $p<0.001$) (Table 4.1).

Table 4.1 Estimated eGFR pre and post MTX treatment by gender and in all patients

	n	Pretreatment eGFR(ml/min/1.73m^2)	Postreatment eGFR(ml/min/1.73m^2)	Difference in eGFR (post minus pre)	95% CI (Diff.)		P value*
Female	194	135	125	-10.0	-14.1	-6	<0.001
Male	220	130	121	-9.03	-13.2	-4.9	<0.001
All	414	133	123	-9.52	-12.4	-6.6	<0.001

Paired t-tests were used to compare pre and post estimated eGFR values.

4.3.2 African Americans appeared to have smaller declines in eGFR than Caucasians

T-tests of post MTX eGFR comparing African American to Caucasian patients demonstrated that after stratifying by risk group, Caucasians in the low cancer risk group had a14 ml/min/1.73m^2 on average greater reduction in post MTX eGFR compared to African American patients (Table 4.2). In other risk (standard and high) categories, there was a small estimated difference between reduction in eGFR post MTX in African American patients in comparison to Caucasians and these effects were not statistically significant.

Table 4.2 Change in eGFR post MTX in African Americans compared to Caucasians by Risk Group

Risk	Num. of Caucasian	Num. of African Am.	Difference in eGFR (ml/min/1.73m^2) in Caucasian patients compared to African Am. patients	P value	95% CI	
Low	153	24	-13.6	0.03	-26.4	-0.98
Standard	94	27	1.31	0.82	-10.3	12.9
High	49	23	3.77	0.61	-11.1	18.7

4.3.3 Individuals in protocol 14 and 15 and those in standard and high risk cancer progression categories experienced greater decline in renal function after MTX treatment

All patients regardless of race were also stratified by risk and protocol. Paired t-test analyses of pre and post treatment eGFR demonstrated that a 10 ml/min/1.73m^2 average decrease in eGFR post MTX among all children in Protocol 14 (p=0.02) and a 11 ml/min/1.73m^2 decline among children in Protocol 15 (P<0.001), were statistically significant (Table 4.3).

Table 4.3 Mean pre and post MTX eGFR and mean change in eGFR (post minus pre MTX) by protocol. (N=414 patients)

Protocol	N	mean pre MTX eGFR (ml/min/1.73m^2)	mean post MTX eGFR (ml/min/1.73m^2)	eGFR change (ml/min/1.73 m^2)	P value	95% CI	
13	103	119	114	-5.13	0.1	-11.1	0.82
14	50	143	132	-10.4	0.02	-18.9	-1.97
15	261	137	126	-11.1	<0.001	-14.7	-7.45

Among patients in protocol 15, standard and high-risk patients experienced an average decrease in eGFR of 11.9 ml/min/1.73m^2 (p< 0.001). Furthermore, children in protocol 14 who were categorized as standard and high risk, experienced an average decrease in eGFR of 15.4 ml/min/1.73m^2 (p< 0.04). The only low risk patients who experienced statistically significant drops in eGFR were those in protocol 15, (10.3 ml/min/1.73 m^2, p = 0.0002). The estimated difference in eGFR was large among standard and high risk children in protocol 14 and showed trends but did not reach statistical significance. Taken together, eGFR appeared to decrease post MTX treatment and the differences were generally most pronounced in protocols 14 and 15 in standard and high risk patients (Table 4.3 and 4.4).

Table 4.4 Difference in creatinine clearance pre and post-treatment in Protocols 13, 14 and 15 among standard/ high and low risk cancer Caucasian subjects.

Protocol	Risk	N	Mean pre MTX eGFR (ml/min/1.73 m^2)	Mean post MTX eGFR (ml/min/1.73 m^2)	Differences in means (post minus pre)	P value	95% CI	
13	Std/high	56	118	112	-5.4	0.09	-11.7	0.92
14	Std/high	24	139	124	-15.4	0.04	-30.0	-0.74
15	Std/high	131	133	121	-11.9	<0.001	-16.8	-6.89
13	Low	47	121	116	-4.8	0.38	-15.8	6.13
14	Low	26	146	130	-5.8	0.22	-15.6	3.90
15	Low	130	141	130	-10.3	0.0002	-15.7	-4.92

An analysis of major risk factors for changes in creatinine clearance post-MTX treatment was conducted. A risk factor that appeared to significantly contribute to altered creatinine clearance was MTX dose (p=0.001, see Table 4.5). eGFR decreased by $3X10^{-5}$ ml/min/1.73m^2 per 100 mg/m^2increase in MTX dose. There was a trend towards a statistically significant association between age and eGFR with an eGFR decrease of 0.53 ml/min/1.73m^2 per year of age (p=0.07) (Table 4.5).

Table 4.5 Risk factors associated with change in creatinine clearance in a repeated measures multivariate model in all patients(N=414)

Variable	Difference in eGFR change (post minus pre MTX eGFR) ml/min/1.73m^2	P value	95% CI	
Male	-4.18	0.106	-9.26	0.89
Cancer Progression Risk	2.36	0.223	-1.43	6.16
Age (months) at visit	-0.53	0.065	-1.10	0.03
Dose per 100mgm/m2	-3X10^-5	0.001	-0.01	0.00

4.3.4 Genetic variation in membrane transporters is associated with decreases in creatinine clearance

Table 4.6 illustrates the cohort based frequencies and rs numbers of SNPs that were found to be significantly associated with changes in eGFR after MTX treatment in the Caucasian group.

Table 4.6 SNPs and population frequencies in the Caucasian subset of the MTX treated cohort. SNPs shown are only the ones associated with significant changes in eGFR.

SNP	Gene	Frequency of SNP in this cohort (N=296)
rs11568643	MRP4	0.081
rs1885301	MRP2	0.458
rs2231138	ABCG2	0.050
rs2231153	ABCG2	0.069
rs2231157	ABCG2	0.400
rs2231162	ABCG2	0.050
rs2274401	MRP4	0.175
rs4149180	OAT3	0.051
rs4149182	OAT3	0.160
rs7324065	MRP4	0.062
rs8187843	MRP1	0.081

4.3.5 A total of eleven SNPs were significantly associated with eGFR among all Caucasian patients

A total of eleven SNPs (Table 4.6) out of 44 SNPs were associated with statistically significant changes ($p \leq 0.05$) in eGFR after MTX treatment among Caucasian patients. These were SNPs in efflux transporters, MRP1 (rs8187843) and MPR4 (rs11568643), which were associated with decreased renal function in the dominant genetic model (42 ml/min/1.73m^2 and 23 ml/min/1.73m^2) (p value 0.04 and 0.02), respectively (Table 4.7a). Two SNPs (rs2231153 and rs2231162) in the ABCG2 transporter were associated with improvement in renal function in the recessive genetic model; they exhibited approximately 11 ml/min/1.73m^{2}and 12 ml/min/1.73m^2 increase in estimated eGFR (p =0.01). rs2231153 was significantly associated with a 7.74 ml/min/1.73m^2 improvement with a p value that met the stringent Bonferroni adjustment (where the P value cut off was set at $P \leq 0.05/44$ SNPs) only after the analyses were run including all races. In the Caucasians sub-sample, the effect was larger (11 ml/min/1.73m^2) but the effect size was also larger (p =0.01) (Table 4.7a). One SNP met

strict Bonferroni criteria when all races were included, i.e., ABCG2 (rs2231153). However, the significance was highly dependent on the model (recessive or dominant).

In the influx transporter OAT3, SNP rs4149180 was associated with a 12 ml/min/1.73m^2 (p= 0.001) decrease in renal function in the recessive model. This SNP met the stringent Bonferroni adjustment (where the P value cut off was set at $p \leq 0.05/44$ SNPs). In the dominant model (14.4 ml/min/1.73m^2); however, the p value did not meet the Bonferroni criteria although it exhibited trends towards significance (p=0.02). Another OAT3 SNP, rs4149182 was associated with a 10 ml/min/1.73m^2 decrease and this exhibited a trend towards significance (p= 0.07). Efflux transporter MRP2 SNP (rs1885301) was statistically significantly associated with improved in renal function associated with 9.4 ml/min/1.73m^2 (p=0.007) in the dominant genetic model. All of these trends were similar, in general, when the same models were run including all races (Table 4.7a).

Table 4.7a: Genotype associations with change in eGFR using the dominant and recessive genetic models in patients

Caucasian Dominant						Caucasian Recessive					
SNP	eGFR (ml/min/1.73m^2) change	P value	95% CI		Gene	SNP	eGFR (ml/min/1.73m^2) change	P value	95% CI		Gene
rs11568643	-23.1	0.04	-45.6	-0.64	MRP4	rs11568643	-0.83	0.83	-8.27	6.61	MRP4
rs1885301	9.43	0.007	2.54	16.3	MRP2	rs1885301	0.33	0.91	-5.5	6.16	MRP2
rs8187843	-42	0.02	-76.3	-7.59	MRP1	rs8187843	-0.38	0.92	-7.96	7.2	MRP1
rs2231153	38.3	0.06	-1.46	78.1	ABCG2	rs2231153	11.52	0.01	2.8	20.3	ABCG2
rs2231157	3.37	0.27	-2.59	9.33	ABCG2	rs2231157	-1.21	0.71	-7.58	5.15	ABCG2
rs2231138	N/A	N/A	N/A	N/A	ABCG2	rs2231138	8.62	0.06	-0.43	17.7	ABCG2
rs2231162	-20.4	0.51	-80.5	39.7	ABCG2	rs2231162	12.68	0.01	2.99	22.4	ABCG2
rs2274401	-5.11	0.49	-19.6	9.41	MRP4	rs2274401	-2.27	0.41	-7.61	3.08	MRP4
rs7324065	2.3	0.86	-22.8	27.4	MRP4	rs7324065	6.26	0.16	-2.52	15	MRP4
rs4149180	-14.4	0.02	-26.6	-2.23	OAT3	rs4149180	-12.16	0.001	-19.61	-4.72	OAT3
rs4149182	-10.78	0.07	-22.30	0.74	OAT3	rs4149182	-2.31	0.39	-7.63	3.01	OAT3
All Races Dominant						**All Races Recessive**					
SNP	eGFR (ml/min/1.73m^2) change	P value	95% CI		Gene	SNP	eGFR (ml/min/1.73m^2) change	P value	95% CI		Gene
rs11568643	-24.1	0.03	-45.6	-2.56	MRP4	rs11568643	0.35	0.91	-5.84	6.54	MRP4
rs1885301	5.85	0.04	0.3	11.4	MRP2	rs1885301	0.62	0.8	-4.09	5.33	MRP2
rs8187843	-21.4	0.09	-46.4	3.6	MRP1	rs8187843	-0.41	0.91	-7.21	6.39	MRP1
rs2231153	5.7	0.15	-2	13.3	ABCG2	rs2231153	7.74	0.001	2.99	12.5	ABCG2
rs2231157	3.1	0.17	-1.3	7.5	ABCG2	rs2231157	0.73	0.81	-5.08	6.54	ABCG2
rs2231138	-25.3	0.41	-84.8	34.3	ABCG2	rs2231138	6.55	0.06	-0.2	13.3	ABCG2
rs2231162	0.9	0.85	-8.2	9.9	ABCG2	rs2231162	6.91	0.01	1.82	12.01	ABCG2
rs2274401	-3.9	0.52	-15.4	7.7	MRP4	rs2274401	-1.43	0.52	-5.72	2.87	MRP4
rs7324065	1.9	0.88	-22.2	26	MRP4	rs7324065	4.09	0.24	-2.7	10.87	MRP4
rs4149180	-4.5	0.14	-10.4	1.4	OAT3	rs4149180	-8.11	0.09	-17.5	1.28	OAT3
rs4149182	-2.31	0.56	-10.1	5.46	OAT3	rs4149182	0.13	0.953	-4.191	4.45	OAT3

Table 4.7b Association between SNPs and eGFR change in patients who are classified in the standard and high-risk cancer progression category. Results for both recessive and dominant models in Caucasians and all races are shown.

Caucasians Dominant SNP	eGFR (ml/min/1.73m^2) change	P value	95% CI		Gene	Caucasians Recessive SNP	eGFR (ml/min/1.73m^2) change	P value	95% CI		Gene
rs11568643	-9.7	0.483	-36.7	17.3	MRP4	rs11568643	-1.46	0.752	-10.5	7.62	MRP4
rs1885301	11.2	0.01	2.69	19.8	MRP2	rs1885301	2.62	0.517	-5.3	10.6	MRP2
rs8187843	-42.0	0.01	-74.1	-10	MRP1	rs8187843	-5.86	0.294	-16.8	5.09	MRP1
rs2231153	43.4	0.024	5.69	81	ABCG2	rs2231153	13.3	0.021	2	24.6	ABCG2
rs2231157	7.00	0.082	-0.9	14.9	ABCG2	rs2231157	4.49	0.308	-4.1	13.1	ABCG2
rs2231138	N/A	N/A	N/A	N/A	ABCG2	rs2231138	16.7	0.01	4	29.4	ABCG2
rs2231162	-21.1	0.473	-78.7	36.5	ABCG2	rs2231162	11.4	0.071	-1	23.8	ABCG2
rs2274401	-4.20	0.669	-23.2	14.9	MRP4	rs2274401	-9.67	0.008	-16.9	-2.47	MRP4
rs7324065	N/A	N/A	N/A	N/A	MRP4	rs7324065	13.1	0.047	0.2	26.1	MRP4
rs4149180	-14.0	0.078	-29.5	1.6	OAT3	rs4149180	-4.31	0.729	-28.7	20.1	OAT3
rs4149182	-8.59	0.26	-23.65	6.46	OAT3	rs4149182	-6.35	0.08	0.83	-13.6	OAT3
All Races Dominant SNP	**eGFR (ml/min/1.73m^2) change**	**P value**	**95% CI**		**Gene**	**All Races Recessive SNP**	**eGFR (ml/min/1.73m^2) change**	**P value**	**95% CI**		**Gene**
rs11568643	-11.2	0.4	-37.4	14.9	MRP4	rs11568643	-1.43	0.723	-9.34	6.47	MRP4
rs1885301	5.30	0.14	-1.7	12.4	MRP2	rs1885301	3.84	0.227	-2.39	10.1	MRP2
rs8187843	-42.5	0.01	-74.1	-10.8	MRP1	rs8187843	-4.85	0.347	-14.9	5.25	MRP1
rs2231153	6.60	0.18	-3	16.2	ABCG2	rs2231153	7.33	0.019	1.19	13.5	ABCG2
rs2231157	4.40	0.14	-1.5	10.3	ABCG2	rs2231157	6.57	0.099	-1.23	14.4	ABCG2
rs2231138	-27.9	0.34	-84.8	29	ABCG2	rs2231138	12.8	0.011	2.9	22.6	ABCG2
rs2231162	2.90	0.61	-8.3	14.2	ABCG2	rs2231162	5.83	0.081	-0.72	12.4	ABCG2
rs2274401	-2.80	0.71	-17.8	12.2	MRP4	rs2274401	-6.30	0.029	-12	-0.63	MRP4
rs7324065	N/A	N/A	N/A	N/A	MRP4	rs7324065	9.08	0.073	-0.85	19	MRP4
rs4149180	-4.46	0.21	-11.5	2.55	OAT3	rs4149180	-6.48	0.254	-17.6	4.66	OAT3
rs4149182	-3.21	0.53	-13.34	6.91	OAT3	rs4149182	-3.20	0.278	-8.99	2.59	OAT3

Repeated measures model controlled for dose, cancer risk, age and sex in Caucasian patients

Table 4.7b gives the results of repeated measures random intercept association analyses in dominant and recessive models in selected SNPs in all races and in only Caucasian patients who were treated as standard and high risk treatment groups. Several SNPs appeared to show statistical significance ($P \leq 0.05$) in the Caucasian sub-sample, but none appeared to make the stringent Bonferroni cut off where the P value cut off was set at $P \leq 0.05/44$ SNPs or $P \leq 0.05/44$ SNPs X 2 models (dominant and recessive). The SNP that showed the most statistical significance was rs2274401 in MRP4 It was associated with decreased renal function and a drop in eGFR of 9.67 ml/min/1.73m^2 in the Caucasian group using a recessive model (p=0.008). Among Caucasian patients in the standard and high-risk protocols, 6 SNPs out of 44 SNPs were statistically significantly associated with changes in eGFR. SNPs rs2274401 (MRP4), rs8187843(MRP1) were associated with worsening of renal function through decreases in creatinine clearance after MTX treatment**.** All 4 others were associated with improvement in renal function. These SNPs were in MRP2 (rs1885301), in ABCG2 (rs2231138, rs2231153) and MRP4 in (rs7324065). These results showed the same trends when the same random intercept repeated measures genetic models (dominant and recessive) were run and the analyses were expanded to include all races (including Caucasians) (Table 4.7b).

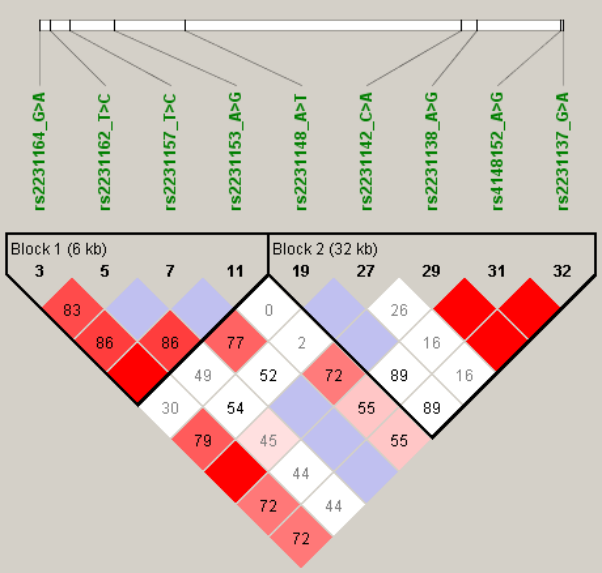


Figure 4.4 Haplotype and blocks of ABCG2 used in haplotype analysis. Tag SNPs used for block 1 were: rs2231164, rs2231162, rs2231157, rs2231153, and for block2 : rs2231148 rs2231142 rs2231138, rs2231137. The plot above is a heat map illustrating the degree of linkage disequilibrium (denoted by D') and correlation (R^2) between SNPs. The color of the squares is the combination of D' and (R^2) with intensity going from red (most linked and highly correlated to white, least linked and correlated). The numbers in the boxes are the D's. The haplotype can be separated into 2 haplotype blocks as shown. SNP rs numbers are denoted, along with SNP identification numbers (i.e. 3,5,7,11) above the haplotype.

4.3.6 Haplotype association analysis identified several haplotypes in ABCG2 linked to improved renal function

Our analysis demonstrated that there were several SNPs in ABCG2 that were associated with changes in eGFR (see Table 4.8 for haplotype frequencies). These SNPs may be correlated due to linkage disequilibrium.

Table 4.8 ABCG2 haplotypes and haplotype blocks correlated to their population frequencies in the Caucasian subset of patients.

Block Type	Haplotype No	Haplotype	Frequency
Blocks 1 and 2	1	TTGTCAG	11%
Blocks 1 and 2	2	TTGTCGA	3%
Blocks 1 and 2	9	CTATCAG	5%
Blocks 1 and 2	11	CTATAAG	7%
Blocks 1 and 2	14	CTAACAG	26%
Blocks 1 and 2	16	CCATCAG	36%
1	1	TTG	15%
1	4	CTA	42%
1	5	CCA	39%
2	1	TCAG	57%
2	3	TCGA	5%
2	4	TAAG	8%
2	7	ACAG	25%

To test this hypothesis, haplotypes were constructed and 2 blocks were identified using Haploview and the LD spline algorithm (Figure 4.4). ABCG2 haplotypes were associated with changes in eGFR. Haplotypes that exerted the greatest influence on change in eGFR were uncovered. Three different haplotype analyses were conducted. Haplotypes were constructed using all of the chosen SNPs in the ABCG2 gene. Haplotypes with greater than 5% population frequency were associated with changes in eGFR. Using an additive genetic model and conducting a repeated measures random intercept model, we determined that haplotype 2 (TTGTCGA) was associated with improvement in renal function; 14.6 ml/min/1.73m^2 increase in the change in eGFR ($p=0.02$) (Table 4.9).

Table 4.9 ABCG2 haplotype and haplotype block associations with eGFR change after MTX treatment in 256 Caucasian individuals. Additive repeated measures random intercept model illustrates the statistically significant association of haplotype TTGTCGA and haplotype blocks TTG and TCGA with change in eGFR post MTX.

Analysis type	Haplotype	eGFR change Post MTX (ml/min/1.73 m^2)	Std (ml/min/1.73m^2).	P>|z|	95% CI	
Both Blocks 1 and 2						
	TTGTCGA	+14.6	6.3	0.02	+2.3	+26.9
Block 1	TTG	+11.3	4.8	0.02	+2.0	+20.7
Block 2	TCGA	-15.6	5.6	0.005	-26.5	-4.6

Secondly, haplotype block analysis demonstrated that the TTG haplotype in haplotype block 1 was associated with an 11.3 ml/min/1.73m^2 increase in the change in eGFR (p=0.02). The TCGA haplotype in haplotype block 2 was associated with a 15.6 ml/min/1.73m^2 decrease in change in eGFR post MTX (p=0.005). When a model considering each block independently was run, block 1 (TTG) and block 2 (TCGA) were independently associated with a +24 and -16.8 ml/min/1.73m^2 change in eGFR post MTX, respectively (Table 4.10). In an interaction model, the interaction of TTG and TCGA haplotypes was not statistically associated with the change in renal function (p=0.348) (Table 4.10). This indicates these two haplotypes appear to influence gene function independently. They were associated with increase in eGFR post MTX of 21.2 (p=0.02) for TTG and 17.6 (p=0.004) for TCGA when they were run in an interaction model.

Table 4.10. The independent association of TTG and TCGA with change in eGFR and as an interaction variable. The results here illustrate that blocks TTG and TCGA act independently in their effects on eGFR change post MTX treatment.

Dominant Model	Coef	Std. Er	P value	95% CI	
TTG	24.0	8.72	0.006	6.95	41.13
TCGA	-16.8	6.08	0.006	-28.73	-4.88
Interaction Model					
TTG	21.2	9.26	0.022	3.03	39.31
TCGA	-17.6	6.14	0.004	-9.65	-5.56
TTGXTCGA	3.92	4.18	0.348	-4.27	12.10

The above model controlled for dose, cancer risk, age and sex

Stratified haplotype analysis was conducted to assess the contribution of combinations of haplotypes as they would behave in two chromosomes. In a dominant model, the subjects having at least one TTG haplotype showed improvement in renal function through an increase (12.7 ml/min/1.73m^2) in eGFR (p= 0.01) (Table 4.11). In block 2, having at least one TCGA haplotype was associated with a 17.7ml/min/1.73m^2 decline in eGFR (p=0.003). To assess whether these haplotypes are independent of each other, haplotypes from both blocks were considered together in the dominant genetic model (Table 4.11). The TTG haplotype in block 1 appeared to be significant and independent of TCAG, TCGA and TAAG in block 2 (See Table 4.11).

Table 4.11 Stratified haplotype analysis to assess the contribution of combinations of haplotypes as they would behave in two chromosomes. Haplotype combinations were associated with change in eGFR post MTX treatment. TTG and TCGA appear to act independently in the stratified haplotype model.

Diplotype Model		Change in eGFR	P Value	95 % CI	
Dominant	**TTG**	12.7	0.01	3	22.4
Dominant	**TCGA**	-17.7	0.003	-29.2	-6.2

Diplotypes in Combination			P Value		
Dominant	**TTG**	24	0.006	6.9	41.1
Dominant	**TCGA**	-16.8	0.006	-28.7	-4.9
Dominant	**TTG**	22.8	0.009	5.7	39.8
Dominant	**TCAG**	0.7	0.9	-7.3	8.6
Dominant	**TTG**	23	0.007	6.2	39.8
Recessive	**TAAG**	40.5	0.2	-15	96.1
Recessive	**CTA**	-4.0	0.454	-14.6	6.5
Dominant	**TCGA**	-16.2	0.008	-28.2	-4.1
Recessive	**CCA**	1.3	0.824	-10.3	12.9
Dominant	**TCGA**	-16.0	0.009	-28.0	-3.9

The above model controlled for dose, cancer risk, age and sex

4.4 Discussion

Summary of Findings

- MTX is associated with decline in eGFR.
- A total of 11 individual SNPs were associated with significant changes in renal function.
- Some of the SNPs the SNPs that were associated with improvement in renal function belonged to efflux transporters, ABCG2, MRP2 and MRP4.
- The strongest predictor of decrease in GFR was SNP rs4149180 in OAT3 that was associated with a 12 ml/min/1.73m^2 decrease in renal function and met the stringent Bonferroni adjustment for multiple testing.

- The second strongest predictor was a polymorphism in the efflux transporter MRP2; rs1885301. This SNP was associated with improved renal function of 9 ml/min/1.73m^2.
- Another SNP rs2231153, a variant in ABCG2 was associated with an 8 ml/min/1.73m2 improvement in eGFR in the entire cohort.
- Our haplotype analysis of ABCG2 revealed that the TTG haplotype in block 1 and the TCGA haplotype in block 2 of ABCG2 exhibited independent and statistically significant associations with improved GFR.

MTX-induced renal dysfunction is a common side effect in acute lymphoblastic leukemia (ALL) which may be severe in some patients despite hydration and urinary alkalinization[21]. In this study, we hypothesized that genetic variation in membrane transporters that control exposure of the kidney to MTX may be determinants of MTX-induced renal dysfunction. Transporters in the kidney act to control drug disposition and cytotoxicity. We analyzed 8 renal transporter genes in the Caucasian subset of 414 children with pediatric ALL to determine whether they were associated with significant changes in creatinine clearance. Organic anion transporters 1[22] , 3, (OAT1 and OAT3) and organic anion transporter polypeptide 1A2[23], efflux transporters multi-resistance proteins 1[24], 2[25] and 4[26], (MRP1, MRP2 and MRP4), the reduced carrier folate transporter (RFC1), the breast cancer resistance protein BCRP also known as ATP-binding cassette transporter subtype G2 (ABCG2) have all been shown to transport MTX in in-vitro studies and for several of these genes, genetic variants have been linked to changes in drug disposition.

4.4.1 Prevalence of decline in eGFR after MTX treatment

In this study, we first examined the cohort to determine the prevalence of MTX-induced renal insufficiency and also to identify non-genetic risk factors that may contribute to MTX-induced renal insufficiency. There was large variation in MTX associated kidney dysfunction. Notably, in 7.4% of the 414 patients, serum creatinine

levels increased greater than 50% after the first MTX treatment, consistent with kidney damage. Analogously, eGFR, estimated by the Schwartz formula, dropped below 75% of baseline after MTX treatment in 28 patients or 6.7% of the cohort, and two patients experienced a severe drop to below 50% of their estimated baseline eGFR. These data suggest that there is substantial variation in MTX associated kidney injury, with some children exhibiting serious kidney damage after treatment. Since there were no apparent differences in race, dose, age, or cancer progression that defined these individuals with large drops in renal function after MTX treatment, genetic effects, may thus, play a role in modulating the renal insufficiency due to MTX treatment.

4.4.2 African Americans with low risk for cancer progression exhibited less decline in renal function than Caucasians

Our analysis indicated that African Americans with low risk cancer progression exhibited less renal dysfunction after MTX treatment than Caucasian patients in the same cancer risk group (Table 4.4). Racial differences in baseline kidney function have been described. In particular, healthy African Americans have greater GFRs than healthy Caucasians of the same age and gender[27]. Our data suggest that African Americans with low risk for progression may also be less pre-disposed to MTX-induced renal dysfunction (Table 4.2).

4.4.3 Dose and exposure to MTX was a non-genetic risk factor for MTX-induced renal dysfunction

A second non-genetic risk factor that was associated with MTX-induced renal insufficiency was MTX protocol. Patients in protocol 14 and 15 who were in the standard and high risk cancer categories exhibited the greatest decrease in mean eGFR. Since

lower dose and duration of MTX is what set protocol 13 apart from protocols 14 and 15, the decline in eGFR associated with protocols 14 and 15 is likely attributed to greater dose and longer exposure to MTX (in protocols 14 and 15, greater dose and longer MTX exposure were administered). The most significant non-genetic risk factor for MTX associated kidney dysfunction was MTX dose (Table 4.5) with greater doses being associated with greater drops in estimated eGFR post-MTX therapy.

4.4.4 SNPs in ABCC2 and OAT3 demonstrated the strongest association with changes in renal function after treatment with MTX, other SNPs in MRP4 and MRP2 may also be associated with reno-protection from MTX treatment

To determine whether genetic variants in renal drug transporters were associated to risk for MTX-induced kidney dysfunction, we performed analyses on all patients and also only in the Caucasian group within the cohort. We found a total of 11 individual SNPs associated with significant changes in renal function using change in eGFR as the outcome (Table 4.6, Table 4.7) and controlling for risk factors such as dose, age, sex and cancer risk in Caucasian patients and in patients of all races. The SNPs that were associated with improvement in renal function all belonged to efflux transporters, ABCG2, MRP2 and MRP4. From our analysis we are not able certain whether these SNPs themselves are causative or in linkage with causative SNPs. Though speculative, reduced function SNPs in efflux transporters would result in a reduced tubular accumulation of MTX and therefore less tubular obstruction. Interestingly, the strongest predictor of change in renal clearance was an OAT3 polymorphism rs4149180, which was associated with a 12 ml/min/1.73m^2 decrease in renal function in a recessive genetic model. This was one of the polymorphisms that we found that met the stringent Bonferroni adjustment for multiple testing for 44 SNPs ($P<0.05/44$ or approximately $P<0.001$). When this analysis was expanded to include all races, the effect size

decreased to -4.5 ml/min/1.73m^2 and the p value decreased in significance. This may be attributed to the fact that African Americans on average are less susceptible to MTX induced kidney toxicity because of perhaps a greater reserve kidney function than Caucasians (Table 4.2)[27]. OAT3 codes for a membrane transporter that is highly expressed in the kidney and is one of the influx transporters that mediates MTX uptake into the renal tubule. rs4149180 is an A to G base pair change that causes a synonymous change but that does not result in a change in the amino acid sequence of the transporter. The SNP rs4149180 is not found in linkage with any non-synonymous variants but is in moderate linkage D'0.72 with rs2187384, an intronic SNP. It is also possible that this SNP is in linkage disequilibrium with another SNP, not in our list, that may be largely responsible for this decrease in renal function. Functional polymorphisms in OAT3 have not only been shown to impact changes in transport but they have also been shown to demonstrate altered MTX transport[28]. A gain of function variant of OAT3 would result in greater tubular accumulation of the drug. In contrast, a reduced function variant in OAT3 may be associated with reduced renal clearance and greater plasma levels of MTX.

Another strong predictor was a polymorphism in efflux transporter MRP2, rs1885301**,** which was associated with improved renal function of 9 ml/min/1.73m2. This was an intronic region SNP, an A to G change that may be in linkage disequilibrium with another SNP in MRP2 that is primarily responsible for improved renal function. The P value (0.007) for this did not meet the most stringent multiple testing criteria which was $P \leq 0.001$. Polymorphisms in MRP2 have been linked to altered MTX excretion as well as variability in MTX pharmacokinetics[13] . Further, another group identified and validated a coding region polymorphism in MRP2 (R412G mutation) in one patient that appeared to cause severe MTX over-dosing and reversible nephrotoxicity that is believed to be caused by a 3-fold reduction in the MTX elimination rate[29]. Table 4.7 also

lists other SNPs that were significantly associated with changes in renal function but did not meet criteria for multiple testing. These SNPs present interesting candidates for studies with a larger cohort of patients.

Another SNP rs2231153, a variant in ABCG2 was associated with an 8 ml/min/1.73m2 improvement in eGFR in the entire cohort. The p value for this SNP also met the Bonferroni adjustment (p=0.001). In Caucasians; however, the effect of the SNP was increased to 11.5 ml/min/1.73m2 but the significance of the p value decreased to 0.01. This reduction in significance is likely due to the fact that the SNP was present at lower population frequency in Caucasians (6% in Caucasians vs. 28% in African Americans). This SNP was found in linkage to rs2231162 and rs2231164, two intronic SNPs in ABCG2.

Mutations in ABCG2, the breast cancer resistance gene have been linked to anti-cancer drug resistance due to its high expression in tumors[30, 31]. More specifically, there have been studies linking genetic variation in ABCG2 to differential MTX transport. In one Korean population, a functional coding region mutation in one person was shown to have up to 40% reduced transport compared to the reference in-vitro. We found a small estimated effect and no statistically significant association with the RFC G80A polymorphism which has most recently been linked to hepatotoxicity in ALL patients with mutations in glutathione S-transferase (GST) genes[32]. Haplotype analyses were conducted with the ABCG2 gene because multiple SNPs within this gene, in individual SNP analysis appeared significant, and because ABCG2 SNPs appeared correlated through linkage disequilibrium. Our haplotype analyses revealed that the TTG haplotype in block 1 of ABCG2 exhibited the strongest association and was an independent predictor for association with improved eGFR.

4.4.5 Conclusions

In this study, we determined that children on MTX may be at risk for MTX associated kidney dysfunction with some children being more susceptible than others despite hydration therapy. Of the non-genetic factors, dose of MTX was the most significantly associated with risk for renal dysfunction. Our genetic analysis indicated that a polymorphism in the influx transporter, OAT3, was associated with MTX-induced kidney dysfunction in Caucasians. This transporter is known to transport MTX and non-synonymous coding region variants have been found to exhibit reduced transport of the drug (unpublished data in our laboratory). Genetic variants in several efflux transporters were significantly associated with protection against MTX-induced kidney injury, though these variants did not reach significant associations in the Caucasian group based upon rigid Bonferroni criteria. Collectively, our studies suggest that germ line polymorphisms in the genes that code for transporters involved in renal exposure to MTX may affect an individual's risk for MTX-induced renal toxicity. Though replication studies are needed, these studies suggest that tailoring drug therapy with such information may decrease MTX associated toxicities.

4.4.6 Appendix: Table 4.A. Genes and SNPs selected for genotyping

SNPS selected	Alias	GENE	Exon/Intron	Change	Position	Coding/NonCoding	Failed Hardy Weinberg	Syn/Non
rs2231137		ABCG2	Exon	G/A	34	Coding		Nonsy
rs2231138		ABCG2	Intron	A/G				
rs2231142		ABCG2	Exon	C/A	421	Coding		Nonsy
rs2231148		ABCG2	Intron	A/T				
rs2231153		ABCG2	Intron	G/A				
rs2231157		ABCG2	Intron	T/C				
rs2231162		ABCG2	Intron	C/T				
rs2231164		ABCG2	Intron	G/A			Yes	
pmt3771_G>A		MRP1	Intron	G/A				
rs35588		MRP1	Intron	A/G				
rs35605		MRP1	Exon	C/T	1684	Coding	Bad DNA	Syn
rs3765129		MRP1	Intron	C/T				
rs8187843	pmt3749_G>A	MRP1	Intron	G/A				
rs8187858		MRP1	Exon	C/T	1704	Coding		Syn
rs1137968		MRP2	Exon	G/T	4290	Coding		Syn
rs1885301		MRP2	Intron	A/G			Bad DNA	
rs2273697		MRP2	Exon	G/A	1249	Coding		Nonsy
rs2804402		MRP2	Intron	A/G				
rs3740066		MRP2	Exon	C/T	1324	Coding		Syn
rs717620		MRP2	Intron	C/T			Bad DNA	
rs7910642		MRP2	Intron	G/A			Bad DNA	
rs8187698	pmt3846_T>C	MRP2	Intron	T/C				
rs8187710		MRP2	Exon	G/A	4544	Coding		Nonsy
rs11568643	PMT4560	MRP4	Intron					
rs11568650	PMT4517	MRP4	Intron					
rs11568656	PMT4557	MRP4	Intron	C/A				
rs11568663	PMT4526	MRP4	Intron	G/A				
rs11568672	PMT4542	MRP4	Intron					
rs1751034	PMT4556	MRP4	Exon	A/G	1116	Coding		Syn
rs2274401		MRP4	Intron	A/G				
rs2274403		MRP4	Intron	T/C				
rs2274407		MRP4	Exon	G/T	912	Coding		Nonsy
rs2274409		MRP4	Intron	G/A				
rs3742106		MRP4	Exon	T/G		3"UTR		
rs3765535		MRP4	Intron	T/C				
rs3818494		MRP4	Intron	C/G				
rs4148437		MRP4	Intron	T/C				
rs7324065		MRP4	Intron	G/A				
rs899494		MRP4	Exon	T/C	669	Coding		Syn
rs11568629		OAT1	Exon	A/G	351	Coding		Syn
rs3017670		OAT1	Intron	T/C				
rs4149171		OAT1	Exon	A/G		5UTR		

SNPS selected	Alias	GENE	Exon/Intron	Change	Position	Coding/NonCoding	Failed Hardy Weinberg	Syn/Non
rs2276299		OAT3	Exon	T/A	723	Coding		Syn
rs4149180		OAT3	Exon	G/A	153	Coding		Syn
rs4149182		OAT3	Intron	G/C				
rs10841789		OATP1A2	Intron	T/G				
rs10841795		OATP1A2	Intron	T/C				
rs3764044		OATP1A2	Intron	A/G				
rs722994		OATP1A2	Intron	G/A				
rs1051266		RFC	Exon	A/G		Coding		Nonsy

4.12 References

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Chapter 5

Finding Inhibitors of Transporters OAT1 and OAT3

Note: What follows is my final chapter, which is a work in progress. We have also identified compounds from this screen that may be possible aggregators and may confound the results of this screen. We are working to identify these aggregators. Please note that the data may change after aggregators are separated out.

5.1 Introduction

Detailed understanding of membrane transporter substrates and inhibitors is integral to all facets of drug development ranging from drug discovery through pre-clinical and clinical drug development. Renal transporters that are highly expressed in the kidney are particularly important in controlling tissue distribution, excretion and nephrotoxicity. In the kidney, members of the organic anion transporter (OAT, SLC22A) family of transporters are responsible for the transport of anionic drugs, toxins and other xenobiotics across excretory and barrier tissues[96, 97]. Difficulty in crystallizing membrane proteins has led to insufficient atomic level information that is important in the prediction and design of appropriate ligands to target the OAT family of transporters[98].

OATs are considered members of the major facilitator superfamily (MFS) proteins, although sequence comparisons demonstrate that similarities approximate 40-60% sequence homology[97]. The OATs all share the MFS hallmarks of 12 transmembrane alpha helices, cystolic N- C- termini, intracellular loop connecting two 6-helix halves, and the RXXXR signature conserved sequence between loop 2 and 3[99]. Yet, to date approximately 100 membrane proteins structures have been structurally characterized of which only a handful are from the major facilitator (MFS) and ATP-binding cassette (ABC) superfamilies[100].

5.2 Computational strategies for predicting ligands that interact with transporters

To predict ligand binding, a variety of strategies such as comparative modeling, ligand-based methods and a combination of in-silico strategies have been applied in the absence of a crystal structure [100]. Comparative modeling takes advantage of existing knowledge at the primary amino-acid sequence level and bioinformatic predictions of secondary structures. It allows one to infer the structure of an unknown transporter by using information from one previously characterized based on sequence identity[101]. Many groups have attempted to use comparative modeling paired with iterative bench experimentation to structurally characterize transporters such as the glucose transporter 1 (GLUT1) and P-glycoprotein (P-gp). Based on high-resolution crystal structures of two major facilitator superfamily (MFS) members (of which OATs belong), LacY (Lac Permease)[102] and GlpT[103] , MFS members are thought to share a similar fold[104]. This led to the development of three-dimensional structural models of organic cation transporters (SLC22A), OCT1(rat)[105] and OCT2(rabbit) [106]. The limitation of predicting substrate binding sites of OATs based on the OCT models was constrained by the alignment of residues within the 12 trans-membrane-spanning domains. Alignment of the trans-membrane domains did not allow inference into other key areas that are also important to substrate binding such as areas that connect trans-membrane domains as well as the amino and carboxy-termini which bear glycosylation sites[104]. This is true for the OCT transporters as well. Low homology among the OCTs limits the predictive ability of homology modeling. Further, because of the relatively low sequence homology (approximately 30%) of the crystal structures of their template, *Lac Permease* and *E. coli* lipid A transporter E. coli MsbA[107], few groups have made great strides in successfully generating knowledge about the structural features and binding characteristics of transporters.

Ligand-based methods such as pharmacophore and QSAR can be used to correlate variations in substrate and inhibitor binding affinities with structural features. Ligand-based methods generally quantitatively group and relate proteins based on chemical similarity of their ligands[108] with the hypothesis that similar proteins are likely to have similar properties and bind to the same group of proteins. Common features of ligands that bind to several proteins are used to develop a pharmacophore model. These can be constructed on distance comparisons (DISCO) - incorporating structural features such as hydrogen bonding, charge capacity, hydrophobic centers[109 110], and the genetic algorithm similarity program (GASP)[111]-that superimposes similar active molecules, or combinations of QSAR and pharmacophore methods, Catalyst/Hypogen (Acclrys, Inc., San Diego, CA) [100, 110]. Inputs required are active compounds that interact with transporters along with measured activities in some cases (Catalyst).

A review of the QSAR models built for the ABCB1 transporter found that all pharmacophore models retrieved were highly predictive for new ligands, but each show clear differences, both in the number and type of features involved, and in the spatial arrangement of these features. This observation implies that there is a multispecificity and diversity of the P-gp-binding site/region/zone. Given the promiscuous nature of the OAT transporters for structurally diverse ligands, this feature of multispecificity is likely, yet there may be recognizable patterns or clusters of preferred ligands that can be identified and improve our current knowledge of OAT substrate interactions.

Other novel ligand based methods have recently been developed. Several groups have proposed various methods for identifying ligands shared among proteins[108, 112, 113] , namely: through creating chemical linkage maps that relate targets[113], development of dendrograms that organize selectivity relationships[112], and identification of the most similar molecules shared by enzymes[114]. Given the relative difficulty and inaccuracy of comparative modeling for membrane transporters we focus on a ligand-

based method for predicting novel transporter inhibitors, similarity ensemble approach (SEA)[108].

5.3 Significance of OAT substrate prediction

Determining new substrates and inhibitors of the SLC22A family of transporters is particularly important because these transporters are expressed in organs that play major roles in drug absorption and elimination, such as the liver and the kidney. The most common method for identifying new substrates for OATs generally entails knowledge of the compound's charge, hydrophobicity and size. This information is then compared to a canonical set of substrates (of certain classes and charge properties) that are known to interact with the transporter. If close, the compound is deemed a potential substrate. As a result, molecules chosen for testing as potential OAT substrates are often biased because scientists' chosen test substrates are generally based on known compounds that are commercially available.

5.3.1 OAT1 and OAT3

OAT1 and OAT3 are homologous genes (approximately 50% amino acid identity)[47] and occur as tightly linked paralogs on human chromosome 11, separated only by 8.3 kb[115]. Both are expressed on the basolateral membrane and encode proteins of approximately 546 (OAT1) and 536 (OAT3) amino acid residues[96]. Substrates of OAT1 include para-aminohippurate, PAH, and the antiviral agents adefovir, cidofovir, tenofovir, zidovudine, and acyclovir[116]. Substrates of OAT3 appear to be broader in specificity. Therapeutic classes of drugs, which are substrates of OAT1 and OAT3 include: non-steroidal anti-inflammatory drugs (NSAIDs), hormone-like derivatives (i.e. prostaglandins), nucleotide/nucleoside analogues, antibiotics, and diuretics. Some model drugs for the OATs include estrone sulfate, ochratoxin A and methotrexate [117, 118]. Translocation of

sulfate or glucuronide conjugates of steroid hormones distinguishes OAT3 from OAT1[117]. Table A in the Appendix illustrates the general substrates and inhibitors known to interact with OAT1, OAT3, OAT4 and URAT1.

5.3.2 Transport mechanisms of OAT1 and OAT3

Transport of organic anions occurs through basolateral influx transporters (such as OAT1 and OAT3) that uptake anions across the membrane for excretion through the tubular lumen. Reabsorption occurs at the luminal membrane through apical transporters[119]. The energy dependence and function of OAT isoforms has been well studied[119]. For human OAT1 (SLC22A6), transport of organic anions across the basolateral membrane into the renal proximal tubular tubule occurs through an electrochemical gradient where anionic substrates are exchanged for intracellular dicarboxylates. The secondary transporter sodium-dicarboxylate (NaDC3) is the primary transporter that maintains an outwardly directed dicarboxylate gradient[119]. Sodium, potassium-ATPase maintains the inward sodium gradient necessary to drive the transport of anionic substrates. Human organic anion transporter 3 (OAT3) or (SLC22A8), functions in a similar manner[119].

5.3.3 Significance of identifying inhibitors for OAT1 and OAT3

Inhibition of the SLC22A transporters, OAT1 and OAT3 has been advocated as a mechanism for protecting against kidney toxicity. There is increasing evidence that some drug-induced nephrotoxicities result from a drug- or metabolite-mediated accumulation mediated by renal transporter systems, such as OAT1 and OAT3[97]. Therefore, interaction with OAT-transporters determines, to some extent, the clinical usefulness, side effects and toxicity risk for nephrotoxicity of drugs that are substrates of these transporters. Differential transport in OAT1 and OAT3 suggests that OAT specific

isoform inhibitors could be developed to determine the in vivo roles of these transporters in drug disposition and elimination. Further, identifying the transporters involved in renal elimination is critical to determining the mechanisms that drive pharmacokinetics, potential drug-drug interactions and the pathways involved in renal toxicity.

5.3.4 Structural models for OAT1 and OAT3

One three dimensional model of OAT1 has been built based on the crystal structure of glycerol-3-phosphate transporter (GLpT)[120] and validation through site-directed mutagenesis. Perry and colleagues found two new residues Y230 and F438 in the electronegative putative active site (830 Angstroms) that are important for altering the affinity of OAT1 for PAH and cidofovir and they suggested that aromatic amino acids are important for substrate recognition by OAT1[120]. Further, models for the substrate recognition site of rat OAT3 (rOAT3) and mutational experiments in flounder OAT support the model that different binding domains within the same binding site can act to recognize charge and translocate substrates.

5.4 Study objective

We focus our study on organic anion transporter 1, OAT1 (SLC22A6) and on its paralog, organic anion transporter 3, OAT3 (SLC22A8) because they are highly expressed in the kidney and are thought to play a major role in the elimination and accumulation of various drugs and environmental toxins in the kidney. Our objective was to discover novel and selective inhibitors of these transporters by high throughput screening of over 4000 diverse compounds, and through development of predictive methods through SAR and computational ligand-based methods. Figure 5.1 represents the use of one computational method (SEA), to cluster transporters according to the chemical fingerprints of known inhibitors. One of the underlying principles of our study is the

hypothesis that chemical similarity is a predictor of biological action. We use this approach to investigate whether we could predict molecular interactors for the OATs and identify chemical features that could be used to distinguish between two homologous transporters such as OAT1 and OAT3. A secondary goal was to find better methods for relating transporters for structurally diverse compounds, quantitatively based on the chemical similarity of their ligands.

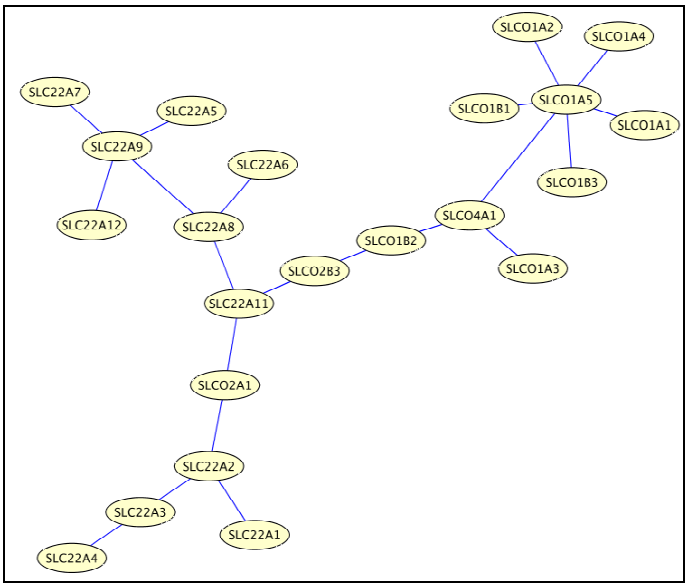


Figure 5.1 Cluster diagram of the SLC22A members by known substrates and inhibitors (from literature) by chemical similarity using similarity ensemble approach (SEA). This illustrates the use of a computational method to identify and predict transporter-drug chemical space.

The use of SEA (see methods below) to cluster the SLC22A family members by their substrates and inhibitors yielded the diagram shown in Figure 5.1. OAT3 (SLC22A8) shares similar ligands with SLC22A11 (OAT4) and SLC22A6 (OAT1). However, OAT1 appears to be more distinct from the other OATs in terms of biological function compared to OAT3, which seems to have more overlap in its biological function with other OATs. These results are consistent with the observation that OAT3 appears to display broader substrate specificity than OAT1.

5.5 Methods

5.5.1 Cell-based screening methods

OAT1 and OAT3 over-expressed HEK cell lines were grown in Dulbecco's modified Eagle medium and supplemented with 10% FBS, 5% penicillin G streptomycin, and 75 μg/μl hygromycin. Cells were plated onto 96 well poly-D-lysine coated black plates (Greiner bioOne) at approximately 6 X 10^4 cells/well and grown for 48 hours until 85% confluent. Experiments were carried out using Biomek FX. Cells were washed 1 time with 100 μl PBS. Residual PBS was removed by inverting the plate and centrifuging out the residual PBS at 650 gs/ min. Cells were then incubated with 100 μl of compound at 50μM and carboxyfluorescein (concentration 15 μM) for 2 minutes washed 3 times with 100 μl PBS. The plate was then centrifuged again to remove residual PBS and lastly, wells were filled with 100 μl PBS. The two outermost columns of the 96 well plate were used as controls. Ochratoxin A was used as a positive control compound for OAT1 and indomethacin was used as a positive control compound for OAT3. Fluorescence was read using a plate reader (HT-Analyst) with excitation and emission wavelengths of 485nm and 530nM, respectively. Two different reads were taken with various integration times at 3000 ms and 30000 ms, Z height was 3mm and each well was read 10-30 times.

5.5.2 Fluorescence high throughput screening

Screening libraries consisted of a total of 4460 compounds, 2161 from the microsource library, 2299 compounds from the chemical diversity library were purchased from the UCSF Small Molecule Discovery Center (SMDC). Calculation of hits and Z factors were generated using pipeline pilot ™ and 3 standard deviations from the mean was the most

stringent criteria used to define a hit. Data were normalized by averaging the positive and negative controls across all the plates and using them to benchmark the amount of inhibition per compound. The second threshold used to determine a "hit" was two inhibition thresholds; inhibition greater than 50% and inhibition greater than 80%. Hits were validated by re-screening, by conducting detailed dose response profiles, and through inhibition of radioligands ([H^3] adefovir for OAT1 and [H^3] estrone sulfate for OAT3). All of the results from the screening can be viewed at druid.ucsf.edu/smdc (Microsource and ChemDiv normalized datasets).

5.5.3 Radioactive secondary screens

After the primary screen of the library, compounds found to be selective inhibitors for OAT1 and OAT3 were selected to be re-screened at three different concentrations using radioactive uptake assays. Cells were plated as described above and grown for 48 hours until 85% confluent. Cells were washed once with 100 μl PBS, residual PBS was removed, cells were incubated with 75 μl of compound in duplicate at 0, 5, 20 and 50 μM compound for 15 minutes. Compound was then removed by carefully pipetting out the compound from each well. 100 μl of radioactive compound (1μM) in 100 μl of PBS was then incubated for 2 minutes. Cells were then washed 3 times with 100 μl PBS, and then lysed with 100 μl 0.1N NaOH/0.1% sodium dodecyl sulfate (SDS). Cellular lysates (75 μl) were then placed into scintillation vials for determination of radioactivity using a scintillation counter.

5.5.4 Computational prediction of OAT1 and OAT3 interacting molecules

Computational prediction was conducted by Mike Keiser in the laboratory of Brian Shoichet. The primary method that was used to predict novel inhibitors was Similarity Ensemble Approach (SEA).

5.5.4.1 Similarity Ensemble Approach (SEA)

Similarity Ensemble Approach or SEA is an algorithm that compares sets without size chemical composition bias by correcting for chemical similarity that is expected between ligand sets at random using a model similar to BLAST[108]. The goal of the technique is to link and compare ligand sets and compare them to protein targets in minimal spanning trees. This allows us to identify clusters of biologically related proteins (transporters) through the chemical similarity of their ligands[108]. When given a set of ligands that are known to interact with OAT1 or OAT3, similarity scores are calculated through Tanimoto coefficients (Tc) for each pair of ligands and raw similarity scores (the expected similarity across all pairs in the set) was calculated to denote the similarity across ligands sets. Keiser and colleagues then developed a statistical model to provide e values and z scores that allow one to compare across ligand sets of different sizes[108].

5.5.4.2 Assembly of OAT1 and OAT3 substrate/inhibitor data from literature

Data on substrates and inhibitors for OAT1 and OAT3 were gathered through an exhaustive literature search. This information was compiled into tables and then converted into simplified molecular input line entry specification or SMILES which is a specification for unambiguously describing the structure of chemical molecules using short ASCII strings for use in SEA.

5.5.4.3 SAR multivariate regression analysis

Data from the primary HTS screen was used in the SAR study. SARVision™ was used to calculate SAR parameters. Multivariate regression analysis with STATA™ was used to conduct the analysis. Two main forms of analyses were used. To determine the

chemical features that identify OAT1 inhibitors and OAT3 inhibitors independent from each other, the inhibition activity was used as the independent variable. To distinguish OAT1 features from OAT3, the difference in OAT1 and OAT3 inhibition activity was used as the independent variable. Covariates included lipophilicity, molecular weight, polar surface area (PSA), number of heteroatoms, hydrogen bond donor activity (HBD), hydrogen bond acceptor activity (HBA), number of rotatable bonds and number of rings.

5.6 Results and discussion

5.6.1 Summary of findings

1) We found molecules that interact with both OAT1 and OAT3 vary enormously in structure

2) We identified several molecules that appeared to selectively inhibit carboxyfluorescein and adefovir in OAT1 expressing cells

3) We identified several molecules that appeared to selectively inhibit carboxyfluorescein and estrone sulfate in OAT3 expressing cells

4) We found that within molecules with similar chemical scaffolds, subtle changes in electrostatic interactions can influence inhibition

5) We observed that changes in steric interactions can abolish the activity in molecules that interact with both OAT1 and OAT3

6) We determined that greater hydrogen bond acceptors and logP values were positively associated with inhibition of OAT1 whereas the number of rotatable bonds and number of rings were negatively associated with OAT1 inhibition and increasing logP values and number of heteroatoms were positively associated with OAT3 inhibition

5.6.2 General hit rate

Of 4460 molecules in the microsource library, there were 10 molecules that inhibited greater than 80% of CF uptake in OAT1 expressing cells and 26 in OAT3 expressing cells. Of the over 2000 molecules in the chemical diversity library, only 1 and 0 compounds for OAT1 and OAT3, respectively, inhibited CF accumulation greater than

80%. At 50% inhibition, 190 and 319 compounds were identified as inhibitors of OAT1 and OAT3, respectively, in the microsource library and 93 and 9, respectively, for the chemical diversity library (Table 5.1)

Table 5.2a and 5.2b is a listing of selective inhibitors for OAT1 and OAT3 demonstrating that in the primary screen, there were a total of 35 selective inhibitors and 14 nonselective inhibitors.

Table 5.1 Number of compounds in two libraries that inhibit CF accumulation in OAT1 and OAT3 expressing cells. The number and percent of molecules that selectively inhibited OAT1 and OAT3 by 80% and 50% from 2 chemical diversity libraries (Microsource and Chemical Diversity) are shown below

Library	Hits >80% Inhibition	Hits >50% Inhibition	N
Microsource- OAT1	10 (0.5%)	190(9%)	2161
Microsource OAT3	26(1%)	319(15%)	
Chem Diversity- OAT1	1(0.04%)	93(4%)	2299
Chem Diversity- OAT3	0(0)	9(0.4%)	

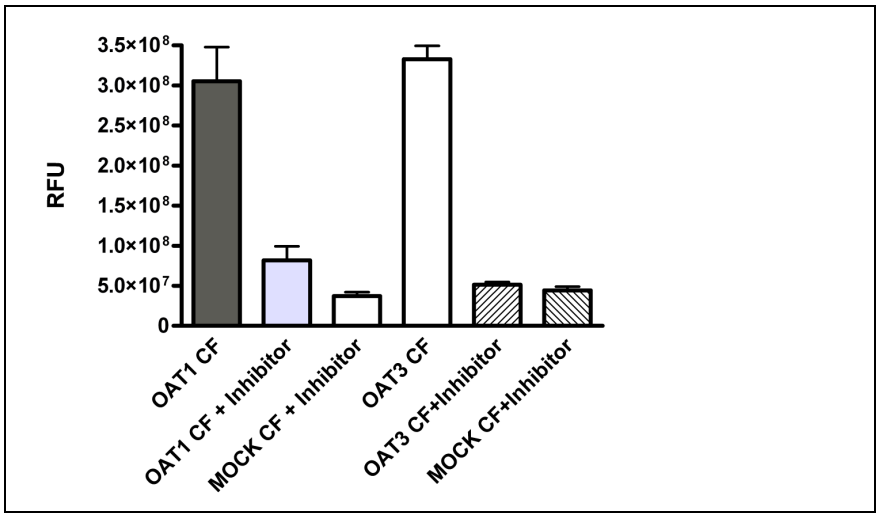


Figure 5.2 Control experiments for the high throughput screening in OAT1 and OAT3 stably expressing HEK293 cells. Data represent the mean ± SEM from 12 replicate determinations of carboxyfluorescein (CF) uptake in cells incubated CF alone or CF plus an inhibitor (either 50 μM ochratoxin for OAT1 or 50 μM indomethacin for OAT3) for 2 minutes.

Figure 5.2 shows the comparison of carboxyfluorescein (CF) uptake in OAT1, OAT3 and Mock transfected cells with and without our positive control inhibitor. Our OAT1 assay demonstrated over 8 fold uptake of CF over our most negative control (mock together with positive control inhibitor) and OAT3 showed over 7 fold uptake over our most negative control.

5.6.3 OAT1 and OAT3 interacting molecules vary enormously in structural diversity

A qualitative overview of the hits for OAT1 and OAT3 suggests that there was a large diversity in the structures of molecules that inhibited OAT1 and OAT3. Figure 5.3 illustrates the large range in structural scaffolds of molecules from the microsource library that showed greater than 50% inhibition of OAT1.

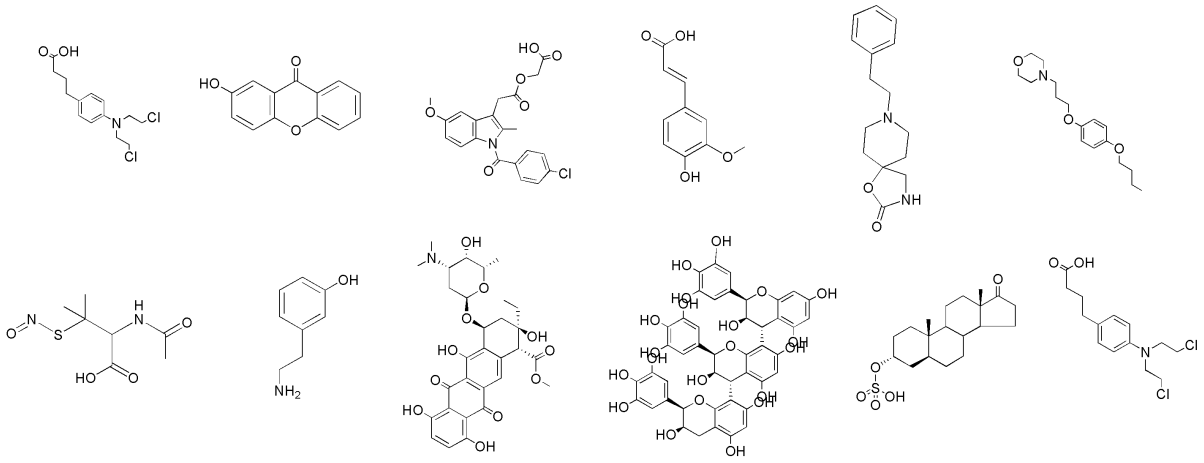


Figure 5.3 Examples of range of molecules from the microsource library that showed greater than 50% inhibition in OAT1 transfected cells. The very wide range in structure demonstrates that molecules that interact with OAT1 are very diverse in structure.

Table 5.2a Non-selective Inhibitors that inhibited carboxyfluorescein uptake in both OAT1 and OAT3 expressing cells

ID*	OAT1	OAT3	ABS Diff.	Library
130123	79.71%	82.92%	3.21%	Microsource
130255	88.21%	89.97%	1.76%	Microsource
130982	82.06%	84.52%	2.46%	Microsource
131132	82.44%	84.48%	2.04%	Microsource
131322	84.23%	88.25%	4.02%	Microsource
197905	57.42%	73.23%	15.81%	Chemdiv
197843	62.72%	68.7%	5.98%	Chemdiv
197794	71.67%	60.22%	11.45%	Chemdiv
197658	55.08%	85.76%	30.68%	Chemdiv
197308	55.75%	75.26%	19.51%	Chemdiv
197272	75.35%	64.64%	10.71%	Chemdiv
197270	70.82%	63.49%	7.33%	Chemdiv
197041	60.39%	74.7%	14.31%	Chemdiv
192300	70.29%	78.6%	8.31%	Chemdiv

* see http://druid.ucsf.edu/smdc OAT data for structures

Table 5.2a lists the library code numbers of molecules that inhibited CF uptake in both OAT1 and OAT3 expressing cells. In addition to discovering general inhibitors for OAT1 and OAT3, our objective was to discover selective inhibitors for OAT1 versus OAT3. Table 5.2b lists identification numbers of the selective inhibitors that were found in both libraries for each transporter where inhibition was greater than 50% for one transporter and less than 50% for the other. Whereas the purpose of finding selective inhibitors for OAT1 and OAT3 may allow us to characterize their substrate and inhibitor discrimination capabilities, identifying overlapping interacting molecules can help elucidate how inhibitors may interact in tissues that express both transporters.

We selected several of these selective hits from our primary screen to conduct a secondary dose response carboxyfluorescein screen as well as a radioactive screen with the OAT1 and OAT3 canonical substrates adefovir and estrone sulfate, respectively. Our secondary screen consisted of a dose response profile of primary screen “hits”. This profile allowed us to select compounds to test for selective inhibition.

We followed this by screening the top 24 chemical diversity library hits as inhibitors of radiolabelled model substrates of the two transporters. Below is one selected example of a secondary screen with carboxyfluorescein and radioactive screen of the hits we uncovered.

Table 5.2b Selective inhibitors that inhibit carboxyfluorescein accumulation by >50% in OAT1 expressing cells and less than 50% in OAT3 expressing cells and vice versa.

Compound Number	ID*	OAT1	OAT3	ABS Diff	library
1	129940	16.76%	67.69%	50.93%	Microsource
2	130016	35.17%	86.33%	51.16%	Microsource
3	130333	38.13%	88.2%	50.07%	Microsource
4	131088	21.67%	79.21%	57.54%	Microsource
5	131090	27.66%	85%	57.34%	Microsource
6	131119	36.66%	96.67%	60.01%	Microsource
7	131291	24.45%	82.51%	58.06%	Microsource
8	131549	26.67%	80.43%	53.76%	Microsource
9	131576	70.89%	20.02%	50.87%	Microsource
10	131732	96.57%	28.44%	68.13%	Microsource
11	131733	23.64%	76.5%	52.86%	Microsource
12	197925	62.8%	11.08%	51.72%	Chem Div
13	197902	61.61%	6.73%	54.88%	Chem Div
14	197275	6.71%	58.16%	51.45%	Chem Div
15	197246	26.62%	77.38%	50.76%	Chem Div
16	197123	61.61%	2.78%	58.83%	Chem Div
17	193281	66.75%	-2.33%	69.08%	Chem Div
18	193239	7.19%	57.54%	50.35%	Chem Div
19	192239	6.61%	56.77%	50.16%	Chem Div
20	192138	81.89%	13.65%	68.24%	Chem Div
21	192137	65.03%	11.16%	53.87%	Chem Div
22	192109	23.59%	79.64%	56.05%	Chem Div
23	192106	23.35%	78.91%	55.56%	Chem Div
24	192105	15.19%	80.52%	65.33%	Chem Div
25	192104	27.85%	81.24%	53.39%	Chem Div
26	192103	24.09%	83.48%	59.39%	Chem Div
27	192102	24.67%	86.29%	61.62%	Chem Div
28	192101	28.98%	87.06%	58.08%	Chem Div
29	192049	61.97%	5.64%	56.33%	Chem Div
30	192000	11.68%	66.12%	54.44%	Chem Div
31	191970	31.35%	-20.68%	52.03%	Chem Div
32	191739	18.49%	71%	52.51%	Chem Div
33	191737	9.09%	90.45%	81.36%	Chem Div
34	191608	4.76%	96.12%	91.36%	Chem Div
35	191607	22.54%	105.46%	82.92%	Chem Div

* see http://druid.ucsf.edu/smdc OAT data for structures

Molecule 191607 was found to be an inhibitor of OAT3 (105% inhibition) but not of OAT1 (22% inhibition) (Table 5.2b). To confirm the selective inhibition results of the primary screen for 191607, a dose response radioactive screen was conducted. Dose response radioactive screen with radioactive estrone sulfate as the probe compound incubated with 191607 demonstrates that this molecule is also a selective inhibitor of OAT3. Figure 5.4 illustrates a clear inhibition response curve for OAT3 while OAT1 transport of adefovir does not appear to decrease with increasing concentrations of 191607. Molecules with a similar scaffold to 191607 demonstrated that changes in the location of the nitrogen and addition of methoxy substituents were sufficient to substantially decrease activity (Figure 5.5).

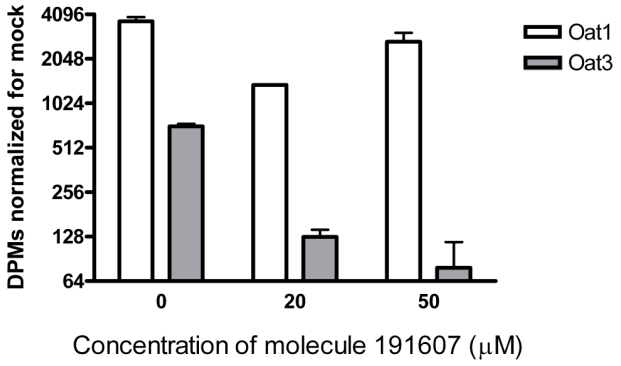
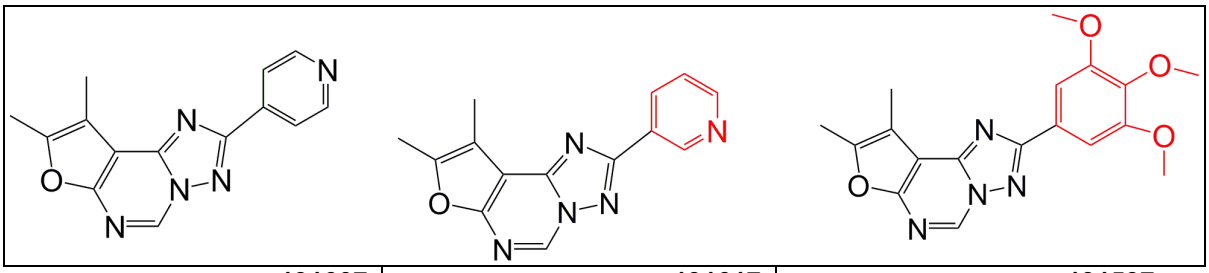


Figure 5.4 Dose response of molecule 191607 with [H^3] estrone sulfate in OAT3 cells and [H^3] adefovir in OAT1 transfected HEK cells. Duplicate wells for the experiment were used for each concentration.



	191607	191617	191597
OAT3	105%	22%	12%
OAT1	22%	27%	32%

Figure 5.5 Percent inhibition of carboxyfluorescein by 191607 and molecules with a similar scaffold in OAT1 and OAT3 transfected HEK cells.

We conducted an analysis of the most common scaffolds in the set of OAT3 interacting molecules that showed greater than 50% inhibition. Determining the most frequent overlaps in substituents and scaffolds in the molecules that exhibited some inhibition activity may identify important chemical groups that are significant for the inhibitor/substrate binding to OAT1 and/or OAT3. The 5 most common scaffolds for inhibition of each transporter are shown in Figure 5.6. Both the transporters seem to favor the presence of heteroatoms, which contain oxygen atoms. The sulfonamides were more selective for OAT3 when compared to OAT1.

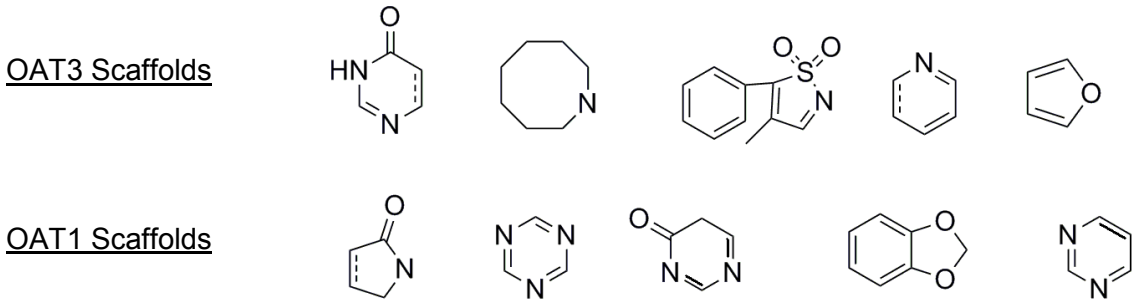
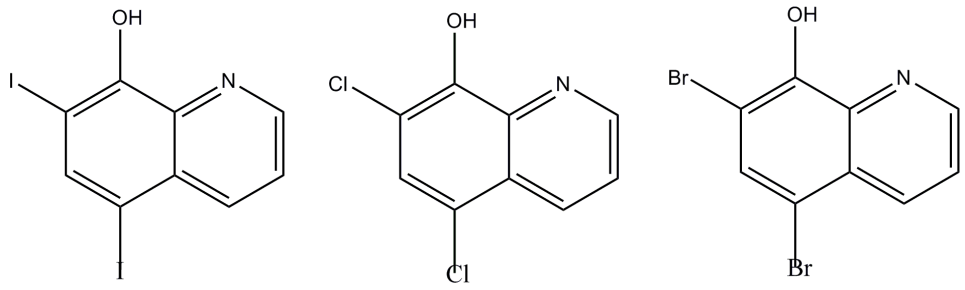


Figure 5.6 Scaffolds common to OAT1 and OAT3 selective inhibitors
From left to right:
OAT3 Scaffolds: Pyrimidone; Azocane, 2-benzyl-4-methyl Isothiazole-1,1-dioxide, pyridine, furanOAT1 Scaffolds: dihydropyrrolidine, triazine, (Pyrimidone) benzodioxolane; 5,6-dihydropyrimidine

5.6.4 Subtle changes in substituents in molecules with similar scaffolds can abolish the activity in molecules that interact with both OAT1 and OAT3

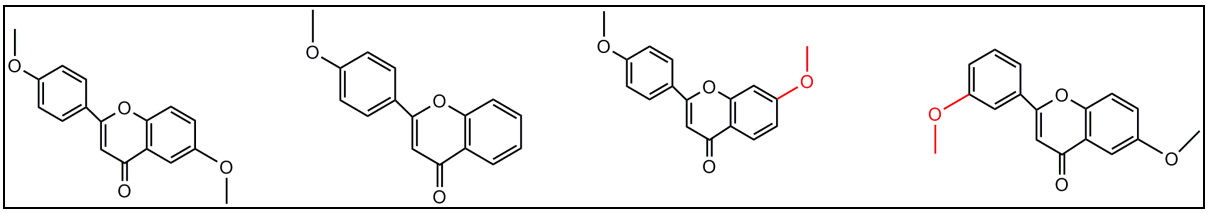
We found several examples where molecules within the same chemical family exhibited vastly different inhibition activity due to the addition of a charged group or the substitution of hydrogen bond acceptor moieties. For example, the substitution of iodide with chloride or bromide reduced the inhibition activity of the reference molecule over 50% for both OAT1 and OAT3 (Figure 5.7). Diiodosubstituted 8-hydroxyquinolines demonstrated significant inhibition of both OAT1 and OAT3 that decreased sequentially with a change of the halogen substitutent to dibromo and then to dichloro. Bulky, hydrophobic substituents are therefore favored on 8-hydroxyquinoline ring.



Molecule 130855			
Percent Inhibition			
OAT1:	**70%**	**14%**	**27%**
OAT3:	**82%**	**27%**	**35%**

Figure 5.7 Percent inhibition of carboxyfluorescein by molecule 130855 and its analogs in OAT1 and OAT3 stably transfected cells. Inhibition activity was vastly reduced by substituting iodide with chloride and bromide.

We also observed that there are instances where not only does the particular charged moiety matter, but the position of R groups could substantially change the inhibition activity (Figure 5.8). In the example, the existence and specific placement of methoxy groups appear to drastically change inhibition activity. Movement or deletion of the methoxy group from the chromenone ring seems to have an impact on the inhibition of transport in case of both transporters; more so for OAT1. These data suggest that the loss of important hydrogen bond interactions with the transporter protein or a highly unfavorable interaction with protein side chains/backbone, leads to adverse interactions with the transporter and inhibitory activity.



Molecule 81318 Percent Inhibition				
OAT1:	**74%**	**24%**	**22%**	**6%**
OAT3:	**71%**	**41%**	**35%**	**30%**

Figure 5.8 Effect of the substituent placement on the inhibition of OAT1 and OAT3. Inhibition activity was vastly reduced by removing or moving the methoxy group around the scaffold

Collectively, these results suggest that the ligand binding sites for OAT1 and OAT3 not only recognize specific pharmacophoric features of ligands but that the placement of these features is important. Kaler and colleagues used COmparative Molecular Field Analysis (COMFA) to distinguish amongst the substrate preferences of mOat1 and mOat6[98]. Their most obvious observation was that doubly charged organic anions were preferred by mOat1 and that steric and electrostatic factors govern the

affinity for mOat1. These findings reinforce our observations about the importance of steric and electrostatic factors in OAT1 molecular interactions.

5.6.5 SAR study

To identify other global chemical features for OAT1 and OAT3 interacting ligands, we used a general multivariate regression model. First, a multivariate regression model with inhibition activity of OAT1 or OAT3 as the independent variable was conducted with several pharmacological features introduced as covariates (Table 5.3). These features were logP, hydrogen bond donor/acceptor, number of heteroatoms, number of rings, polar surface area, number of rotatable bonds and molecular weight. For the model of OAT1 activity, only molecules with less than 50% inhibitory activity for OAT3 activity were considered in order to ensure OAT1 selectivity. Similar considerations were used for OAT3. On average, the features that produced a statistically significant change in inhibition activity for OAT1 were hydrogen bond acceptor (HBA), number of rotatable bonds, lipophilicity and number of rings. Hydrogen bonding acceptor (0.73% inhibition) activity and logP (0.74% inhibition) were associated with increased inhibition (P=0.001 and P=0.002) while, OAT1 preferred few rotatable bonds (-0.32% inhibition) and few rings (-0.63% inhibition) (P=0.003 and P=0.04, respectively). Likewise OAT3 inhibitor activity was associated with few rotatable bonds (0.32% inhibition, P=0.021), and an increase in logP (1.02% inhibition, P=0.001). An increase in the number of heteroatoms (atoms that are not carbon or hydrogen) was also preferred by OAT3 although statistical significance was not achieved (0.56, P=0.053). Truong and colleagues conducted a quantitative structure analysis of discrimination between nine antiviral drugs within organic anion transporters 1,3 and 6[121]. They found that the antiviral preference of mouse Oat1 could be explained by Oat1 affinities for ligands with high polar surface areas, and that mouse Oat3 preferred hydrogen bond

acceptors and low rotatable bond numbers[121]. With the exception of Oat3 preference of few rotatable bonds, our results differ from those of Truong. These differences are likely because our analysis confers a more global approach by considering a large range of molecules without specifying class and considers only the human ortholog.

Table 5.3 Significant SAR features found in primary HTS assay of OAT1 and OAT3

	Feature	Coef.	Std. Err.	P value	95% CI	
OAT1	Hydrogen Bond Acceptor	0.73	0.21	0.001	0.31	1.16
	Num rotatable bonds	-0.32	0.11	0.003	-0.54	-0.11
	logP	0.74	0.24	0.002	0.26	1.21
	Num of rings	-0.63	0.31	0.040	-1.25	-0.02
OAT3	Num rotatable bonds	-0.32	0.14	0.021	-0.60	-0.05
	log P	1.02	0.30	0.001	0.42	1.62
	Num of Heteroatoms	0.56	0.29	0.053	-0.01	1.13

The above model controlled for logP, hydrogen bond donor/acceptor, number of heteroatoms, number of rings, polar surface area, number of rotatable bonds and molecular weight

To determine inhibitor specificity differences for OAT1 and OAT3, we used multivariate regression analysis. Our analysis suggested that a 1 heteroatom increase in OAT3 inhibitors was associated with a positive 2% difference in inhibition activity between OAT1 and OAT3 (P=0.05). Hydrogen bond acceptors are associated with 2% increase in inhibition activity of OAT1 over OAT3 (P=0.03). Other features were not significantly different. The differential features of the molecules that interact with OAT1 and OAT3 suggest that there are distinct binding sites between the two transporters despite their sequence homology. These data may be used to inform a more rational approach to drug design for OAT1 and OAT3 selective inhibitors (Table 5.4).

Table 5.4 SAR features that distinguish OAT1 from OAT3

Difference	Coef	P value	95% CI	
Number of Heteroatoms	-2.03	0.05	-4.03	-0.02
HBA	2.08	0.03	0.23	3.92

The above model controlled for logP, hydrogen bond donor/acceptor, number of heteroatoms, number of rings, polar surface area, number of rotatable bonds and molecular weight

5.6.6 Lead Identification: Tools for the future

While our chemo-informatic analysis of our hits is ongoing, we have identified several selective inhibitors for targeting OAT1 and OAT3. Further analysis and experimentation must be conducted to ascertain whether in fact they have all of the drug-like properties required to be move forward as a lead compound. If they can progress to leads, there could be several applications for these leads. Selective inhibitors may be potential pharmacological agents targeting OAT1 and OAT3. For example, probenecid (tradename: Benuryl), is a known inhibitor of OAT1 and OAT3 and has been used to decrease cidofovir toxicity in to the kidney in patients with cytomegalovirus retinitis [122] [123, 124]. Further, probenecid has been investigated as a potential agent to double Tamiflu plasma concentrations in the case of an influenza pandemic[125].

Another use of selective inhibitors, regardless of their use as a drug, is their ability to be used as tools for drug discovery and preclinical drug dvelopment[126]. To probe the mechanisms of drug-drug interactions, we have identified several molecules where subtle changes in the structural features of the molecule create large changes in inhibition activity. These molecules can be exploited in further biophysical studies to answer questions about the active site(s) of transporters, the mechanism of substrate binding and may even be used as a tool in crystallography. OAT1 and OAT3 are key organic anion transporters in the kidney and are involved in active tubular secretion of many clinically used drugs. Selective inhibition may be used to identify which of these transporters is responsible for drug elimination and importantly to identify the mechanisms of drug-drug interactions. Selective chemical agents with different lengths, different stereochemistries and different side-chain specificities in the same or different oligomuric specifications to crosslink/immobilize proteins can be utilized as biochemical

tools to increase the stability of transporters in certain conformational relationships for crystallography. Determining which molecules or class of molecules are likely inhibitors of OAT1 and OAT3 also allows us to determine what areas of chemical space to avoid and how to prioritize classes of molecules when scientists make choices to functionally screen libraries. Our characterization of selective inhibitors and their analogs and correlation with their inhibitory activities will allow us to move ahead with making more rational decisions when developing drug molecules to target OAT1 and OAT3 or developing other types of crystallographic tools to probe their active sites.

5.6.7 Appendix

Table 5.A: Known substrates and inhibitors of OAT1, OAT3, OAT4 and URAT1

SLC22A6	SLC22A8	SLC22A11	SLC22A12
solute carrier family 22 (organic anion transporter) member 6	solute carrier family 22 (organic anion transporter) member 8	solute carrier family 22 (organic anion/cation transporter) member 11	solute carrier family 22 (organic anion/cation transporter) member 12
OAT1,hPAHT	**OAT3**	**OAT4**	**RST, OAT4L, URAT1**
acetazolamide	azidothymidine	dehydroepiandrosterone sulfate (DHEA-S)	furosemide
6-carboxyfluorescein	bumetanide	prostaglandin F2alpha	lactate
PMEDAP	cAMP	tetracycline	nicotinate
PMEG	cefadroxil	prostaglandin E2	phenylbutazone
acetaminophen	cefamandol	ochratoxin A	probenecid
acetylsalicylate	cefazolin	estrone sulfate	salicylate
acyclovir	cefoperazone	estradiol 3,17-disulfate OR 66430	sodium nitrate
adefovir	cefotaxim	dehydroepiandrosterone-3-sulfate	sulfinpyrazone
adefovir	ceftriaxone	17-beta-estradiol-3-sulfate	benzbromarone
allopurinol	cephalexin	beta-estradiol-3-sulfate	benzylpenicillin
azidothymidine	cephaloridine	Phorbol myristate acetate	bumetanide
benzbromarone	cephalotin	phorbol 12,13-dibutyrate (PDBu)	indomethacin
bumetanide	chlorothiazide	6-Carboxyfluorescein	losartan
captopril	cimetidine	glutarate	urate
cefadroxil	cyclothiazide	pranlukast	sodium nitrate
cefamandole	dehydroepiandrosterone sulfate (DHEA-S)	zafirlukast	
cefazolin	diclofenac	losartan	
cefoperazone	estradiol glucuronide	benzbromarone	
cefotaxime	estrone sulfate	sulfinpyrazone	
ceftriaxone	ethacrynate	Cholecalciferol sulfate	
cephaloridine	famotidine	betamipron	

cephalotin
cephradine
chlorothiazide
cidofovir
cimetidine
cinoxacin
cyclothiazide
diclofenac
diflunisal
doxycycline
ethacrynate
etodolac
flufenamate
fluorescein
flurbiprofen
fluvastatin
furosemide
ganciclovir
ganciclovir glutarate
hydrochlorothiazide
ibuprofen
indole acetate
indomethacin
indoxyl sulfate
ketoprofen
losartan
loxoprofen
mefenamate
methazolamide
methotrexate
minocycline
nalidixate
naproxen
ochratoxin A
oxytetracycline
p-aminohippurate
phenacetin
phenylbutazon
piroxicam
PMEDAP
PMEG

flufenamate
furosemide
ganciclovir
glutarate
hydrochlorothiazide
indomethacin
ibuprofen
ketoprofen
loxoprofen
mefenamate
methazolamide
methotrexate
naproxen
ochratoxin A
phenacetin
phenylbutazone
piroxicam
prostaglandin E2
prostaglandin F2alpha
ranitidine
salicylate
salicylate
simvastatin
sulindac
taurocholate
tetracycline
trichlormethiazideacetylsalicylate
urate
valacyclovir
zidovudine
acetazolamide
acyclovir
azathioprine
benzylpenicillin
p-aminohippurate
pravastatin
probenecid
valproate

diethylpyrocarbonate
hydrochlorothiazide
lactate
nicotinate
pyrazinoate
oxypurinol
bumetanide
azidothymidine

pravastatin
probenecid
prostaglandin E2
prostaglandin F2alpha
ranitidine
salicylate
simvastatin
sulindac
tenofovir
tetracycline
trichlormethiazide
zalcitabine
zidovudine
benzylpenicillin

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Chapter 6

Discussion

The objective of this dissertation is to define the role of transporters in nephrotoxicity. Studies in this dissertation highlight the importance of transporters as primary modulators of drug disposition and elimination, as genetic targets for pharmacogenetic interventions, and drug targets for influencing drug safety and efficacy. We assess how genetic variation in transporters may play a role in disease through epidemiological studies and through functional cellular studies. We also use a chemocentric and informatics perspective to identify and predict selective inhibitors with the ultimate goal of using transporter as targets for discovering novel therapeutics and as tools for drug discovery.

6.1 Summary of findings

In Chapter 2 we conducted an epidemiological analysis of tenofovir associated renal dysfunction using a nation-wide prospective cohort of women with HIV, the Women's Interagency HIV Study (WIHS). The study is unique because it is the first to investigate the effect of tenofovir over time in a large population of US women living with HIV. Our analysis suggests that the prevalence of tenofovir-associated renal insufficiency in women infected with HIV appears to be in the range of that reported in mostly male cohorts. After controlling for a number of covariates, tenofovir use was associated with a 2.9% decrease in GFR from baseline. Women with healthy renal function at baseline experienced an average decline in GFR of 5.6% ($P < 0.001$). We also found that consistent with what has been observed in other cohorts, where patients with prior renal dysfunction experienced improvements in renal function, women with baseline renal function grade 2 experienced an average improvement in renal function of

9.3% after tenofovir exposure. This improvement was also reflected in patients on abacavir. We concluded that it is possible that improvement in HIV due to any HIV treatment may lead to improvement in renal function.

In Chapter 3 we use functional experimental studies to determine which transporters may drive adefovir and tenofovir associated cytotoxicity. We looked also at the effect of genetic variation in transporters on adefovir and tenofovir transport and cytotoxicity. We found that OAT1 was a primary transporter for adefovir and tenofovir and that cytotoxicity was 28 fold greater in OAT1 expressing cells than in mock-transfected cells. Through site-directed mutagenesis and stable transfection, we created seven known non-synonymous variants of OAT1 and tested them to determine if these variants showed differential transport and cytotoxicity compared to reference OAT1. We found that one OAT1 variant, a singleton, the R454Q variant, was nonfunctional ($P<0.001$) in its transport of adefovir and tenofovir. The base change resulted in protection from tenofovir-associated cytotoxicity. The other variants did not show a statistically significant difference in transport or cytotoxicity compared to wild type. Western blot suggested that the nonfunctional variant showed reduced expression compared to reference. Immunohistochemistry and GFP-tagged constructs of the R454Q variant suggested that the nonfunctional variant had a lower expression at the plasma membrane, which explained in part its reduced function. This analysis demonstrated that organic anion transporters can play a major role in modulating adefovir and tenofovir associated transport and cytotoxicity and that genetic variation in these transporters may contribute to differences in adefovir and tenofovir transport and cytotoxicity. Detailed investigation of transporter(s) role in modulating transport and cytotoxicity may provide insights into how transporters can influence adefovir and tenofovir associated nephrotoxicity and determine whether genotyping for genetic

polymorphisms may identify patients who are at risk for adefovir and tenofovir associated nephrotoxicity.

Chapter 4 is an analysis of a genetic epidemiology study of methotrexate (MTX)-induced renal dysfunction. Our study focuses on a cohort of 556 pediatric leukemia patients from St. Jude's Children's Research Hospital. Our observational analysis of patient response to MTX treatment is indicative that some patients experience renal dysfunction after treatment. We hypothesize that polymorphisms in renal transporters may contribute to the renal dysfunction seen in these patients. A total of 11 individual SNPs were associated with significant changes in renal function. The outcome used was a change in creatinine clearance and our analysis controlled for risk factors such as dose, age, sex and cancer risk in Caucasian patients. The SNPs that were associated with improvement in renal function all belonged to efflux transporters, ABCG2, MRP2 and MRP4. SNPs in other genes were associated with decreased renal function. Interestingly, the strongest predictor of change in GFR was an OAT3 polymorphism rs4149180 associated with a 12 ml/min decrease in renal function. This was the only polymorphism that we found that met the stringent Bonferroni adjustment for multiple testing. The second strongest predictor was a polymorphism in efflux transporter MRP2, rs1885301. This SNP was associated with improved renal function of 9 mls/min. Several other SNPs were also found to be associated with changes in renal function. We identified these as interesting candidates for detailed experimental characterization. Since we found that multiple SNPs within the ABCG2 gene were associated to changes in outcome, we conducted haplotype analyses on ABCG2. Our haplotype analyses revealed that the TTG haplotype in block 1 of ABCG2 exhibited the strongest association. This haplotype appears to be an independent predictor for association with improved GFR. The TCGA haplotype in block 2 also exhibited significant association. Similarly, haplotype CACA was an independent predictor associated with decreased

GFR. Of the 4 SNPs that were significantly associated in single SNP analyses, SNPs rs2231162 and rs2231153 appeared to be independently associated with change in GFR. This is consistent with a strong effect of the TTG haplotype in block 1. SNPs rs2231138 and 2231157 appeared to exhibit effects only in correlation with other SNPs. Our analysis does not determine which SNP drives the association, but two SNPs rs2231162 and rs2231153, as well as the TTG haplotype, are presented as strong candidates for future studies. Our candidate gene analysis in a cohort of 414 ALL patients suggests that single nucleotide polymorphisms in 5 renal transporters known to interact with MTX may modulate changes in GFR and thus, impact renal insufficiency due to methotrexate.

Chapter 5 is a high-throughput screening study that takes a chemoinformatics approach to identifying selective inhibitors for OAT1 and OAT3. One of the underlying principles of our study is the hypothesis that chemical similarity is a predictor of biological action. We use this approach to investigate whether we can better predict molecular interactors for the OATs and also identify chemical features that can be used to distinguish between two homologous transporters such as OAT1 and OAT3. We also wanted to determine whether we could find better methods for relating transporters quantitatively based on the chemical similarity of their ligands. While our chemoinformatic analysis of our hits is ongoing, we have identified several selective inhibitors for targeting OAT1 and OAT3. Our analysis yielded several findings. OAT1 and OAT3 interacting molecules vary enormously in structure. We found interacting molecules that appear to selectively inhibit OAT1 and OAT3 and some that inhibited both. We also observed that very subtle changes in electrostatic interactions and changes in the steric position of charged substituents could significantly influence inhibition. Further analysis and experimentation must be conducted to ascertain whether in fact they have all of the drug-like properties required to be move forward as a lead

compound. We conclude by commenting on how such a study and the information gained from our screen for selective inhibitors can be used in identifying lead compounds as drug targets and tools for use in drug discovery and drug development.

6.2 Final commentary

The different chapters of this thesis explore the role of transporters in nephrotoxicity through several perspectives: chemoinformatics, functional studies and population based studies. Our chemoinformatics and high-throughput screening study expands the existing knowledge about the chemical space in which transporters interact. Previous structure activity studies of molecular interactors with transporters generally consisted of very small sets of compounds and thus provide limited insights into transporter molecule interactions. Our screening study of thousands of compounds from random diversity libraries for molecules that interact with transporters allows us to more objectively define classes of molecular interactors, distinguish scaffolds that are preferred among the OAT transporters, distinguish among those selective between homologous transporters, and also identify chemical signatures and features that are important for a whole dynamic range of activity. In general, this screening study provides a broad birds-eye view of the landscape of OAT1/OAT3 transporter-inhibitor interactions that will hopefully expand the scope of understanding of transporter substrate/inhibitor interactions.

Moving from transporter chemical space, the other chapters of this thesis explore how genetic variation in membrane transporters may contribute to drug associated renal dysfunction in genotype to phenotype investigations. We look at two drug-induced nephrotoxicities, tenofovir associated renal insufficiency and MTX-induced renal insufficiency in special subpopulations, women and children. In each study we survey the epidemiology of renal disease in the context of the drug by asking about the

prevalence of the toxicity in the population and its risk factors, including transporter germline polymorphisms. We then stratify the population at risk into those that may have genetic risk factors. Our tenofovir study attempted to draw associations between known polymorphisms in OAT1 and those at risk for tenofovir associated renal insufficiency. We conducted a detailed genotype to phenotype study where a candidate gene was chosen (OAT1) and functional studies of polymorphisms in OAT1 were tested to determine whether they may be associated with differential transport and cytotoxicity. Our hypothesis was that tenofovir-associated nephrotoxicity might be modulated by polymorphisms in the OAT1 transporter; however, in this study, we were limited by access to DNA samples and the ability to carefully select patients in order to conduct a comprehensive genetic epidemiological study complete with functional studies. In a genetic epidemiological study of MTX-induced nephrotoxicity; however, access to DNA samples and detailed patient information was not a limiting factor. Here we identified polymorphisms in major renal transporters that were strong candidates influencing changes in MTX elimination. We genotyped patients for these SNPs in these candidate genes and drew correlations between the phenotype (change in GFR) and the frequencies of these polymorphisms. Together, the studies of tenofovir and MTX - induced nephrotoxicity are two example studies that couple epidemiological analysis with genetic associations studies and functional studies (where known coding DNA sequences are tested for functional significance in cellular assays). This provides a more mechanistic pharmacogenetic view of drug-induced nephrotoxicity.

The studies in this dissertation provide a unique look at drug-induced nephrotoxicity from the vantage of pharmacogenetic variation in the OAT1 and OAT3 transporters. They set the stage for investigations where other genes involved in the nephrotoxicity pathways of these drugs may be investigated. They also provide transporter/molecule interaction information that may be used to shed new light on the

mechanism of OAT1 and OAT3. These insights can be further extended to other transporters in the other families. Altogether, this dissertation has enhanced our knowledge of the role of organic anion transporters in drug-induced nephrotoxicity and provides a framework to enhance our ability to understand and predict drug toxicity.

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