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Pharmacodynamic Effects of Xenobiotic ABC Transporters in Peripheral Tissues

Author
Chinn, Leslie

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Pharmacodynamic Effects of Xenobiotic ABC Transporters in Peripheral Tissues

by

Leslie W. Chinn

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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in the

GRADUATE DIVISION

of the

A c k n o w l e d g e m e n t s

The writing of a thesis seems to be nothing more than the gargantuan task of compiling years of living, breathing scientific research into a neat package, contained within several hundred pages bound together and smelling of book adhesive. Science, by its very nature, is a messy process, and best suited for those persistent foragers who don't mind slogging through fields of unwieldy data in search of even a glimpse of scientific enlightenment. Yet this process inevitably raises more questions to be answered, more sparks of curiosity, more hypotheses to be tested and experiments to do. And so science takes a breath, expands, trying to break free of the measured text which restrains it on the page.

The ability to know when to let a project expand, when to follow its offshoots, and when to let a project go is something that must be learned, and I've been lucky enough to be guided by some of the best scientists in the field. Dr. Deanna Kroetz, my thesis advisor, has been a constant source of encouragement and intellectual stimulation, always challenging me to think more deeply about a topic, and supporting my attempts at experimental validation, no matter how overly optimistic I might be. It has been a privilege to learn from someone so motivated and passionate about science, and the clinical collaborations described in this dissertation would certainly not have been possible without her well-placed connections. Many thanks also to the other members of my thesis committee, Dr. Kathy Giacomini and Dr. David Bangsberg, for taking time out of their busy schedules to think about my project and give me invaluable feedback.

The recruiting of subjects, collection of samples, and the compilation of clinical data are daunting tasks which were performed by a number of individuals, including

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Our time in San Francisco was made infinitely more enjoyable by the presence of our friends: Jeff and Molly Kraft, with whom many a bad movie has been groaned at; Eric and Leah Lagpacan Peters, for assistance with forays into population genetics and/or the sale racks at H&M; Tom Urban, for always having the bourbon ready; Jason Gow, for

somehow making Tom Petty sing about subcloning; Jimshima, for his ability to cook not one, but two turkeys at the same time; and many others who have joined us for various gatherings and celebrations over the years. As one of the last to graduate, I can say that it was much more fun when all of you were around.

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A b s t r a c t

The ATP-binding cassette (ABC) superfamily consists of energy-dependent transporters which in many cases play crucial roles in physiological processes; indeed, mutations in several ABC transporter genes has been shown to cause inherited diseases such as cystic fibrosis. The involvement of ABC transporters in systemic xenobiotic protection has led to an examination of the influence of these transporters on drug pharmacokinetics (plasma levels), including a number of clinical studies in which transporter expression levels were associated with drug plasma levels in healthy subjects. Researchers have also demonstrated correlations between the presence of genetic polymorphisms in ABC transporters and altered drug pharmacokinetics. The impact of ABC transporters and their naturally occurring genetic variants on drug pharmacodynamics (pharmacological action) has been characterized to a lesser extent, in part because of the difficulties of quantifying the pharmacological actions of many drugs. In this dissertation, we focus on the pharmacodynamic effects of ABC transporter expression and function in peripheral, non-pharmacokinetic tissues such as adipose and lymphocytes, especially with respect to anti-HIV therapies.

First, we describe the expression of a putative splice variant of the ABCB1 (P-glycoprotein) transporter in lymphocytes. This half-sized protein functioned similarly to the classic full-size P-glycoprotein, but displayed altered immunoreactivity. ABCB1 RNA transcripts of approximately half the length of normal ABCB1 transcripts were found in lymphocytes from healthy subjects. The putative P-glycoprotein splice variant was detected in both HIV-negative and -positive subjects, indicating that it could influence the pharmacodynamics of anti-HIV drugs which have been classified as

substrates of P-glycoprotein. We also investigated the effects of exposure to the HIV protease inhibitors atazanavir and saquinavir on the expression of lymphocyte P-glycoprotein. We found that in several subjects, the amount of P-glycoprotein expression increased substantially, suggesting that while there is no detectable generalized change in lymphocyte P-glycoprotein expression following atazanavir/saquinavir exposure, there may be genetic or environmental influences which affect the extent of lymphocyte P-glycoprotein induction in certain individuals.

We also examine the roles that polymorphisms in candidate genes play in patient response to anti-HIV medications. These genes include the drug-metabolizing enzyme *cytochrome P450 2B6*, the xenobiotic transporters *ABCB1* and *ABCC4*, and the inflammatory cytokine *TNF*α. We performed this study in HIV-infected populations in San Francisco and Uganda, and found no significant associations between any of the polymorphisms investigated and patient virologic or immunologic response to antiretroviral therapies. We also characterized the ancestral admixture of the San Francisco cohort and determined that the minor allele frequencies of several of the polymorphisms differed between ethnicities.

Last, we describe the effects of the adipose RNA expression of the transporter genes *ABCC4* and *SLC29A1*, which regulate cellular exposure to the nucleoside analogue-based HIV reverse transcriptase inhibitors, on the development of lipodystrophy, a fairly common side effect of this class of anti-HIV therapies. Lipodystrophy is thought to result from mitochondrial toxicity, specifically the inhibition of the mitochondrial DNA polymerase. Indeed, we found that the nucleoside analogue fialuridine inhibited mitochondrial DNA synthesis *in vitro*; the effects of transporters in

this system remain inconclusive. While we did not see any correlation between adipose transporter expression and the occurrence of lipodystrophy, we identified a genetic polymorphism in *ABCC4* that was significantly associated with the development of lipodystrophy following stavudine treatment in a San Francisco population of HIV-infected individuals. It is possible that screening for this polymorphism may help to predict which patients will develop lipodystrophy following nucleoside analogue exposure.

In general, the results described in this dissertation indicate that ABC transporters in lymphocytes and adipocytes may have important pharmacodynamic functionalities, as they modulate the intracellular concentrations of certain drugs. In particular, we have focused on HIV antiretroviral therapies because they exert pharmacological effects (beneficial or detrimental) in these cell types. Finally, we show that genetic variation in ABC transporters may be an important factor which determines how a patient responds to an antiretroviral regimen. Future studies should expand on this work with mechanistic studies to further unravel the role that transporters play in antiretroviral action, as well as carefully designed clinical studies to ascertain the validity of the pharmacogenetic associations reported here.

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Chapter 1. ABC Transporter Function and Clinical Relevance of Genetic Variation

1.1. Overview

The ATP-binding cassette (ABC) proteins transport a wide variety of endogenous and exogenous compounds across cell membranes, utilizing the energy released from ATP hydrolysis [1]. ABC transporters have many physiological roles, ranging from the maintenance of bile acid homeostasis to the protection of sensitive tissues from toxins [2]. Impaired of ABC transporters has been associated with a wide array of inherited diseases, including cystic fibrosis, macular dystrophy, and a connective tissue disorder [2]. Despite the broad range of ABC transporter substrates and physiological functions, all of the proteins share the same mechanism of action and can be identified by specific amino acid sequences and structural features [3].

Members of the ABC superfamily are found in organisms from three major groups: bacteria, archae, and eukaryota [4]. The numbers of ABC transporters in the genomes of bacteria (*B. subtilis* and *E. coli*) and eukaryotes (*S. cerevisiae, C. elegans, D. melanogaster, M. musculus,* and *H. sapiens*) are shown in Table 1.1. The presence of ABC transporters in evolutionarily ancient species, and their continued existence in a large number of eukaryotic genomes, indicates the importance of ABC transporters in a variety of biological processes which are essential for the survival of an organism [5].

Table 1.1. ABC transporters in the genomes of a variety of organisms[1]

Species	Genome size (Mb)	Total transporter proteins	Transporters per Mb genome	ABC transporter proteins
B. subtilis	4.2	297	70.71	82
E. coli	4.6	354	76.96	69
S. cerevisiae	13	318	24.46	24
C. elegans	97	656	6.76	48
D. melanogaster	120	604	5.03	51
M. musculus	2667	630	0.24	52
H. sapiens	3150	770	0.24	48

[1] These data were compiled using the TransportDB, FantomDB, and NCBI Genomes websites and published data [1, 6-9].

1.2. Organization

In humans, there are forty-eight members of the ABC superfamily [1]. These proteins are grouped into seven families based on their structure (half transporters are comprised of one transmembrane domain (TMD)/nucleotide-binding domain (NBD) functional subunit; full transporters consist of two TMD/NBD subunits) and their amino acid homology [2] (see Table 1.2). ABC transporters use the energy released from ATP hydrolysis to translocate a substrate molecule across a membrane [2]. This function may be traced back into a series of evolutionarily conserved amino acid sequences, representing the parts of the protein to which ATP binds and is hydrolyzed, as well as the interface between the NBD and TMD domains [3, 10]. These conserved sequences include the Walker A (GNSGCGKST), linker peptide (LSGGQ), and Walker B (ILLLD) amino acid sequences, with the most strongly conserved amino acid sequences in mammalian *ABCB1* orthologues listed here [11, 12]. The transmembrane domains of ABC transporters are responsible for substrate binding [13], and therefore tolerate more amino acid variability between superfamily members and between species than do the

nucleotide binding domains, as is evidenced by the wide range of ABC transporter substrates but an identical mechanism of transport action [14, 15]. A list of ABC transporters with drug substrates is shown in Table 1.3.

Table 1.2. Summary of ABC transporter families

Gene family	Members	General Substrates	Structure[1]
ABCA	1-13	cholesterol	[TMD-NBD]-[TMD-NBD]
ABCB	2,3,5,7-10	peptides, metal ions	[TMD-NBD]
	1,4,6,11	various drugs, bile salts	[TMD-NBD]-[TMD-NBD]
ABCC	1-3,6,7-10	various drugs, chloride ions	[NBD]-[TMD-NBD]-[TMD-NBD]
	4,5,7,11,12	various drugs, nucleosides	[TMD-NBD]-[TMD-NBD]
ABCD	1-4	fatty acids	[TMD-NBD]
ABCE	1	oligoadenylate binding protein	[NBD]-[NBD]
ABCF	1-3	not known	[NBD]-[NBD]
ABCG	1,2,4,5,8	drugs, sterols	[NBD-TMD]

[1] Subunit structure predictions [16, 17].

Table 1.3. ABC transporters involved in xenobiotic transport[1]

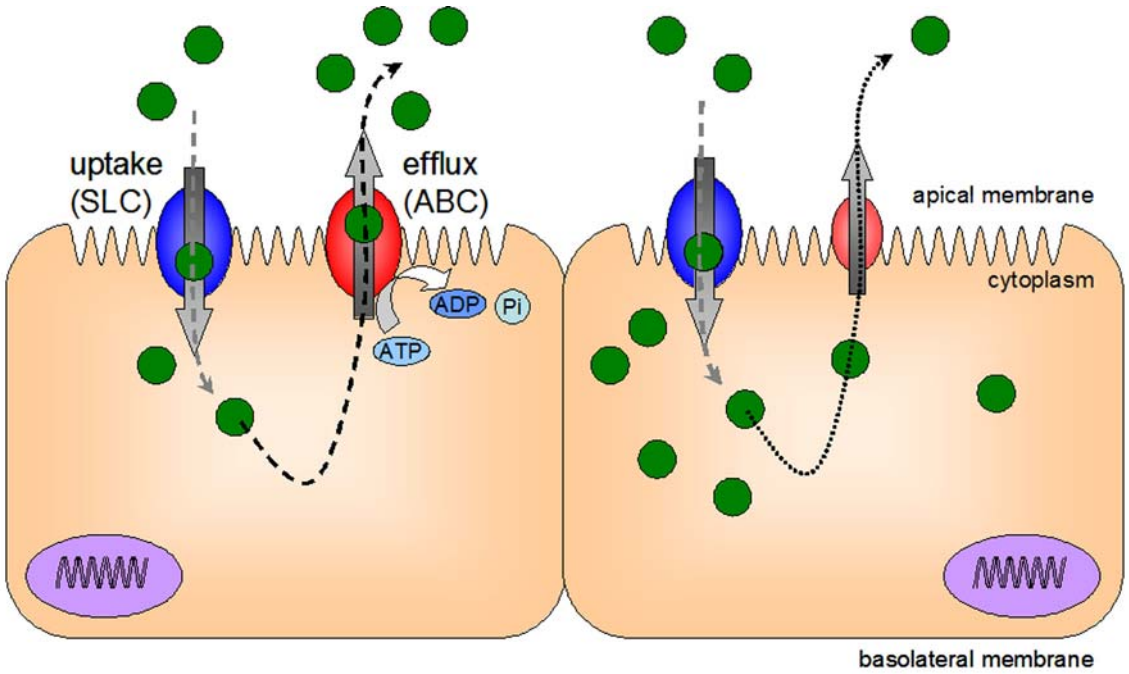
Gene	Protein	Primary Tissue Distribution	Cellular Localization	Drug Substrates
ABCB1	MDR1, P-glycoprotein	kidney, liver, intestine, brain, lymphocytes	apical	digoxin, cyclosporine, paclitaxel, vinca alkaloids, loperamide, erythromycin, HIV protease inhibitors
ABCB11	BSEP	liver	apical	vinblastine, tamoxifen citrate
ABCC1	MRP1	ubiquitous	basolateral	vinca alkaloids, methotrexate, etoposide
ABCC2	MRP2	liver	apical	vinca alkaloids, methotrexate, pravastatin, irinotecan (SN-38), cisplatin
ABCC3	MRP3	liver, kidney, small intestine	basolateral	doxorubicin, vincristine, methotrexate, cisplatin
ABCC4	MRP4	prostate, liver, brain, kidney	basolateral	nucleoside analogs (PMEA, AZT-monophosphate), nucleobase analogs (6-MP, methotrexate)
ABCC5	MRP5	brain, heart, placenta	apical	nucleoside analogs (PMEA, cladarabine, gemcitabine, d4T-monophosphate), nucleobase analogs (5-FU, 6-MP)
ABCG2	MXR, BCRP	placenta, liver, small intestine	apical	mitoxantrone, doxorubicin, topotecan, methotrexate, irinotecan (SN-38)

[1] Data were compiled from published work [17-20].

1.3. ABCB1

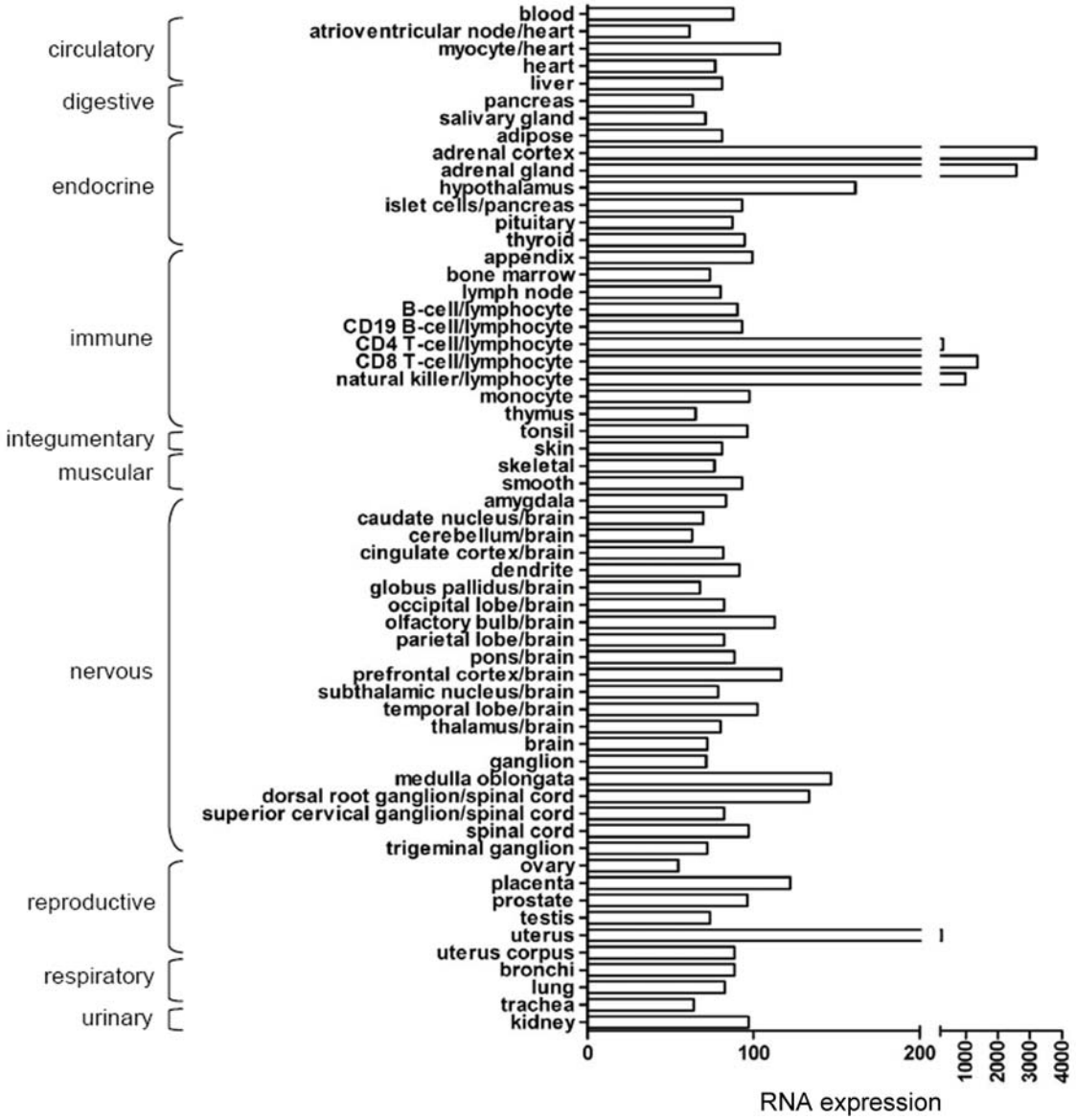
The most widely studied human ABC transporter is *ABCB1*, which encodes P-glycoprotein, a xenobiotic efflux transporter located on the apical membrane of cells in a range of tissues (a depiction of P-gp transport function is shown in Figure 1.1). In 1976, Juliano and Ling reported the surface expression of a 170 kDa protein in CHO cells which exhibited colchicine resistance [21]. Four years later, after further characterization of P-glycoprotein (so named because of the change in cell permeability the protein effected), this protein was also shown to be a factor in resistance to adriamycin, actinomycin D, daunorubicin, vincristine, and vinblastine, among other drugs [10]. Overexpression of P-glycoprotein coincided with an increase in transcripts of a gene called *MDR1* (for MultiDrug Resistance) and was shown to be the gene product of *MDR1* in 1986 [22].

Figure 1.1. Schematic of cellular transport mechanisms at the apical membrane. Intestinal epithelial cells are shown, with the apical membrane facing the lumen and the basolateral surface facing blood. P-gp (red) is expressed on the apical membrane and modulates the amount of substrate absorbed from the lumen, thereby regulating its entry into blood. On the right, an ABC transporter with reduced function is displayed; consequently, the intracellular substrate concentration is increased and more substrate will reach the bloodstream. In some cases, ABC efflux transporters act in concert with uptake transporters (e.g. members of the solute carrier, or SLC, superfamily); substrates may also be passively absorbed into cells.



Subsequently, P-glycoprotein expression was investigated using monoclonal antibodies and was found to be fairly widespread [23]. More recently, microarray technologies have been used to describe ABCB1 RNA expression in a number of cell types and tissues (see Figure 1.2). From expression studies, it became clear that P-gp acted as a protective mechanism in tissues with excretory functions (intestine, liver, kidney) and barrier functions (placenta, blood-brain barrier) and needed to be considered outside of the field of multidrug resistance [23]. The expression of ABCB1/P-gp in excretory tissues led to investigations of its effects on the pharmacokinetics of many common medications. The involvement of P-gp in the bioavailability of the cardiac glycoside digoxin and the immunosuppressant cyclosporin A has been demonstrated both in humans [24, 25] and in *mdr1a*-/- mice [26]. ABCB1 expression and/or function may explain some of the interindividual variability in the bioavailability and clearance of other drugs as well, and examining drug-transporter interactions has become an important step of the drug development process [27].

Figure 1.2. ABCB1 tissue distribution. Human RNA expression data were extracted from microarray data compiled by the Genomics Institute of the Novartis Research Foundation [28, 29]. Tissues are grouped by biological system. Each bar represents the ABCB1 RNA expression value in a given tissue, which was determined using the GC Robust Multi-array Average (GCRMA) method to adjust for background signal [30].



As the initial discovery of P-glycoprotein in cancer cells suggests, the role of this transporter as a cellular gatekeeper should not be ignored. Transport mechanisms are an obvious explanation for drug resistance at the cellular level, and ABCB1 expression on cells which are the sites of action for drugs is often studied in cases of drug resistance. For example, about a quarter of epilepsy cases are refractory, meaning they do not respond to different antiepileptic treatments [31]. Tishler *et al.* found increased expression of ABCB1/P-gp in 11 of 19 brain specimens from patients with refractory epilepsy, and suggested that overexpression of this transporter may contribute to the drug-resistant form of this disease [32]. While the results of several studies have supported this hypothesis, others have not, and the role of ABC transporters in refractory epilepsy continues to be debated [33].

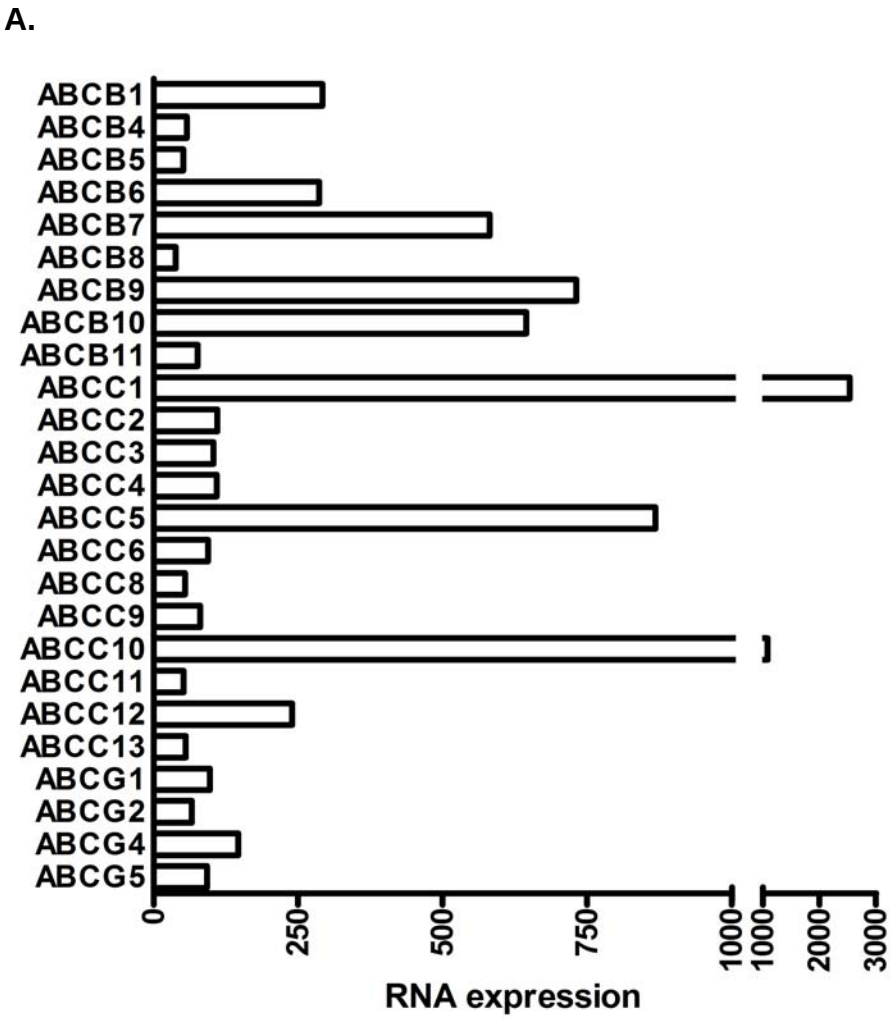
Other tissues which serve as pharmacological drug targets – whether an intentional drug effect (efficacy) or an inadvertent one (toxicity) – have some degree of ABC transporter expression, yet transporter effects on drug accumulation in these tissues remain, to a large degree, unexamined. The roles of transporters in two of these – adipose and lymphocytes – will be studied in greater detail in the following sections.

1.4. ABC transporters in lymphocytes

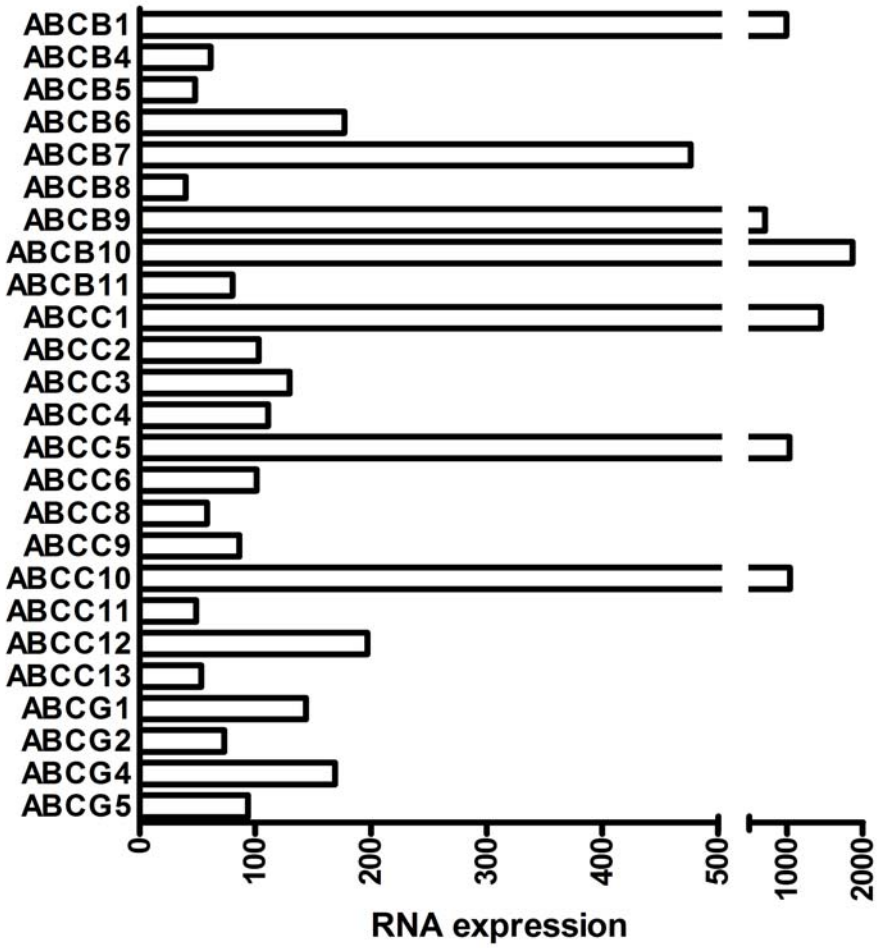
As an integral part of the immune system, lymphocytes are involved in the recognition and destruction of pathogens. There are three types of lymphocytes: T-cells, B-cells, and natural killer (NK) cells [34]. T- and B-cells are elements of the adaptive immune (antigen-specific) response. Once they have detected foreign pathogens or antigens, B-cells produce antibodies which neutralize bacteria and viruses, while CD4+

(helper) T-cells generate cytokines to guide the immune response, and CD8+ (killer) T-cells induce the death of infected, damaged, or dysfunctional cells [34]. Natural killer cells (CD16+/CD56+) are part of the innate immune (non-specific) response and destroy cells which express subnormal levels of MHC class I molecules [34]. The expression of xenobiotic ABC transport families in CD4+ T-cells and NK cells is displayed in Figure 1.3.

Figure 1.3. ABC transporter expression in lymphocytes. Human RNA expression data for CD4+ T-cells (A) and natural killer cells (B) were extracted from microarray data compiled by the Genomics Institute of the Novartis Research Foundation [28, 29]. Members of ABC families known to be involved in xenobiotic transport are shown. Each bar represents the RNA expression value of a given transporter, which was determined using the GC Robust Multi-array Average (GCRMA) method to adjust for background signal [30].



B.


ABCB1
ABCB4
ABCB5
ABCB6
ABCB7
ABCB8
ABCB9
ABCB10
ABCB11
ABCC1
ABCC2
ABCC3
ABCC4
ABCC5
ABCC6
ABCC8
ABCC9
ABCC10
ABCC11
ABCC12
ABCC13
ABCG1
ABCG2
ABCG4
ABCG5
0
100
200
300
400
500
1000
2000
RNA expression

Imbalances in the immune system can lead to significant problems: immunodeficiency disorders occur when the immune system is less active than usual, while autoimmune disorders are a consequence of a hyperactive immune system attacking normal tissues. One such imbalance may be caused by leukemia [35]. Leukemia is characterized by an excessive increase of abnormal blood cells, either immature (acute leukemia) or mature (chronic leukemia) [36]. Leukemic white blood cells may be suppressed, reducing the ability to mount an immune response [37]. While chronic leukemias progress slowly and require less aggressive treatment, acute leukemias tend to spread more quickly, and the time to treatment efficacy can be crucial to improve the chances of survival [38]. The effects of ABC transporters on patient response to anti-leukemia chemotherapies are discussed in the next few paragraphs.

There are two types of acute leukemias: lymphocytic (ALL) and myeloid (AML). Both ALL and AML patients may receive the anthracycline daunorubicin during the course of their treatment [39, 40]. Because ABCB1 overexpression was linked to daunorubicin resistance in cell culture [10], it was proposed to be a factor in drug-resistant acute leukemia cases only a few years after its initial discovery. Indeed, Sato *et al.* described ABCB1 RNA expression in leukemic cells from acute leukemia patients, most frequently from those who did not respond to chemotherapy [41]. *In vitro* support of this hypothesis includes a study in which the treatment of ABCB1-expressing leukemic cells with the P-gp inhibitor cyclosporin A led to an increase in intracellular daunorubicin accumulation [42]. In clinical analyses, ABCB1 expression has repeatedly been correlated with multidrug resistance in acute leukemia patients [43, 44],

demonstrating the importance of ABC transporter lymphocyte expression in the treatment of leukemia.

ABC transporters have also been associated with the development of resistance to drugs used to treat the human immunodeficiency virus, or HIV. HIV infects CD4+ lymphocytes, leading to the depletion of these cells through apoptosis as well as the targeting of infected cells by CD8+ killer T-cells [45]. The decline of CD4+ cells severely impacts the functioning of the immune system, leaving HIV patients susceptible to opportunistic infections [45]. Because the virus replicates in CD4+ cells before inducing apoptosis to advance infection, CD4+ cells are a target for anti-HIV drugs [45].

There are three major classes of antiviral therapies used in the treatment of HIV: the protease inhibitors (PIs), the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), and the non-nucleoside reverse transcriptase inhibitors (NNRTIs), all of which act on viral machinery [46]. There are also three newer classes of drugs: integrase inhibitors, which also interfere with a viral protein, and the fusion inhibitors and chemokine coreceptor agonists, which impede viral binding with CD4+ cells [47]. Several of the protease inhibitors are substrates [48] or inducers [49, 50] of ABCB1, and recent reports have suggested that P-gp and MRP4 may moderate the intralymphocytic concentrations of NRTIs [51]. Another study examined the effects of integrase inhibitors on P-gp and found that they are in fact substrates of this transporter [52]. All of these reports indicate that the expression of ABC transporters on lymphocytes may affect the efficacy of anti-HIV medications. However, the majority of work examining the relationship between transporter lymphocyte expression and antiviral drug accumulation has been done *in vitro*, and not in the context of clinical phenotypes; the latter has been

investigated in a number of pharmacogenetic studies (correlating expression to phenotype indirectly, using genotype as a proxy), as described later in this chapter.

1.5. ABC transporters in adipose tissue

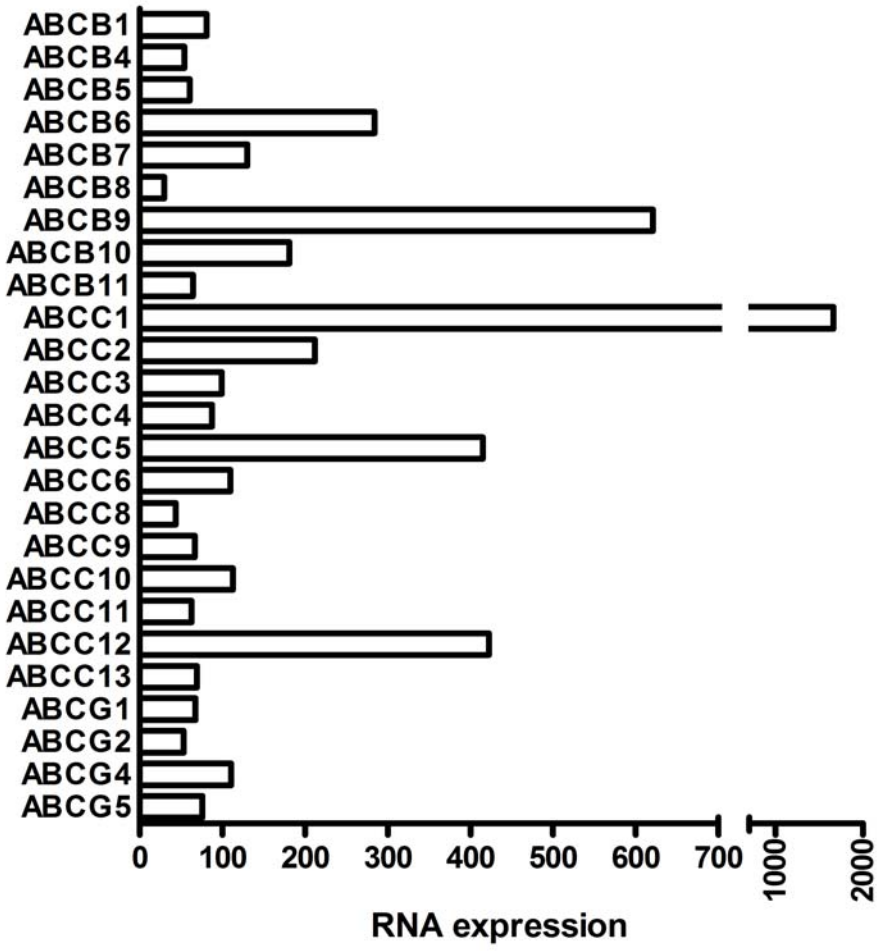
The primary role of adipose tissue is to store energy, although it also serves as an endocrine organ, producing hormones such as leptin and cytokines like TNFα [53]. Because of its highly lipophilic nature, adipose tissue effectively serves as a depot for drugs with high partition coefficients, and the amount of adipose tissue present can significantly affect the volume of distribution for these drugs [54]. Although there are very few, if any, drugs which act on targets in adipocytes, the accumulation of drugs in adipose tissue may lead to unintended adverse effects.

For example, the HIV antiretroviral drugs – specifically the NRTIs – have been implicated in lipoatrophy, a form of lipodystrophy that is manifested in the loss of subcutaneous fat [55]. This fat loss occurs in the face and limbs, leading to what HIV-positive individuals feel is a markedly disproportionate appearance that identifies them as undergoing treatment for HIV [56]. Lipoatrophy is thought to be a result of NRTI mitochondrial toxicity (specifically the inhibition of the mitochondrial DNA polymerase γ), leading to downstream cellular damage and adipocyte apoptosis [57]. Indeed, NRTI exposure was associated with decreased levels of mitochondrial DNA in adipose tissue from HIV-infected patients with lipoatrophy [58], and these findings have been replicated in several studies [59, 60].

Microarray expression data demonstrates that a range of xenobiotic ABC transporters are expressed in adipocytes (Figure 1.4). Recent publications indicate that

the ABC transporters P-gp and MRP4 may be involved in NRTI transport [51, 61]. Therefore, the influence of adipocyte ABC transporter expression on the development of HIV NRTI-associated lipodystrophy poses an intriguing question.

Figure 1.4. ABC transporter expression in adipocytes. Human RNA expression data were extracted from microarray data compiled by the Genomics Institute of the Novartis Research Foundation [28, 29]. Members of ABC families known to be involved in xenobiotic transport are shown here. Each bar represents the RNA expression value of a given transporter in adipocytes, which was determined using the GC Robust Multi-array Average (GCRMA) method to adjust for background signal [30].



1.6. Genetic polymorphisms in ABC transporters

The role of ABC transporters in the pharmacokinetics and pharmacodynamics of drugs, as well as in various physiological processes, can be difficult to elucidate. Such studies usually rely on measuring gene expression or function, which can be difficult to quantify and is limited by the sensitivity of the measurement. An alternative method for determining the importance of a gene in a pathway is to do a pharmacogenetic study, in which a genetic polymorphism (or a series of them) is correlated with a phenotype. The genotype then serves as a surrogate for the expression or function measurement and can be used in statistical analyses to calculate the significance of a gene on a pharmacokinetic, pharmacodynamic, or clinical phenotype. Examples of these analyses in the context of ABC transporters are discussed below.

The introduction of high-throughput DNA sequencing technologies has enabled researchers to study the effects of genetic variation on ABC transporters; the first paper to describe variation in ABC transporter genes was published in 2003 [62]. The ultimate goal of these genotype-to-phenotype studies is to be able to predict an individual's response to and toxicity associated with a drug, which would provide clinicians with the means to tailor drug therapy. The concept of personalized medicine relies upon understanding the effects of genetic variation through studies such as these.

At the same time, researchers interested in diseases such as cystic fibrosis have used forward genetic methods to implicate ABC transporters [63]. Studies have demonstrated that dysfunction in ABC transporters can have severe chronic effects, underlining the role of these proteins in the transport of endogenous compounds – a role necessary for the homeostasis of various physiological processes [63-66]. Genetic

polymorphisms in ABC transporters have been associated with disease states through genotype-to-phenotype studies, and in some cases further research has identified the mechanism through which the polymorphism is leading to transporter dysfunction and disease [63, 67-72].

Both genotype-to-phenotype and phenotype-to-genotype studies have shown that genetic polymorphisms in ABC transporters can have a multitude of effects. The severity of the effect depends on a number of factors, including where a polymorphism is located in the gene (exon, intron, regulatory region), if it changes an amino acid (i.e. is non-synonymous), and the significance of the amino acid change. For example, a SNP in exon 4 of *ABCG2* encodes a premature stop codon at residue 126 (376T>C, Q126stop); this SNP abrogates MXR-mediated methotrexate transport [73]. In another example, the Q141K SNP substantially decreases MXR ATPase activity, thereby rendering the protein incapable of transport function [74].

Polymorphisms located throughout the gene may also influence transporter expression at both the mRNA and protein levels. SNPs found in regulatory regions such as the promoter may impact transcriptional regulation, as is the case with the -14 C>T polymorphism in the gene encoding the cholesterol transporter ABCA1, in which the T allele was found to have higher transcriptional activity *in vitro* [75]. Splice sites are another regulatory factor which may be affected by SNPs; deletions in *ABCA4* have been associated with alternative splicing, resulting in expression of shortened mRNA transcripts and defective retinoid transport [76].

In some cases, polymorphisms have been associated with clinical phenotypes, although the molecular mechanisms which cause these phenotypes hav not been

identified. Indeed, these associated polymorphisms may not be causative of the phenotype, but may exist in linkage disequilibrium with the causative SNP or SNPs; one way to circumvent such misleading SNP associations is to perform haplotype association studies, rather than focusing on a single SNP [77]. It has become clear that determining why a SNP in an ABC transporter leads to a clinical phenotype, whether it be a disease state or a pharmacokinetic (e.g. plasma drug concentration) or pharmacodynamic (e.g. drug response) parameter is a challenging and time-consuming process, albeit one that is crucial to advancing our understanding of ABC transporter biology.

1.6.1. Polymorphisms in ABC transporters: risk for disease states

Polymorphisms in ABC transporters have been associated with predisposition to complex clinical conditions, listed in Table 1.4. One of the diseases examined is Parkinson's disease. Several studies have reported that the *ABCB1* 3435TT genotype is associated with early-onset Parkinson's disease, although these did not reach statistical significance [78, 79]. Another group found that the *ABCB1* 3435TT genotype was associated with late-onset Parkinson's disease, but not early-onset [80]. Because haplotypes containing the 3435T allele have been linked to decreased ABCB1 expression and function *in vitro* [81], it was hypothesized that 3435T carriers would have decreased P-gp at the blood-brain barrier and thereby increased neuronal exposure to potentially toxic xenobiotics, predisposing them to Parkinson's disease. While there is some evidence to support this hypothesis, research in this area is preliminary and remains inconclusive at this time.

A similar hypothesis has been proposed for inflammatory bowel disease, or IBD – decreased intestinal ABCB1 expression may in turn have a weaker physiological defense against intestinal bacteria or toxins, leading to IBD. In support of this hypothesis, it has been shown that *mdr1a*-/- mice develop intestinal inflammation similar to IBD [82]. SNPs in *ABCB1* have been associated with susceptibility to IBD: an intronic SNP (rs3789243) and haplotypes including this SNP were tightly correlated with incidence of IBD compared to healthy controls [83]. The *ABCB1* 3435TT genotype has also been correlated with ulcerative colitis, a type of IBD [84-88], and with a specific subset of Crohn's disease, another type of IBD [89]. However, other studies have not found an association between *ABCB1* genotype and incidence or response to treatment of IBD [90-92]. These conflicting reports may be due to the complex pathophysiology of IBD, and the difficulty in phenotyping and subphenotyping patients with different forms of IBD.

Table 1.4. Polymorphisms in ABC transporters associated with disease predisposition.

Gene	Polymorphism	Population	Outcome marker	Finding	Ref
ABCB1	3435C>T	157 patients	Gastric cancer	TT genotype associated with lower risk of gastric cancer	[93]
ABCB1	3435C>T	113 Caucasian children	Acute lymphocytic leukemia	TT genotype associated with ALL	[94]
ABCB1	3435C>T	198 patients	Endometrial cancer	T allele associated with endometrial cancer	[95]
ABCB1	3435C>T	30 early-onset, 77 late-onset (59 pesticide-exposed) patients	Age at onset of Parkinson's disease	CT associated with a 3X and TT associated with a 5X increased disease risk compared to CC genotype	[78]
ABCB1	3435C>T	149 UC, 126 CD patients	UC, CD susceptibility	TT genotype associated with UC	[88]
ABCB1	3435C>T	135 CD, 123 UC Caucasian patients	UC, CD susceptibility	No statistical association	[96]
ABCB1	3435C>T	66 Japanese UC patients	Age at onset of UC	T allele associated with late onset UC	[97]
ABCB1	3435C>T	45 matched tumor and normal colon tissue samples	Colorectal cancer susceptibility	No statistical association	[98]
ABCB1	3435C>T	179 patients	Clear-cell renal carcinoma	T allele associated with tumor occurrence	[99]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	244 German CD and 144 UC patients	UC, CD, age of onset	2677GG/3435TT genotype associated with early-onset UC	[100]

ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	411 drug-naïve HIV+ patients	HIV progression	No statistical association	[101]
ABCB1	-1517T>C, -41A>G, -129T>C, 2677G>T/A (Ala893Ser/Thr), 3435C>T	121 Japanese schizophrenia and 62 mood disorder patients	Schizophrenia or mood disorders	-1517T, -41A, -129T, and 2677A alleles associated with mood disorders; 129T/2677A/3435C haplotype associated with mood disorders	[102]
ABCB1	-129T>C, 2677G>T/A (Ala893Ser/Thr), 3435C>T	25 early-onset, 70 late-onset Parkinson's disease patients	Age at onset of Parkinson's disease	No statistical association	[79]
ABCB1	-41G>A, -145G>C, -129C>T, 1236C>T, 2677G>T/A, 3435C>T, 4036G>A	206 Chinese patients	Parkinson's disease	1236, 2677, 3435 genotypes associated with Parkinson's disease; 2677T and 3435T alleles associated with late onset	[80]
ABCB1	5'UTR SNP, -129T>C, 5 intronic SNPs, 1236C>T, 2677G>T, 3435C>T	144 UC, 163 CD patients	UC, CD, refractory CD	1236T/2677T/3435T (and 2 intronic SNPs in LD with this haplotype) alleles associated with refractory CD and UC (only significant as haplotype)	[87]
ABCB1	intronic SNP rs3789243	249 UC, 179 CD patients	UC, CD	G allele associated with UC	[83]
ABCB1	Promoter +8T>C	139 patients	Osteogenic sarcoma	CT genotype more frequent in patients than controls	[103]
ABCB11	Various mutations	19 PFIC patients	PFIC occurrence	Mutations found in 10 of 19 PFIC patients	[104]
ABCB11	10 mutations	25 PFIC families	PFIC occurrence	Ten causative mutations found in PFIC patients	[105]

ABCC2	12 polymorphisms	94 Korean patients	Toxic hepatitis	-1774delG associated with cholestatic hepatitis; -1549G>A, -24C>T, 285C>T, 3972C>T associated with hepatocellular hepatitis	[106]
ABCC6	Various mutations	166 PXE patients	PXE occurrence	188 mutations associated with PXE	[107]
ABCG2	421C>A (Glu141Lys)	200 patients	Nonpapillary RCC susceptibility	A allele associated with nonpapillary RCC	[108]

Abbreviations used are: CD, Crohn's disease; UC, ulcerative colitis; CCRC, clear-cell renal carcinoma; DJS, Dubin-Johnson syndrome; PXE, pseudoxanthoma elasticum; and PFIC, progressive familial intrahepatic cholestasis.

The phenotypes of several other inherited diseases are more clearly defined, including the progressive familial intrahepatic cholestases (PFIC), in which bile flow is impaired [69]. Families with a certain type of PFIC have heterogeneity on chromosome 2; detailed mapping demonstrated the existence of mutations in *ABCB11*, which encodes the bile salt export pump (BSEP) [105]. BSEP is localized to the canalicular membrane of hepatocytes and plays a crucial role in bile production [109]. Immunohistochemistry has shown that liver specimens from PFIC patients with mutations in *ABCB11* had decreased protein expression [104]. *In vitro*, PFIC-associated ABCB11 mutations impair the trafficking and/or transport function of BSEP [70, 110-112]. Case reports continue to be published describing novel *ABCB11* mutations in PFIC patients.

BSEP maintains bile acid homeostasis along with other transporters such as MRP2 (encoded by *ABCC2*), which is also expressed on the canalicular membrane of hepatocytes, but while BSEP transports bile salts, MRP2 effluxes anionic conjugates such as reduced glutathione, creating a driving force for bile flow [109]. Interestingly, the role of MRP2 in an inherited disease called Dubin-Johnson syndrome (DJS) was uncovered through rat studies, in which a strain deficient in the canalicular multispecific organic anion transporter, or *cMOAT*, presented with impaired hepatobiliary transport of a number of compounds [113, 114]. The human orthologue of *cMOAT*, *ABCC2*, was screened for mutations in DJS patients [72, 115]. The presence of mutations in *ABCC2* (e.g. premature stop codon) confirmed that reduced expression and/or function of this transporter leads to DJS [72]. As with PFIC, the process of identifying the *ABCC2* mutations which cause DJS is ongoing.

Another rare disorder whose pathophysiology involves mutations in transporter genes is pseudoxanthoma elasticum, or PXE. Individuals with PXE experience mineralization of elastic fibers in the skin, eye, and cardiovascular system [68]. Genetic analysis of PXE families narrowed the causative region to approximately 500 kb on chromosome 16 [68], and mutations in one of the genes in this region, *ABCC6*, were found in PXE patients [67, 116]. In cell culture, PXE-associated *ABCC6* mutations abolished transport activity [117]. The endogenous substrate of MRP6 remains unknown, and it is unclear why mutations in *ABCC6* cause PXE [71].

Mutations in another ABC transporter gene, *ABCC7*, cause a more common inherited disorder, cystic fibrosis (CF). An old folk saying that "a child that tastes salty when kissed will soon die" is now thought to refer to the abnormally high salt concentration in the sweat of children with CF [118]. CF was later associated with decreased chloride ion conductance across the membranes of epithelial cells [119]. In 1989, Riordan *et al.* used reverse genetics approaches to identify, clone, and characterize the cystic fibrosis gene, now designated *CFTR* (for cystic fibrosis transmembrane conductance regulator) or *ABCC7* [119], which encodes a chloride ion channel [120]. The authors also reported that the deletion of a single amino acid, the phenylalanine at position 508 located in the first nucleotide binding domain, was commonly found in patients with CF [119]. Over a thousand additional mutations in *ABCC7* have since been associated with CF [121]. The discovery of these mutations has allowed genetic testing for CF in neonates, with screening being mandatory in a number of states in the U.S. [122].

1.6.2. Polymorphisms affecting drug pharmacokinetics

Polymorphisms in ABC transporter genes have been implicated in a number of serious disorders, as reviewed above. However, changes in these genes may not lead to an obvious phenotype, as demonstrated with the creation of the *mdr1a-/-* mouse. *Mdr1a-/-* mice appeared physiologically normal until substrate drugs were administered, at which time significant alterations in pharmacokinetic parameters were observed [123]. In the late 1990s, researchers began examining the effects of genetic polymorphisms on the pharmacokinetics of ABC transporter substrate drugs in humans, hoping that these polymorphisms would help to explain interindividual variability in dosing requirements and pharmacological response. Table 1.5 contains a list of the ABC transporter polymorphisms whose effects on drug pharmacokinetics have been studied.

Table 1.5. Polymorphisms in ABC transporters associated with drug pharmacokinetics.

Gene	Polymorphism	Population	Outcome marker	Finding	Ref
ABCB1	3435C>T	62 patients	Fluvoxamine C_{ss}	CT/TT>CC (concentration:dose ratio, 200 mg/d dose)	[124]
ABCB1	3435C>T	14 healthy Caucasians	Digoxin C_p	TT genotype associated with increased digoxin levels	[125]
ABCB1	3435C>T	118 HIV+ patients	Atazanvir C_p	CC>CT/TT C_{min}, T allele associated with decreased C_p	[126]
ABCB1	3435C>T	92 Turkish renal transplant patients	Tacrolimus dose and C_{min}	TT genotype associated with higher dose, CC<CT/TT (C_{min})	[127]
ABCB1	3435C>T	42 Japanese patients	Cyclosporin A C_p	No statistical association	[128]
ABCB1	3435C>T	24 healthy volunteers	Digoxin C_p, AUC_{0-4h}	T allele associated with increased AUC_{0-4h} and C_{max}	[129]
ABCB1	3435C>T	14 healthy subjects	Cyclosporin C_p, AUC	No statistical association	[130]
ABCB1	3435C>T	102 children	Etoposide CL	CC genotype associated with increased CL	[131]
ABCB1	3435C>T	123 HIV+ patients	Antiretroviral C_p	CC>CT>TT	[132]
ABCB1	3435C>T	32-43 HIV+ patients	Lopinavir and efavirenz C_p	No statistical association	[133]
ABCB1	3435C>T	97 Caucasian cancer patients	Paclitaxel C_p, AUC, CL	No statistical association	[134]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	29 carbamazepine- and 15 phenytoin-treated patients	Carbamazepine and phenytoin PK	2677 minor alleles associated with lower phenytoin dose	[135]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	69 renal transplant patients	Cyclosporin A C_p, AUC	3435CC>CT/TT (AUC)	[136]

ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	26 healthy males	Amlodipine C_p, AUC	2677GG/3435CC>CT/CT>TT/TT (C_{max}), 2677TT/3435TT>CT/CT>GG/CC (CL)	[137]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	88 Iranian renal transplant	Cyclosporin A C_p	3435TT genotype associated with higher concentration:dose ratio immediately after transplant	[138]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	32 healthy subjects	Digoxin AUC	TT genotype associated with increased AUC	[139]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	15 healthy subjects	Digoxin bioavailability	2677TT/3435TT>GT/CT>GG/CC	[140]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	33 healthy Korean males	Fexofenadine AUC	2677AA/3435TT associated with decreased AUC	[141]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	52 children	Vincristine PK	No statistical association	[142]
ABCB1	1199G>A (Ser400Asn), 1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	104 pediatric renal transplant patients	Cyclosporin PK	2677TT>GT>GG (bioavailability); 1199C/1236C/2677G/3435C haplotype associated with decreased bioavailability	[143]
ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	195 elderly European patients	Digoxin C_p	T alleles and TTT haplotype associated with higher digoxin serum levels	[144]

ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	37 Caucasians, 23 African Americans	Fexofenadine AUC	CC/GG/CC>TT/TT/TT	[145]
ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	66 cancer patients	$CL_{1.5h}$	2677T and 3435T alleles associated with decreased elimination	[146]
ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	28 Caucasian patients with solid tumors	Tipifarnib AUC	1236T allele associated with increased AUC	[147]
ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	65 cancer patients	Irinotecan and SN-38 AUC	1236T allele associated with increased AUC	[148]
ABCC2	-24C>T	35 HIV+ patients	Antiretroviral PK	T allele associated with higher indinavir CL	[149]
ABCG2	421C>A (Glu141Lys)	12 cancer patients	Topotecan bioavailability	CA>CC	[150]
ABCG2	421C>A (Glu141Lys)	84 Caucasian cancer patients	Irinotecan and SN-38 PK	No statistical association	[151]
ABCB1 and *ABCG2*	3435C>T 195769-72del	29 Asian nasopharyngeal carcinoma patients	Irinotecan PK and metabolism	3435CC genotype associated with lower C_{max}; ABCG2 deletion associated with decreased irinotecan conversion to SN-38	[152]

ABCB1 was one of the first genes to be fully sequenced in coding and regulatory regions, and several groups published reports describing the extent and nature of sequence variation in *ABCB1* [145, 153]. The effects of *ABCB1* SNPs on the pharmacokinetics of certain drugs with narrow therapeutic ranges, such as the cardiac glycoside digoxin, were of particular interest because of their clinical relevance. The first report to investigate *ABCB1* pharmacogenetics in relation to digoxin pharmacokinetics was published in 2000 by Hoffmeyer *et al*. The findings of this study indicated that the *ABCB1* 3435C>T synonymous SNP was correlated with lower duodenal P-gp expression and higher digoxin plasma levels [125]. Kim *et al.* examined *ABCB1* 2677G>T, a non-synonymous polymorphism that frequently occurs in a haplotype with 3435C>T, and found that it led to increased digoxin efflux *in vitro* [145], in contrast to the results in the Hoffmeyer study [125]. The *ABCB1* 2677T and 3435T alleles have also been associated with increased digoxin plasma levels [139, 140, 154], suggesting reduced ABCB1/P-gp expression and/or function, as well as with decreased digoxin plasma levels and increased duodenal ABCB1 mRNA expression [155], indicating the opposite. Other groups found no effect of *ABCB1* SNPs on digoxin pharmacokinetics [156, 157].

Pharmacogenetic studies of fexofenadine pharmacokinetics are also conflicting, with *ABCB1* 2677T/3435T-containing haplotypes correlating with increased [141] or decreased plasma levels [145], or having no effect [158]. The immunosuppressant cyclosporin A, often administered following organ transplantation, is also a substrate of P-gp [159]. While some researchers found that transplant patients with the 3435T allele or haplotypes containing that allele had higher dose-adjusted cyclosporin A plasma levels [160-163], others reported the opposite [136, 164] or found no effect [165, 166]. The

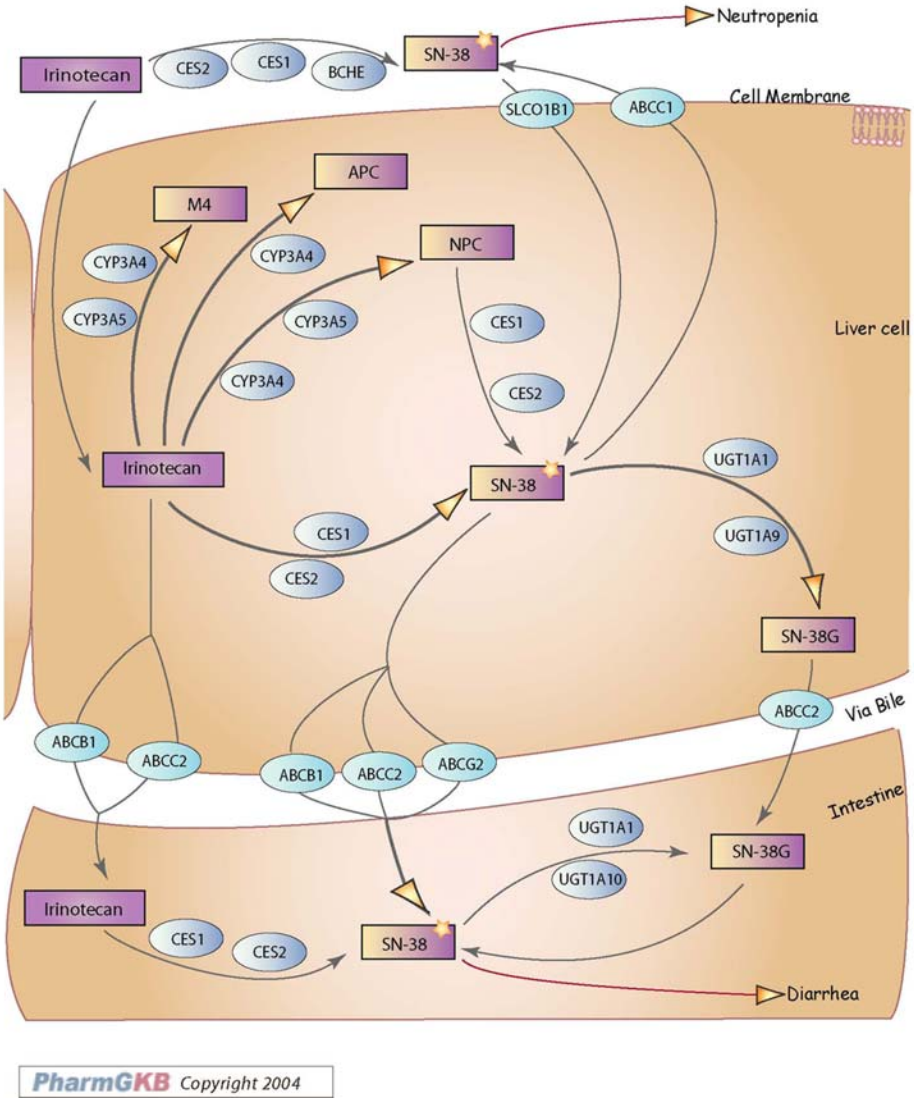
reasons for these conflicting results may be due to differences in phenotyping methods, study design (e.g. controlling for population admixture or ethnicity), degree of statistical power, or some sort of underlying linkage disequilibrium that has not been taken into account.

Polymorphisms in the hepatic organic anion transporter MRP2 have been shown to cause the genetic disorder Dubin-Johnson syndrome (described above), but more recently they have been associated with interindividual differences in irinotecan pharmacokinetics. The active metabolite of irinotecan, SN-38, is glucuronidated by uridine diphosphate glucuronosyltransferases (UGTs), and SNPs in *UGT1A1* have been associated with the rate of SN-38 glucuronidation [167] and irinotecan toxicity [168]. However, both irinotecan and SN-38 are transported from hepatocytes into the bile by MRP2 [169]. A haplotype consisting of six SNPs in *ABCC2*, designated *ABCC2*2*, was significantly associated with lower irinotecan clearance and an increased risk of irinotecan-induced diarrhea, suggesting impaired MRP2 transport function and elevated irinotecan plasma levels leading to toxicity [170].

MXR has also been implicated in the efflux of SN-38 into the bile, and the effect of *ABCG2* 421 C>A on irinotecan pharmacokinetics has been investigated, since this SNP was shown to decrease MXR expression *in vitro* [171]. Several studies did not find statistically significant associations between the 421A allele and SN-38 plasma levels [172-174], although one group reported that carriers of the 421A allele had elevated plasma concentrations of topotecan, another topoisomerase I inhibitor and anticancer drug [150]. The example of irinotecan pharmacogenetics underscores the importance of considering polymorphisms in multiple genes in a pharmacokinetic pathway, including

metabolizing enzymes as well as transporters; such pathway genotyping may become crucial in the clinical application of pharmacogenomics. As illustrated in Figure 1.5, at least four ABC transporters and one SLC transporter have been implicated in irinotecan disposition (www.pharmgkb.org) [175].

Figure 1.5. Irinotecan disposition in a hepatocyte. This schematic also demonstrates the involvement of transporters in intestinal epithelial cells in irinotecan pharmacokinetics (www.pharmgkb.org) [175].



At times, however, the proteins in a pharmacokinetic pathway are unknown; in these cases, animal models can help elucidate which proteins may be important. For example, the creation of the *Abcc2-/-* mouse led the conclusion that MRP2 plays a role in pravastatin disposition. Pravastatin plasma levels were significantly elevated in *Abcc2-/-* mice in comparison to wild-type mice [176]. Subsequently, a number of researchers investigated the effects of *ABCC2* polymorphisms on pravastatin pharmacokinetics. Niemi *et al* found an association between the *ABCC2* 1446C>G SNP and reduced pravastatin exposure [177]. In contrast, Ho *et al.* did not find any statistically significant associations between *ABCC2* SNPs and pravastatin pharmacokinetics [178]. It should be noted that polymorphisms in *SLCO1B1*, which encodes for an uptake transporter, have also been correlated with pravastatin disposition and activity [178, 179], again demonstrating the importance of considering the entire pharmacokinetic pathway of a drug rather than focusing on a single component.

1.6.3. Polymorphisms affecting drug pharmacodynamics

Even when the pharmacokinetic pathway of a drug is well-established, the proteins involved in the pharmacodynamics, or pharmacologic activity, of a drug may be unknown. In some cases, the plasma levels of a drug have no effect on its activity because its site of action is physiologically protected, often by ABC efflux transporters. Studies examining the effects of SNPs in ABC transporter genes on the pharmacodynamics of drugs targeting peripheral tissues have become increasingly common (see Table 1.6.3.1). For example, resistance to chemotherapy is a major cause of treatment failure in leukemia patients; a number of anti-leukemia drugs are also

substrates for P-gp, and the consequences of *ABCB1* SNPs on remission and survival in acute myeloid leukemia (AML) patients have been investigated. These SNPs include *ABCB1* 1236C>T, 2677G>A/T, and 3435C>T, all of which have been correlated with decreased P-gp function [81]. AML patients who carry the minor alleles would therefore be predicted to have increased intralymphocytic drug concentrations and experience a more favorable outcome. However, conflicting data have been reported: one study described a correlation between the *ABCB1* 1236, 2677, and 3435 minor alleles and significantly increased survival rate and decreased risk of relapse [180], while a second study demonstrated an association between the 2677 and 3435 minor alleles and a decreased probability of AML remission [181].

Table 1.6. Polymorphisms in ABC transporters associated with drug pharmacodynamics.

Gene	Polymorphism	Population	Outcome marker	Finding	Ref
ABCB1	3435C>T	313 patients with *H. pylori* infection	Chemotherapy response	CC>CT>TT	[182]
ABCB1	3435C>T	44 Caucasian leukemia patients	Chemotherapy response	No statistical association	[183]
ABCB1	3435C>T	113 Caucasian children	Acute lymphocytic leukemia outcome	TT genotype associated with increased probability of survival	[94]
ABCB1	3435C>T	53 patients	Leukemia outcome	No statistical association	[184]
ABCB1	3435C>T	123 HIV+ patients	CD4 cell increase	TT>CT>CC	[132]
ABCB1	3435C>T	340 HIV+ patients	Virologic response	TT genotype associated with increased virologic response	[185]
ABCB1	3435C>T	149 HIV+ patients	CD4 cell increase	No statistical association	[186]
ABCB1	3435C>T	31 drug-naïve HIV+ patients	Virologic and immunologic response	No statistical association	[187]
ABCB1	3435C>T	461 drug-naïve HIV+ Caucasians	Time to virologic or immunologic failure	No statistical association	[188]
ABCB1	3435C>T	68 patients treated preop with anthracyclines with or without taxanes	Response to preoperative chemotherapy	TT genotype associated with response to chemotherapy	[189]
ABCB1	3435C>T	200 drug-resistant, 155 drug-responsive patients	Refractory epilepsy	CC genotype associated with refractory epilepsy	[190]
ABCB1	3435C>T	230 drug-resistant, 170 drug-responsive patients	Refractory epilepsy	No statistical association	[191]
ABCB1	3435C>T	401 drug-resistant, 208 drug-responsive patients	Refractory epilepsy	No statistical association	[192]

ABCB1	3435C>T	63 drug-resistant, 108 drug-responsive Korean patients	Refractory epilepsy	No statistical association	[181]
ABCB1	2677G>T	30 leukemia patients	Survival	GG and TT genotypes associated with decreased survival	[193]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	26 patients	Paclitaxel response and toxicity	3435CC associated with reduced risk of neuropathy; 2677TT/3435TT haplotype associated with neutropenia	[194]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	127 Chinese lupus patients	Steroid toxicity (ONF)	TT genotypes associated with lower incidence of osteonecrosis of femoral head (ONF)	[195]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	69 Chinese non-small cell lung cancer patients	Vinorelbine response	3435CC genotype and 2677G/3435C haplotype associated with better response	[196]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	170 heart transplant patients	Rejection	3435CC associated with rejection episodes	[197]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	101 Korean leukemia patients	Remission, survival	2677G and 3435C alleles associated with remission and 3-year event-free survival	[198]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	54 small cell lung cancer patients	Etoposide-cisplatin response	3435CC genotype and 2677G/3435C haplotype associated with better response	[199]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	52 children with leukemia	Vincristine side effects	No statistical association	[142]
ABCB1	1236C>T, 2677G>T (Ala893Ser), 3435C>T	89 healthy Caucasians	Mefloquine neuropsychiatric side effects	Minor alleles or the 1236T/2677T/3435T haplotype associated with adverse events in women	[200]

ABCB1	1236C>T, 2677G>T (Ala893Ser), 3435C>T	405 leukemia patients	Leukemia outcome	3435CC genotype associated with decreased survival and increased risk of relapse	[180]
ABCB1	1236C>T, 2677G>T (Ala893Ser), 3435C>T	Retrospective study in HIV+ patients	Immunologic recovery	Marginal association with 1236 genotype	[201]
ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	210 patients	Refractoriness of epilepsy	1236C/2677G/3435C associated with increased resistance	[202]
ABCB1	-129T>C, 2677G>T/A (Ala893Ser/Thr), 3435C>T	25 drug-resistant, 20 drug-sensitive Turkish patients	Acute leukemia chemotherapy response	No statistical association	[203]
ABCB1	3435C>T, intron 26 +80T>C	28 HIV+ patients	Intralymphocytic nelfinavir levels	3435TT>CT>CC, +80CC>CT>TT	[204]
ABCB1 and *ABCC4*	*ABCB1* 2677G>A/T, *ABCC4* 3724G>A, 4131T>G	35 HIV+ patients	Antiretroviral levels and viral response	*ABCB1* 2677TT>GT>GG viral response; *ABCC4* 4131G allele associated with increased lamivudine-triphosphate intralymphocytic levels	[149]
ABCB1, *ABCC1*, *ABCC2*, *ABCG2*	9 SNPs	914 ovarian cancer patients	Platinum chemotherapy response and toxicity	No significant associations	[205]

ABC transporters expressed on the cell surface of lymphocytes have also been implicated in limiting the intracellular concentration of antiretroviral drugs used to treat HIV. Fellay *et al.* reported an association between the *ABCB1* 3435TT genotype and increased immunologic response following protease inhibitor-based therapy, indicating that this genotype may allow more drug to accumulate inside target lymphocytes, thereby increasing its efficacy [132]. This genotype has also been correlated with higher levels of efavirenz efficacy [185, 187]. Other researchers, however, have been unable to consistently reproduce any pharmacogenetic associations between *ABCB1* 3435 genotype and HIV antiretroviral efficacy [186, 188], although these studies were conducted in antiretroviral-inexperienced patients, unlike the Fellay report.

Another example of the pharmacodynamic effects of transporters occurs at the blood-brain barrier. Anti-epileptic drugs act in the brain, but because of the existence of the blood-brain barrier, plasma levels do not reflect drug levels in the brain. It follows that the xenobiotic transporters localized to the blood-brain barrier may therefore play an important role in the pharmacodynamics of anti-epileptic drugs, as well as in the development of drug-resistant epilepsy.

The first investigation into *ABCB1* pharmacogenetics and drug-resistant epilepsy found that patients with drug-resistant epilepsy were less likely to have the *ABCB1* 3435TT genotype [190]. This finding agreed with an earlier report that lymphocytes from individuals with the *ABCB1* 3435TT genotype accumulated more rhodamine 123 (a P-gp substrate) than did carriers of the C allele [206], indicating that the TT genotype conferred reduced P-gp function, and would therefore result in a decreased incidence of drug resistance compared to the 3435CC genotype. Other researchers described a similar

finding, in which the *ABCB1* 3435T allele was less frequent in a group of patients with drug-resistant epilepsy [201, 207, 208], although several groups found the opposite [202, 209]. However, an equal number of researchers found no association between *ABCB1* 3435 genotype and drug-resistant epilepsy [181, 191, 192, 210-212]. Soranzo *et al.* suggested that one or more intronic SNPs in *ABCB1*, which are strongly associated with 3435C>T, may be *responsible* for multidrug-resistant epilepsy; in fact, one of these intronic SNPs was significantly associated with multidrug resistance in epilepsy patients [213].

1.6.4. Conclusions

The controversy surrounding the role of *ABCB1* polymorphisms in response to anti-epileptic drugs speaks to the complex nature of the disease at hand. The difficulty of replicating pharmacogenetic findings is often amplified by the necessity of phenotyping a complicated disorder at different locations by different medical professionals, as is the case with refractory epilepsy. In general, pharmacogenetic associations should be able to withstand the rigor of thorough replication studies before they are considered real.

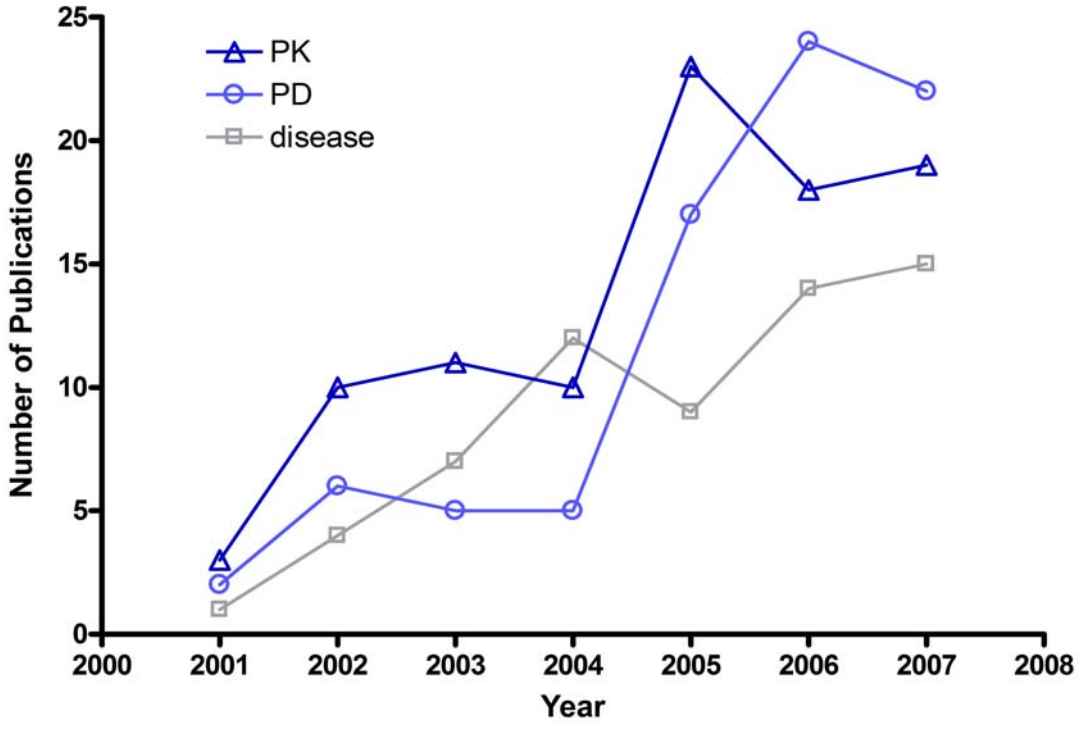
Recently, the frequency of reports describing intriguing pharmacogenetic associations has increased. For example, a SNP in the 5'-untranslated region of *ABCC4* was associated with intralymphocytic concentrations of the anti-HIV drug lamivudine [149]. There has also been a recent report that *ABCG2* 421C>A is associated with the pharmacokinetics of the anticancer drug gefitinib [214]. These accounts, while exciting, are generally described as preliminary or pilot investigations and should be considered as such until follow-up studies can be performed. The same can be said of *in vitro* or animal

studies which show transporter genotype-phenotype associations: replication *in vivo* is crucial, as is an exploration into the mechanism through which the polymorphism is exerting its effects.

As the field of *ABCB1* pharmacogenetics expands, so too do the number and diversity of clinical phenotypes examined. Pharmacogenetics studies are now being used as a tool to elucidate the role that a transporter plays in drug response or disease susceptibility, rather than characterizing polymorphism effects on patient exposure to drugs which are known substrates of that transporter. In this way, researchers hope to learn more about the underlying transporter biology – does it have endogenous substrates, and what are they? This shift from pharmacokinetic phenotypes to pharmacodynamic and disease susceptibility phenotypes is displayed in Figure 1.6. After the initial characterization of *ABCB1* genetic variation, researchers tended to focus more on pharmacokinetic effects on known drug substrates, attempting to explain interindividual variability in these parameters. More recently, the number of studies examining drug response and toxicity phenotypes started to increase.

With the increasing ease with which genetic data are available, pharmacogenomics is being used more often as a tool to investigate biological and pharmacological mechanisms of action. However, the interpretation of pharmacogenetic data must be done in a careful and thorough manner so that the results are as unambiguous as possible. The consideration of other variables – environmental exposures, genetic background and ethnicicty, concomitant medications – is a crucial step in understanding the results of pharmacogenetic studies, and in being able to apply them in a clinically relevant way.

Figure 1.6. The changing focus of *ABCB1* pharmacogenetic studies. The number of publications examining the effects of *ABCB1* polymorphisms on disease susceptibility or drug pharmacokinetics (PK) or pharmacodynamics (PD, including response, resistance, or adverse events) were counted following a literature search using NCBI PubMed. Only reports based on human *in vivo* results were included; reviews and meta-analyses were excluded. Publications were grouped based on stated study aims (not positive findings), and could be included in more than one group.



1.7. Focus of dissertation

The influence of xenobiotic ABC transporters on the pharmacokinetics of drugs has been widely studied. However, less is known about the significance of ABC transporter effects on drug pharmacodynamics. The goal of this dissertation is to examine the expression and function of ABC transporters and their genetic variants in tissues that are sites of pharmacological action, either intentional (efficacy) or inadvertent (toxicity), and to study the consequences of these transporters on drug effects. Specifically, the aims of the dissertation include:

1. Characterize the ABCB1/P-glycoprotein expressed in lymphocytes (Chapter 2). Expression of ABC transporters in lymphocytes has been associated with lower intracellular drug concentrations, as well as unfavorable clinical outcomes in leukemia and HIV patients. Here we describe the expression of a half-size P-gp with altered reactivity but typical function compared to classic full-size P-gp, and examine lymphocyte ABCB1 RNA transcripts in an effort to identify the mini P-gp.

2. Investigate the effects of the HIV protease inhibitors atazanavir and saquinavir on lymphocyte ABCB1/P-gp expression (Chapter 3). The ASPIRE II pharmacokinetic study included the collection of lymphocytes following atazanavir/saquinavir administration. Here we examine the effects of HIV protease inhibitor treatment on lymphocyte ABCB1 and P-gp expression *in vitro* and in ASPIRE II lymphocyte samples.

3. Evaluate the effects of polymorphisms in ABC transporter genes, and in other pharmacokinetic/pharmacodynamic genes, on the toxicity and efficacy of HIV antiviral therapies (Chapter 4). Here, we use two HIV+ populations, REACH (San Francisco) and UARTO (Uganda), for pharmacogenetic analyses, with an emphasis on immunologic response and peripheral neuropathy outcome events. The role of ethnicity in pharmacogenetic analyses is examined in greater detail.

4. Study the role of transporters in adipose tissue on the occurrence of HIV NRTI-induced lipodystrophy (Chapter 5). The nucleoside analogue reverse transcriptase drugs, especially stavudine (d4T), have been associated with clinical lipodystrophy, which is thought to be caused by mitochondrial toxicity. In this chapter, we investigate the influence of two transporters, ABCC4/MRP4 and SLC29A1/ENT1, on NRTI toxicity both *in vitro* and in adipose samples from HIV+ patients.

The results of these studies will provide insight into the importance of transporters expressed in peripheral tissues (lymphoid, adipose) and help to explain how these transporters influence the pharmacodynamics of certain drugs.

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Chapter 2. Altered P-Glycoprotein Immunoreactivity but Normal Function in Human Peripheral Blood Mononuclear Cells

2.1 Abstract

The xenobiotic efflux transporter P-glycoprotein (P-gp, the product of the *ABCB1* gene) has been extensively characterized in its roles in multidrug resistance and drug pharmacokinetics. Fewer studies have focused on its expression and function in cells which are drug targets, where it may exert pharmacodynamic effects. Here we report the presence of an approximately half-size P-gp with altered immunoreactivity in human peripheral blood mononuclear cells (PBMCs), targets for leukemia and HIV drugs. This 75 kDa P-gp exhibits functional properties similar to those of the classic 170 kDa transporter. Cloning of ABCB1 transcripts from PBMC RNA led to the identification of three putative splice variants with the sequence and structural characteristics of an ABC half-transporter. These results indicate that PBMC P-gp may play a role in the efficacy of leukemia and HIV therapies, and suggest that P-gp may not be identical in all tissues.

2.2 Introduction

The membrane transporter P-glycoprotein (P-gp), encoded by *ABCB1*, was identified in the 1970s as a cellular mechanism of multidrug resistance in Chinese hamster ovary cells [1]. Since then, P-gp expression has been localized to the apical membrane of cells in tissues such as the gut, liver, and kidney [2], where the transporter acts as an efflux pump to prevent xenobiotics from reaching the bloodstream. Through inhibition studies, P-gp has been shown to influence the pharmacokinetics of a wide range of drugs, including the cardiac glycoside digoxin, the anticancer drug paclitaxel,

and cyclosporine, an immunosuppressant [3-5]. To date, most of the research involving P-gp has focused on the role of P-gp in drug pharmacokinetics – a fairly straightforward field of study, since plasma levels provide an easily quantifiable phenotype. In contrast, less work has been done on the contribution of P-gp to drug pharmacodynamics, in part because pharmacodynamic pathways and endpoints tend to be more complex than their pharmacokinetic equivalents.

P-gp is expressed in several tissues that play a role in the pharmacodynamics of several classes of drugs. For example, there have been reports that P-gp expression at the blood-brain barrier limits the access of anti-epileptic drugs [6], and on peripheral blood mononuclear cells (PBMCs), P-gp influences the intracellular accumulation of anti-leukemia and anti-HIV drugs [7, 8]. Cell surface expression of P-gp has been associated with decreased rates of remission in leukemia patients who were receiving daunorubicin [9]. Using cultured human lymphocytes, P-gp was shown to modulate the intracellular levels of the HIV protease inhibitors saquinavir [10], ritonavir [11], and lopinavir [12], as well as several HIV integrase inhibitors [13]. PBMC ABCB1 RNA expression has also been inversely correlated with intracellular ritonavir accumulation in HIV-infected patients [14], although no such correlation was found with the protease inhibitor nelfinavir [7]. Based on these and other published reports, the statement that PBMC P-gp expression influences the intracellular concentrations of drug substrates, thereby modifying their efficacy, is reasonable and clinically relevant.

Peripheral blood mononuclear cells, while serving as pharmacological targets themselves, are also often used to represent other less accessible tissues which are sites of drug action. For example, it has been proposed that PBMC P-gp may be used as a

surrogate to represent P-gp in other tissues such as liver and intestine, samples of which are difficult to collect in patient populations [15]. However, a report published in 2001 by Trambas *et al.* provided evidence that cultured natural killer cells, a subset of PBMCs, expressed "mini" P-gp instead of the classic 170 kDa form of P-gp [16]. In general, immunologic cells experience a higher degree of RNA transcript modification, including splicing, due to their inherent need to adapt to threats to the immune system [17]. In the present study, we examine the hypothesis that ABCB1 is expressed as a splice variant in PBMCs, and investigate its function in comparison to that of the classic full-length P-gp.

2.3 Materials and Methods

PBMC collection and storage

Whole blood was collected from healthy subjects under the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) protocol at UCSF, or from HIV-positive individuals under the Biobank for HIV Evaluation (BHIVE) protocol at Pacific Horizon Medical Group. Both studies were approved by the internal review boards at the relevant institutions. Peripheral blood mononuclear cells were isolated using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), according to manufacturer's instructions, and stored at -80˚C for less than six months, or in N_2 for long-term storage. PBMC samples from six SOPHIE and six BHIVE participants were used in this study.

Evaluation of PBMC P-gp function

PBMCs were incubated for 30 min at 37˚C in 100 nM calcein AM (Sigma-Aldrich) or 0.5 μg/mL rhodamine 123 (Sigma-Aldrich), with or without 100 nM GF120918 (a kind gift

from GlaxoSmithKline, Research Triangle Park, NC) or 100 nM verapamil (Sigma-Aldrich). Cells were incubated for 30 min on ice in the dark with CD56 antibody (BD Biosciences, San Jose, CA) followed by an APC-conjugated secondary antibody (BD Biosciences). Intracellular fluorescence was analyzed on a FACSCalibur flow cytometer (BD Biosciences). The PBMC population was gated based on forward- and side-scatter signals on a linear scale. Calcein and rhodamine 123 fluorescence was measured in channels FL-1 and FL-2, respectively, on a logarithmic scale. CD56 signal was measured in channel 4 (FL4, λ_{max} 661 nm), also on a logarithmic scale. Intracellular calcein and rhodamine 123 fluorescence was evaluated in a CD56+ PBMC subpopulation (indicating natural killer cells) in at least 3000 cells per sample. Statistical significance was determined using a repeated measures ANOVA followed by Bonferroni t-tests, with a cutoff of p=0.05 (GraphPad Prism, San Diego, CA).

Western blotting of PBMC lysates

PBMCs were collected by centrifugation and lysed in 150 mM NaCl, 1% Igepal CA-630 (Sigma-Aldrich) for 30 min on ice. Lysates were centrifuged at 12,000x*g* at 4˚C for 20 min and supernatants were collected. Proteins were separated by gel electrophoresis using 8% Tris-HCL gels (ProtoGel, National Diagnostics, Atlanta, GA) or 4-12% Bis-Tris gels (NuPage, Invitrogen, Carlsbad, CA) and transferred to 0.2 μm nitrocellulose membranes (BioRad, Hercules, CA). Blots were probed with C494 or C219 mAb (Covance Research Products, Princeton, NJ) and an HRP-conjugated anti-mouse secondary antibody (Invitrogen), and detected using a chemiluminescent ECL detection system (Millipore, Billerica, MA).

RNA isolation and cloning

RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and was reverse transcribed using Superscript reverse transcriptase (Invitrogen). Platinum High Fidelity Taq polymerase (Invitrogen) was used to amplify ABCB1 transcripts, with the following primers: sense 5'-GGATGGATCTTGAAGGGGACC-3' and antisense 5'-AGTCAGAGTTCACTGGCGCTTTG-3'. cDNAs were cloned into pGEM-T vector (Promega, Madison, WI) and subcloned into the pcIneo (Promega) or pcDNA3.1 (Invitrogen) mammalian expression vectors for *in vitro* studies.

Visualization of cDNA transcripts

cDNA transcripts reverse transcribed from PBMC RNA were separated on a 1% agarose gel and transferred to a Hybond-N nylon membrane (Amersham Biosciences, Waukesha, WI) using an overnight upward transfer. DNA was crosslinked to the membrane (Stratalinker, Stratagene, La Jolla, CA) and incubated with a biotinylated DNA probe (Perkin Elmer, Waltham, MA) synthesized using the ABCB1 sense and antisense primers listed above, resulting in a 4 kB probe. Transcripts were visualized using the Detector HRP chemiluminescent blotting kit (KPL, Gaithersburg, MD).

Secondary structure predictions

The transmembrane topology images were generated using TOPO software (S.J. Johns, University of California, San Francisco, CA and R. C. Speth, Washington State

University, Pullman, WA), which displays secondary structure predictions of transmembrane proteins. The software is available at the University of California, San Francisco Sequence Analysis Consulting Group web site [18].

In vitro expression of splice variant cDNA clones

The human embryonic kidney cell line HEK293T was a kind gift from Dr. Warner Greene (Gladstone Institute, San Francisco, CA). Cell medium consisted of minimum essential medium supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin. HEK293T cells were transiently transfected with plasmid cDNA using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions.

2.4. Results

Expression and function of PBMC P-glycoprotein in healthy subjects

Western blots of lysates of primary PBMC samples collected from healthy subjects showed expression of an approximately half-sized P-gp which was detected by the C494 antibody, but not by C219 (Fig. 2.1). In contrast, both antibodies detected the full-sized 170 kDa P-gp in human kidney microsomes. The expression of the 75 kDa P-gp was seen in a panel of PBMC lysate samples from six participants in the SOPHIE study (Fig. 2.2). These six PBMC samples were used to evaluate P-gp transport of the fluorescent model substrates calcein and rhodamine 123 (Fig. 2.3); representative flow cytometry histograms of intracellular fluorescence are shown in Figures 2.3A and 2.3C. The P-gp-specific nature of this transport is demonstrated by the statistically significant increase in intracellular fluorescence (i.e. a reduction in transport function) following the

addition of the P-gp inhibitors GF120918 and verapamil (Figures 2.3B and 2.3D). These data provide evidence that although PBMC P-gp appears to be a half-sized transporter with altered immunoreactivity, it functions similarly to classic 170 kDa P-gp with respect to the substrates tested.

Figure 2.1. Altered immunoreactivity of P-glycoprotein in peripheral blood mononuclear cells. Human kidney microsomes (HKM) and PBMC lysates were separated by electrophoresis, and blots were probed with the P-gp antibodies C219 (A) or C494 (B). Full-length 170 kDa P-gp was expressed in kidney and detected by both antibodies, but PBMC P-gp was detected only by C494 and had a molecular weight of approximately 75 kDa. (C494 also detects the 130 kDa protein pyruvate carboxylase [19], as seen in the HKM lane.)

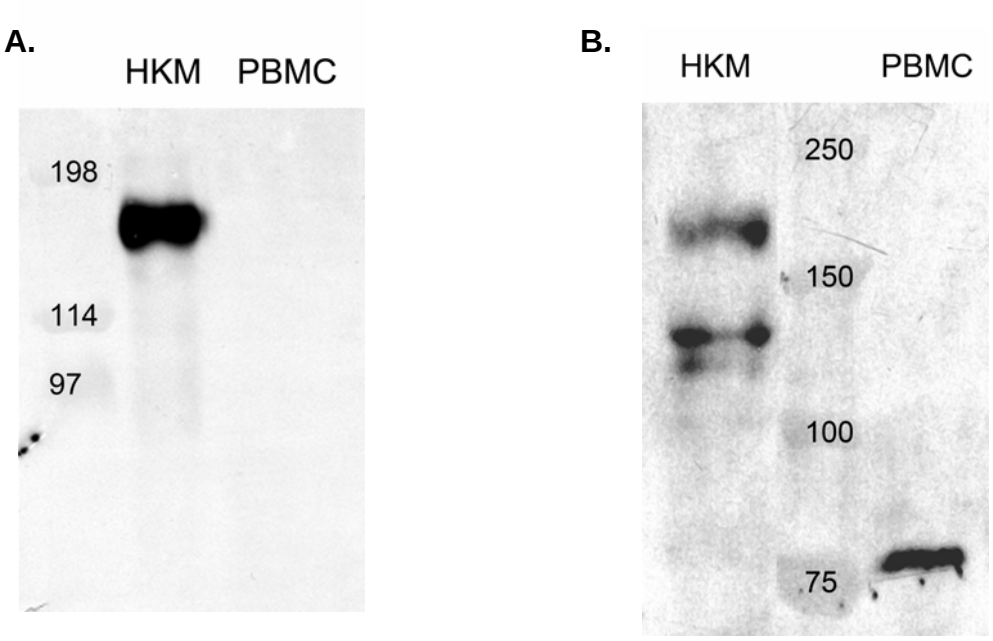


Figure 2.2. P-glycoprotein expression in peripheral blood mononuclear cells from healthy volunteers. Fifty μg of total protein per lane was separated on an SDS-PAGE gel, then transferred to nitrocellulose membrane and probed with the P-gp antibody C494.

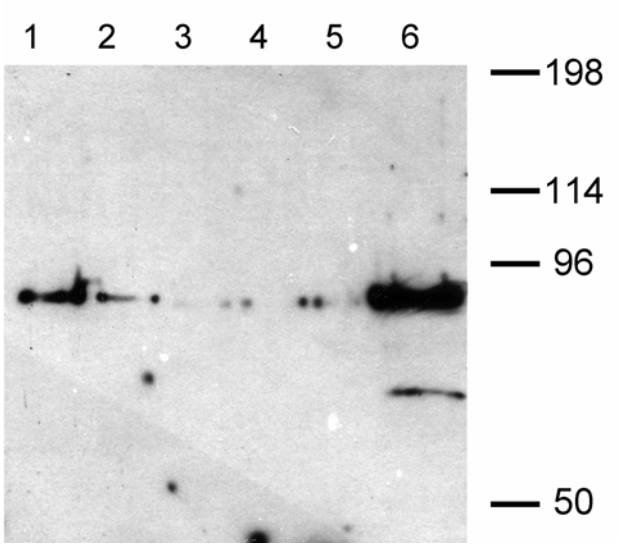
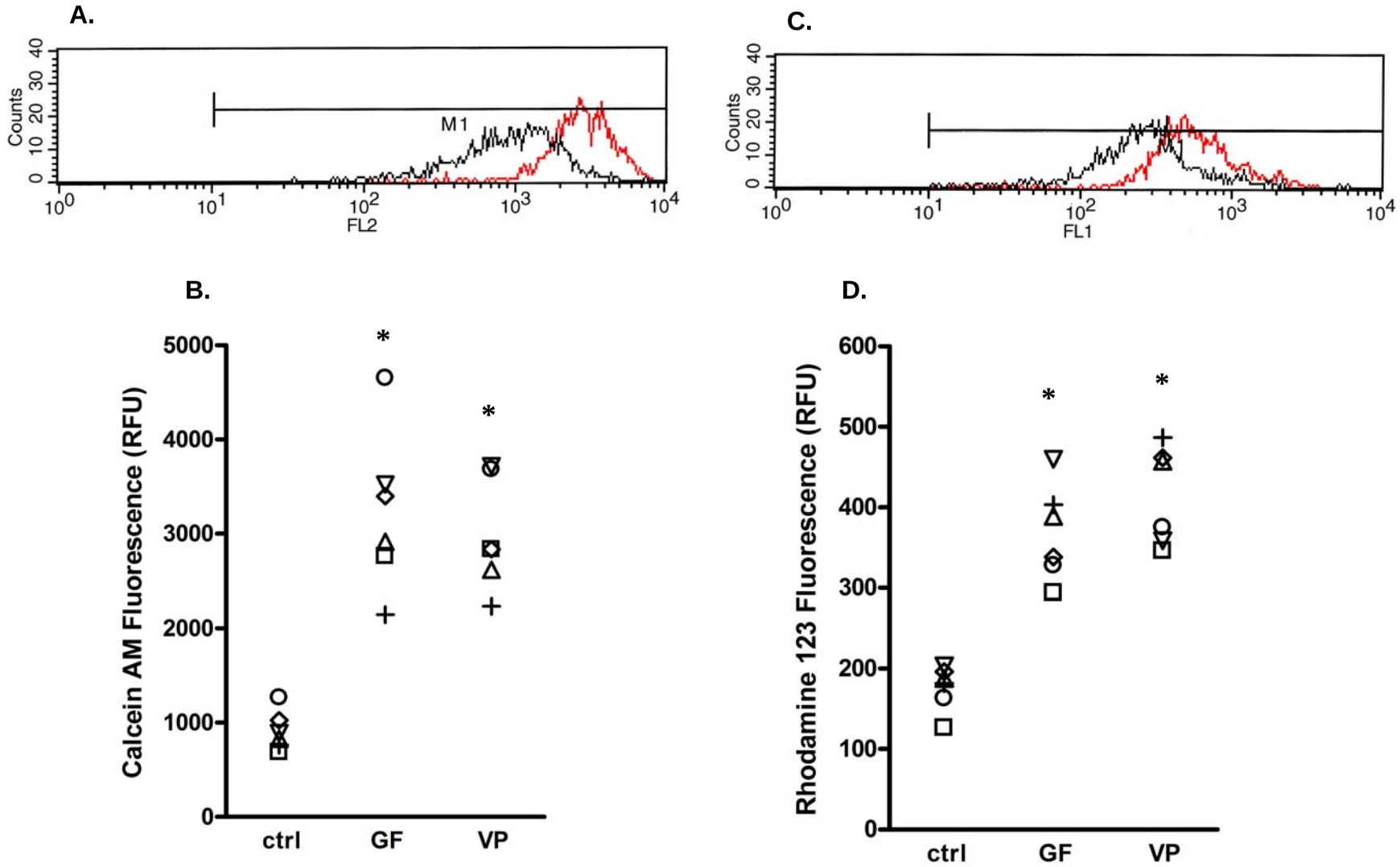


Figure 2.3. Function of peripheral blood mononuclear cell P-glycoprotein. The P-gp substrates calcein AM and rhodamine 123 were used to evaluate P-gp efflux activity in PBMC from healthy volunteers. An increase in fluorescence following the addition of the P-gp inhibitors GF120918 (GF) or verapamil (VP) demonstrated P-gp specific transport. A representative histogram showing intracellular calcein accumulation in PBMCs is shown (A). Intracellular calcein fluorescence reflects the amount of calcein AM effluxed from the cell by P-gp. PBMCs were incubated in calcein AM in the absence (black) or presence (red) of GF120918. Intracellular calcein fluorescence in six healthy volunteers is shown in panel B. A representative histogram showing intracellular rhodamine 123 accumulation in PBMCs is shown in panel C. Intracellular rhodamine 123 fluorescence reflects the amount effluxed from the cell by P-gp. PBMCs were incubated in rhodamine 123 in the absence (black) or presence (red) of GF120918. Intracellular rhodamine 123 fluorescence in six healthy volunteers is shown in panel D. * indicates $p < 0.001$ compared to the control group.


A.
Counts
0 10 20 30 40
M1
10^0 10^1 10^2 10^3 10^4
FL2
C.
Counts
0 10 20 30 40
10^0 10^1 10^2 10^3 10^4
FL1
B.
Calcein AM Fluorescence (RFU)
5000
4000
3000
2000
1000
0
ctrl
GF
VP
D.
Rhodamine 123 Fluorescence (RFU)
600
500
400
300
200
100
0
ctrl
GF
VP

Expression and cloning of PBMC ABCB1 RNA transcripts

Amplification of ABCB1 RNA transcripts using primers over the transcription start and stop sites demonstrated the presence of two transcripts – one approximately 4 kB in length, and a second which is about 2.5 kB in size (Fig. 2.4). The latter is consistent with a translated protein which is roughly half the size of a full-length transporter. Cloning of PBMC ABCB1 cDNA led to the identification of ten transcripts (data not shown). Based on sequencing data, five of these were approximately 2 kB in length, and of the five, three contained the Walker A, linker peptide, and Walker B sequence motifs required for ABC transport function. An amino acid sequence alignment of these putative splice variants, designated SV1, SV2, and SV3, with the two halves of full-length P-gp is shown in Figure 2.5. Figure 2.6 depicts the predicted secondary structures of P-glycoprotein and SV1-3. While P-gp contains two subunits of six transmembrane domains and a nucleotide-binding domain, the putative splice variants consist of a single TMD-NBD subunit.

Transient transfections of HEK293T cells with SV1 and SV2 resulted in SV RNA expression verified by Northern blotting (Fig. 2.7A), and SV2 protein was detectable on a Western blot probed with C494 (Fig 2.7B). Subcloning of SV3 into a mammalian expression vector had not yet been completed when the Western and Northern blotting experiments were carried out, although its expression and function were later assayed by flow cytometry. Interestingly, protein expression was highest at 24 hours post-transfection, when two bands are visible, possibly representing proteins with different glycosylation states. In contrast, no SV1, SV2, or SV3 expression was detected on the cell surface 24 hours after transfection, as evidenced by flow cytometry data on live cells

stained with the P-gp antibody UIC2, which is used to detect PBMC P-gp (Fig. 2.7C). Likewise, the intracellular accumulation of several well-known P-gp substrates (calcein AM, vinblastine, paclitaxel, and prazosin) was not significantly different in SV-transfected cells versus vector-transfected, although ABCB1-transfected cells exhibited a decrease in intracellular fluorescence, indicative of efflux activity (Fig. 2.8).

Figure 2.4. ABCB1 cDNA transcripts in peripheral blood mononuclear cells. PBMC ABCB1 RNA transcripts were reverse transcribed and amplified using ABCB1-specific primers; an identical reaction mix containing no cDNA template served as a negative control. Transcript expression was examined using a biotinylated DNA probe against the full-length transcript of ABCB1.

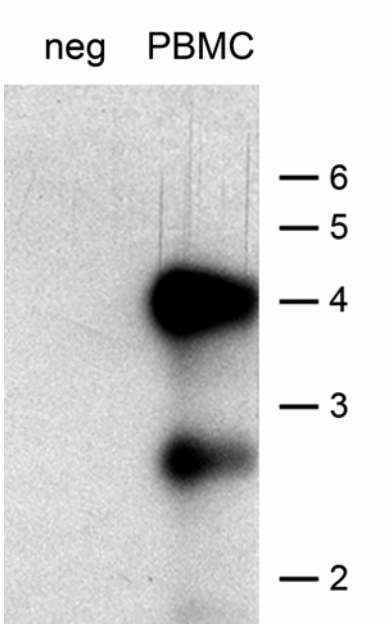


Figure 2.5. Amino acid sequence alignment of P-glycoprotein and cloned putative splice variants. The two halves of P-glycoprotein (residues 1-640 and 641-1280) and SV1, SV2, and SV3 amino acid sequences were aligned using ClustalW software. Identical residues in all five sequences are indicated by red asterisks, highly conserved positions are indicated by two blue dots, and somewhat conserved residues are indicated by one black dot. The Walker A, linker peptide, and Walker B sequences are outlined by boxes and labeled.

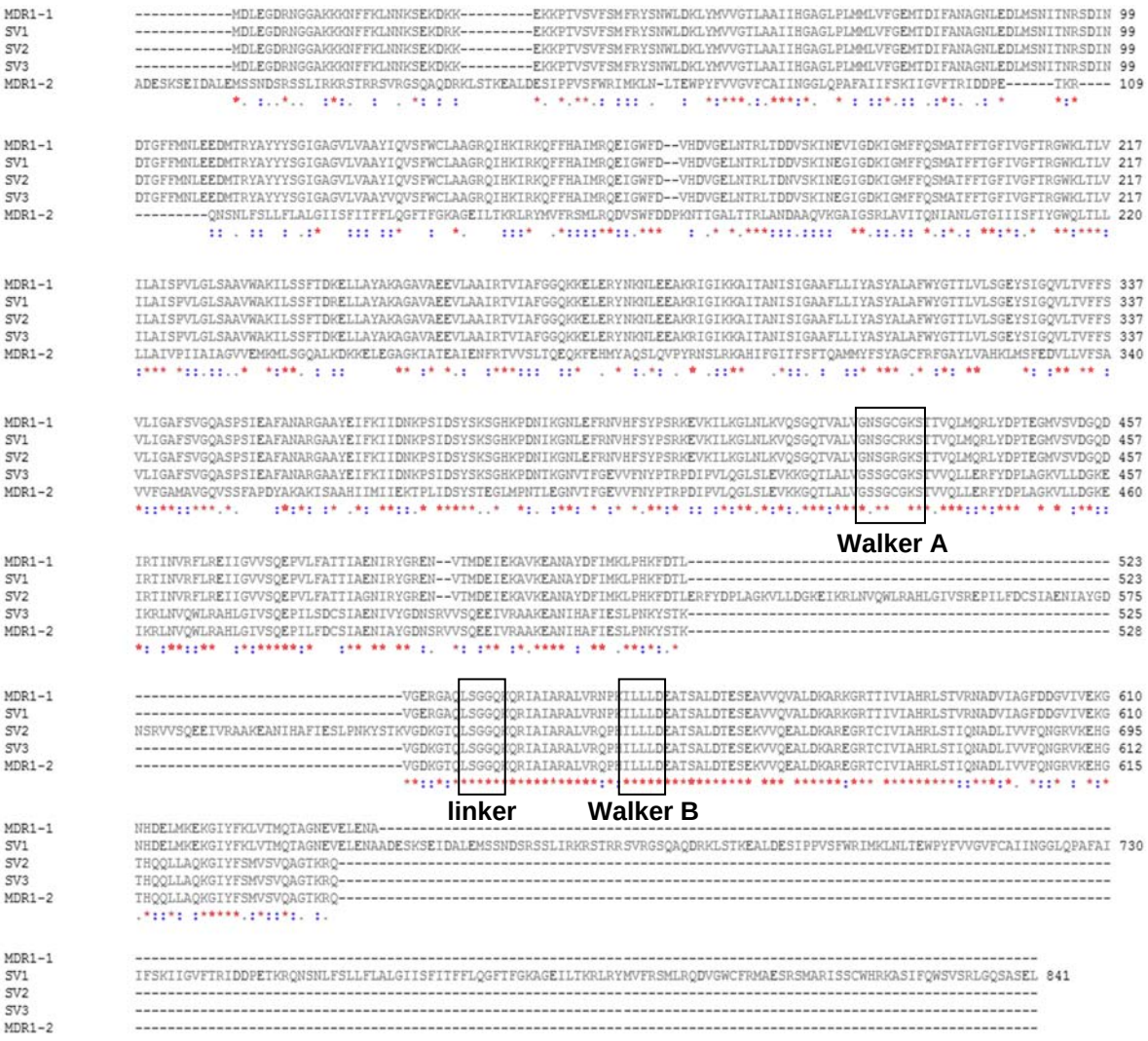
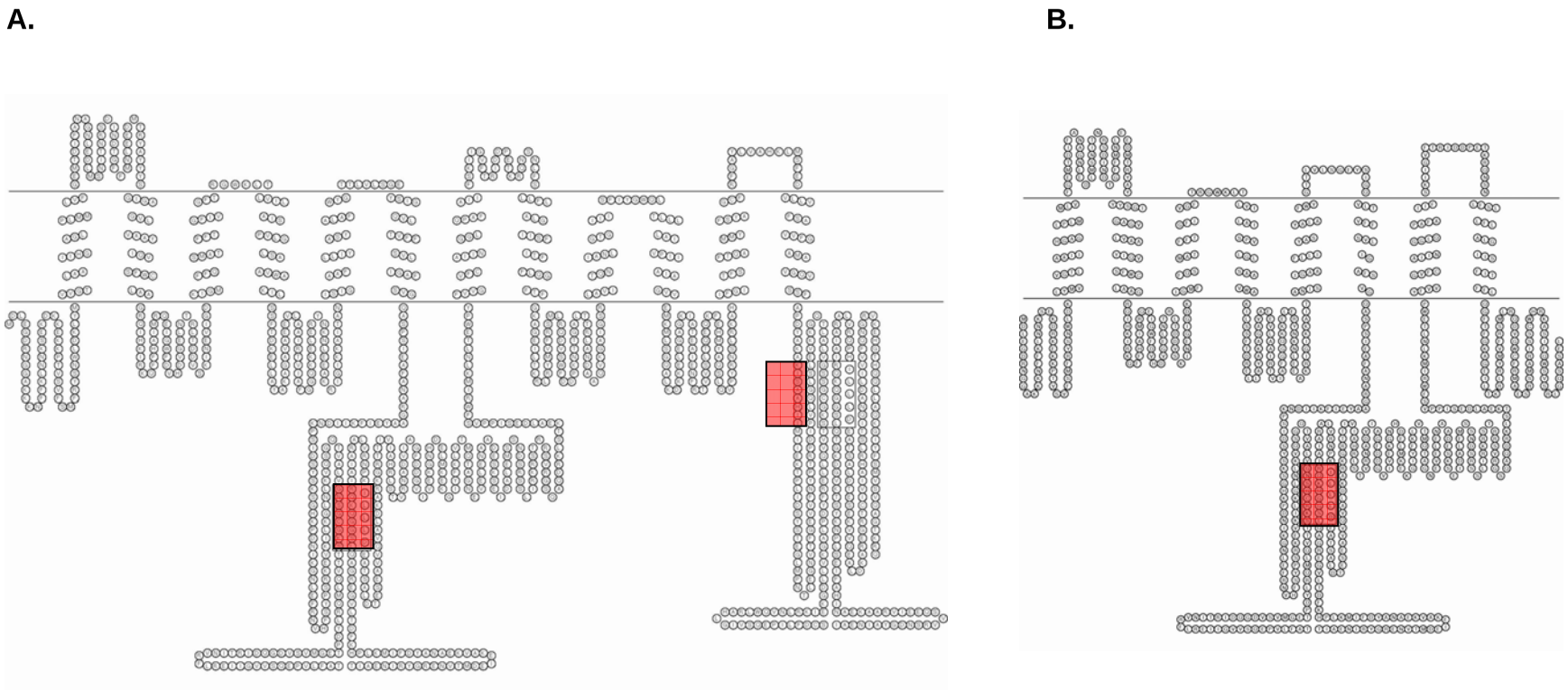
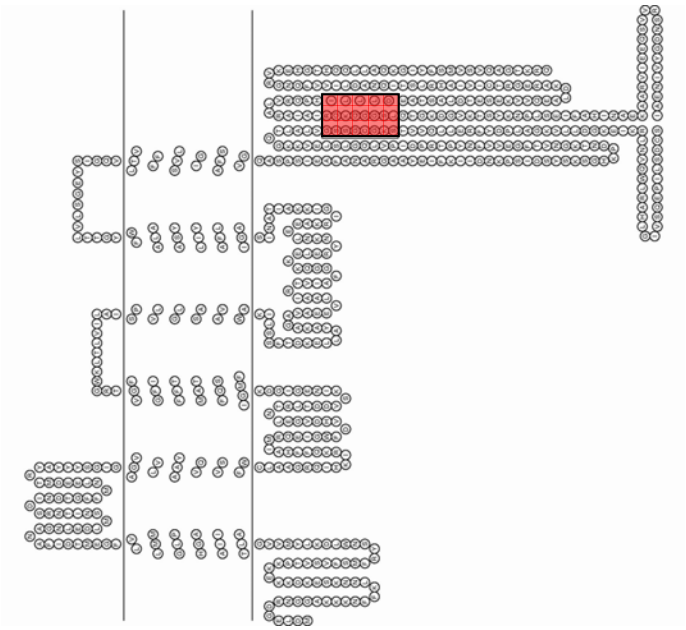


Figure 2.6. Predicted secondary structures of cloned ABCB1 putative splice variants. The ATP-binding site is depicted as a shaded red box, including the Walker A and B and linker peptide sequences. Predicted structures are shown for P-glycoprotein (A), SV1 (B), SV2 (C), and SV3 (D).



D.



C.

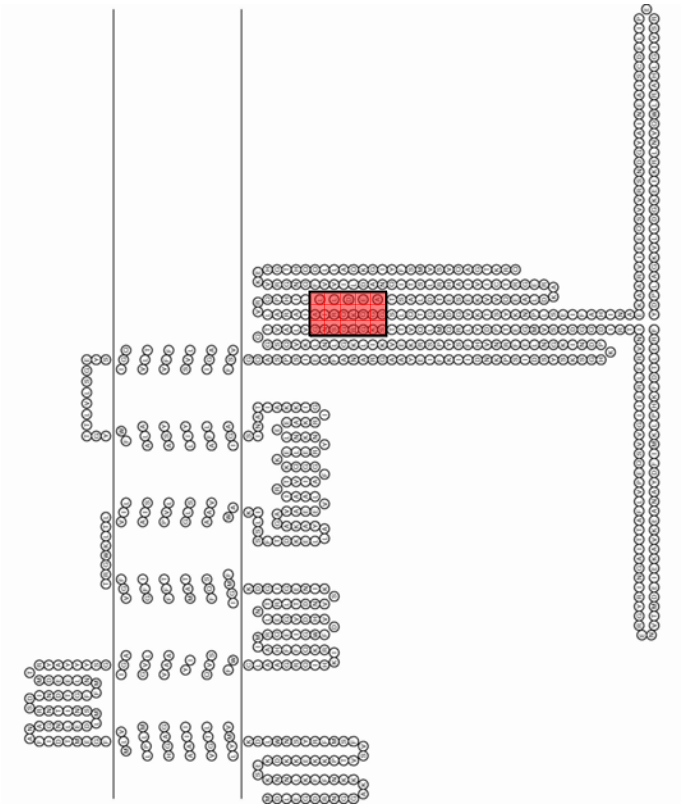
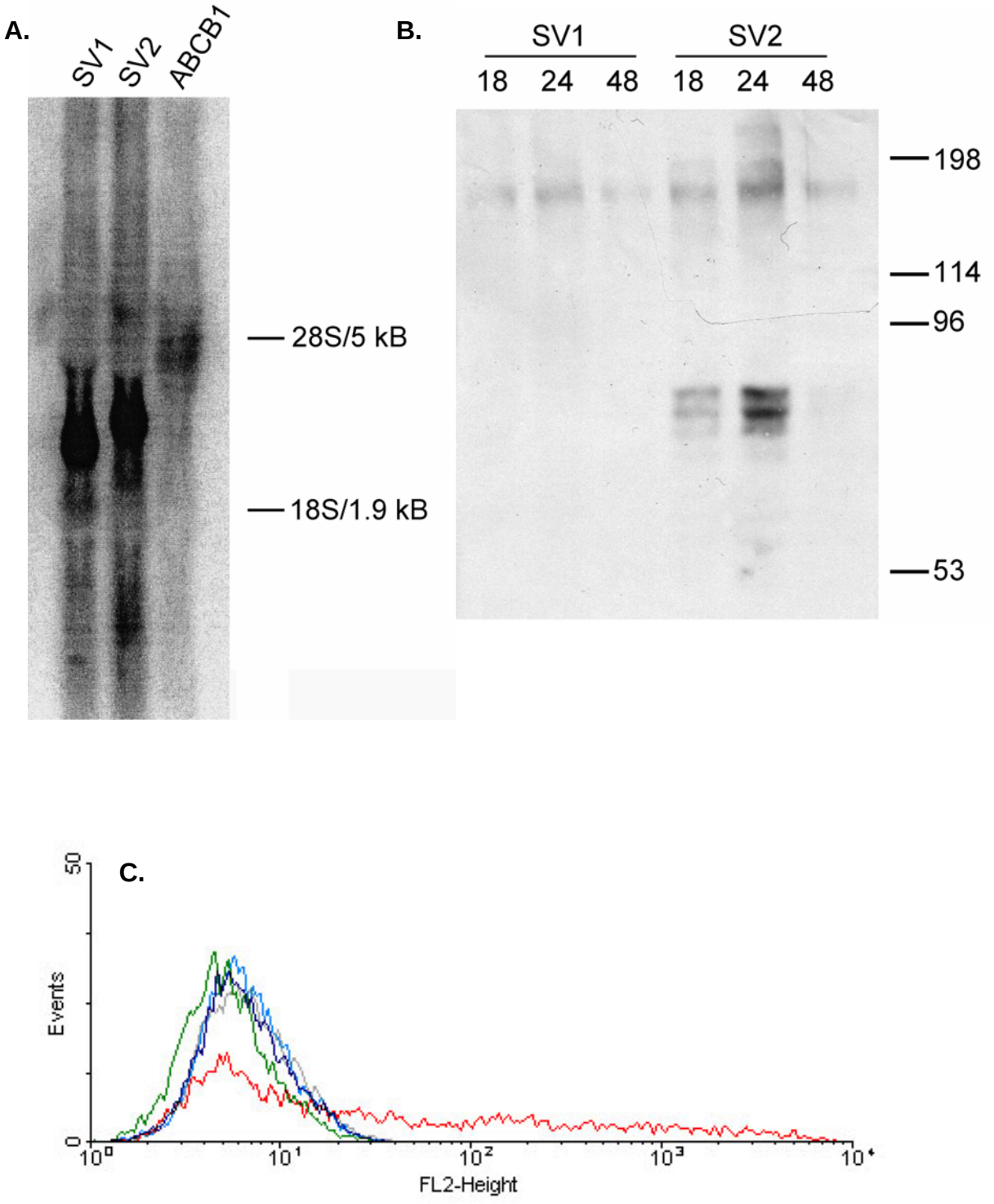
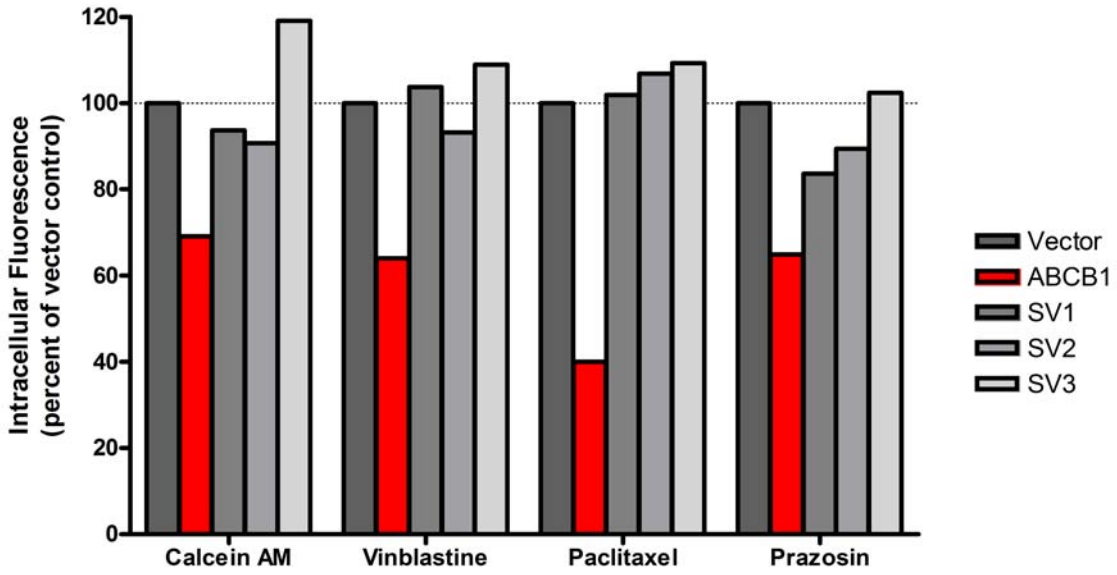


Figure 2.7. *In vitro* expression of cloned splice variants in transiently-transfected HEK293T cells. Plasmids containing the SV1 and SV2 constructs were transfected into HEK293T cells and allowed to express for 18, 24, or 48 hours. Expression levels were investigated by Northern and Western blotting. RNA extracted from transiently transfected cells 25 hours post-transfection was separated on a formaldehyde gel and transferred to a nylon membrane, which was subsequently probed by a full-length construct of reference ABCB1 cDNA labeled with ^{32}P-dCTP (A). Whole cell lysates from HEK293T cells transfected with cloned putative splice variants or reference ABCB1 were separated at 18, 24, or 48 hours post-transfection on a Western blot and probed with the C219 antibody (B). HEK293T cells transfected with SV1, SV2, or reference ABCB1 were incubated with the P-gp antibody UIC2, which recognizes extracellular epitopes of the protein (C). P-gp expression is indicated by positive fluorescence (i.e. FL2 above 30). Red: reference ABCB1, black: SV1, green: SV2, blue: SV3, purple: vector control.


A.
SV1
SV2
ABCB1
28S/5 kB
18S/1.9 kB
B.
SV1
SV2
18
24
48
18
24
48
198
114
96
53
C.
50
Events
0
10⁰
10¹
10²
10³
10⁴
FL2-Height

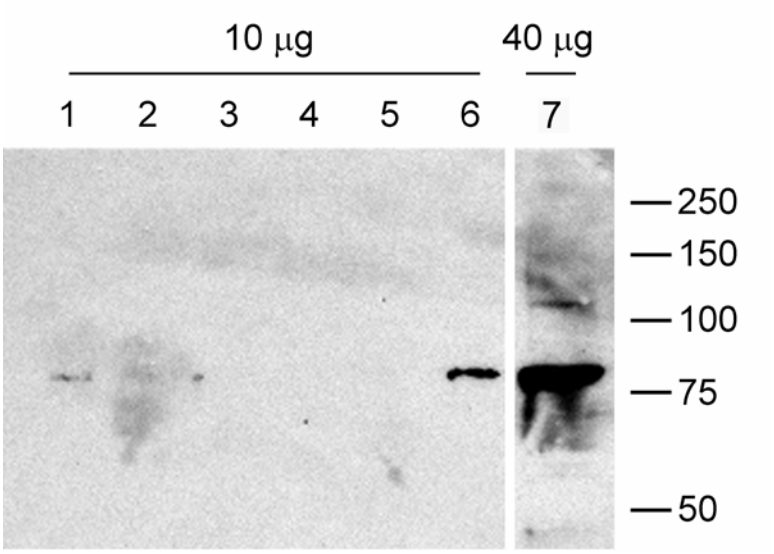
2.8. Transport properties of SV-transfected cells. HEK293T cells were transfected with SV1-3 or ABCB1 and assayed for efflux transport 24 hours post-transfection. Cells were incubated with fluorescent substrate for 30 minutes, and the intracellular fluorescence of the cells was measured by flow cytometry. A decrease in intracellular fluorescence indicates the presence of a functional efflux transporter. Each bar represents the intracellular fluorescence of a single transfected sample, shown normalized to the vector control (indicated by the dotted line at 100%). The fluorescence of ABCB1-transfected cells (shown in red) is substantially lower than that of vector- or SV-transfected cells for all compounds tested. These data are representative of three separate experiments.



Expression of PBMC P-glycoprotein in HIV-positive patients

PBMC samples from six participants in the BHIVE study were examined for P-gp expression by Western blotting. These patients were HIV-positive but were not receiving antiretroviral therapy at the time of PBMC collection, nor had they been exposed to antiretrovirals in the previous six months. Expression of the 75 kDa P-gp was detectable by the C494 antibody in two out of six samples (Fig. 2.9). As a result of the immunodeficiencies caused by HIV, fewer PBMCs were available from these individuals than from the healthy subjects (Fig. 2.2); accordingly, less total protein was loaded (10 μg versus 50 μg), which may explain the weak signal. Note that lane 7 contains 40 μg of total protein, improving the signal strength substantially.

Figure 2.9. P-glycoprotein expression in peripheral blood mononuclear cells from HIV-positive subjects. Western blotting of PBMCs collected from HIV-positive patients demonstrated the presence of an approximately 75 kDa protein detected by the C494 antibody, but no 170 kDa P-gp. Lanes 1-5 contain PBMC lysates from five different BHIVE subjects, and lanes 6 and 7 contain PBMC lysate from a sixth BHIVE subject. In all cases, 10 μg of total protein was loaded per lane, with the exception of lane 7, in which 40 μg total protein was loaded onto the gel.



2.5. Discussion

The presence of a 75 kDa "mini P-gp" has previously been reported in natural killer cells [16]; however, because the natural killer cells in the earlier investigation were immortalized, the question remained of whether the smaller protein was a result of the cell immortalization process, or if it was also present in primary lymphocytes. In the present study, we attempted to resolve this uncertainty by characterizing P-glycoprotein in primary lymphocytes.

Western blots of PBMCs isolated from healthy individuals revealed altered molecular weight and immunoreactivity of PBMC P-gp. A 75 kDa protein was detected only by the C494 mAb, but not by the C219 mAb. These results may be explained by an examination of the epitopes of these antibodies; C219 binds to two epitopes, one in each half of P-gp (568-VQVALD-573 and 1213-VQVELD-1218), while C494 recognizes a single epitope in the second half of P-gp (1028-PNTLEGN-1034) [20]. It follows that a splice variant transporter consisting of one half of P-gp may contain the C494 binding site, but not the two binding sites required for C219 recognition, and therefore no signal is detected on a Western blot when probed with the latter antibody (it should be noted, however, that if large amounts of PBMC lysate are used for Western blotting – e.g. greater than 75 μg – the 170 kDa P-gp band can be detected). Because the C494 antibody detects PBMC P-gp, we attempted to use this antibody to isolate the protein by immunoprecipitation, but were unsuccessful in our efforts (data not shown).

An assessment of the ABCB1 transcripts expressed in PBMCs also supports the existence of a splice variant. Following cDNA synthesis from PBMC RNA transcripts, PCR amplification and subsequent detection, two prominent transcripts were detected.

One transcript was 4 kB in length, and the other approximately half the length, or about 2.5 kB. Cloning of these PCR products resulted in the isolation of three putative splice variant cDNAs which had the sequence and structural characteristics of an ABC half-transporter: six transmembrane domains and a single nucleotide-binding domain, the latter containing the Walker A, linker peptide, and Walker B amino acid sequence motifs which are a defining feature of ABC proteins. Transient transfection into HEK293T cells resulted in expression of SV RNA transcripts (as detected by Northern blot), but no cell surface protein expression or efflux function was observed. These results may indicate a protein folding defect, incorrect trafficking, or protein instability, although we cannot rule out the possibility that the inability to detect P-gp splice variant protein is a function of the expression system, and that a different *in vitro* expression system may facilitate cell surface protein expression. Consequently, we are unable to conclude whether the half-size P-gp expressed in PBMCs is the gene product of any of these splice variant clones. There also remains the possibility that one or more P-gp splice variant proteins may homo- or heterodimerizes to form a functional transporter (a common mode of action for a number of ABC transporters, including ABCG2 [21]), which could be tested by simultaneously expressing multiple SV transcripts *in vitro*.

Although Trambas *et al.* reported that natural killer cell P-gp lacked the ability to transport the model substrates calcein AM or daunorubicin [16], our experiments demonstrated that the intracellular accumulation of calcein AM and another substrate, rhodamine 123, increased significantly in PBMCs in the presence of the P-gp inhibitors GF120918 and verapamil (both of which are P-gp-specific inhibitors at the concentrations used [22, 23]). These findings indicated that the intracellular

concentrations of these substrates are modulated by P-gp – in other words, PBMC P-gp functions as an efflux transporter in a manner similar to that of classic 170 kDa P-gp, thereby modulating the accumulation of xenobiotics inside the cell. The 75 kDa P-gp is also expressed in PBMCs collected from HIV-positive patients, indicating that the transport function of this protein may be a factor in the intracellular accumation and efficacy of anti-HIV drugs.

Alternative splicing of P-glycoprotein has been observed in cells from other species as well. The expression of a 2.3 kB splice variant of the hamster *pgp1* gene was demonstrated in Chinese hamster lung cells [24], which, when transfected into multidrug-resistant Chinese hamster lung cells, seemed to interfere with the drug resistance-causing properties of full-length pgp1 [25]. A 65 kDa P-gp, corresponding to a 2.4 kB RNA transcript, has been detected in P388 murine leukemia cells which were resistant to adriamycin or vincristine; this smaller protein corresponds with levels of drug resistance *in vitro* [26]. In humans, the ABC superfamily includes many functional half-transporters, with several known to contribute to multidrug resistance (e.g. MXR or BCRP [27]). The findings of the present study suggest that a half-sized P-gp is expressed in human peripheral blood mononuclear cells, and that it likely functions as an efflux transporter similarly to classic 170 kDa P-gp. Accordingly, care should be taken when using PBMC P-gp as a marker of P-gp expression and function in other cells and tissues. Subsequent investigations could examine the effects of this transporter on various phenotypes such as the clinical efficacy of leukemia and HIV chemotherapies.

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Chapter 3. Interindividual Variability in the Effect of Atazanavir and Saquinavir on the Expression of Lymphocyte P-glycoprotein[1]

3.1. Abstract

ABCB1 encodes the efflux transporter P-glycoprotein (P-gp), which regulates the intracellular concentration of many xenobiotics, including several HIV protease inhibitors. Exposure to some xenobiotics, such as the antibiotic rifampicin, increases P-gp expression. In the present study, we investigated the effect of the HIV protease inhibitors saquinavir and atazanavir on the expression and function of ABCB1 and P-gp in lymphocytes, as well as the molecular interactions between these drugs and P-gp. ABCB1 and P-gp expression and function were examined in lymphocyte samples from healthy subjects before and after atazanavir-boosted saquinavir treatment, and in cultured lymphocytes following exposure to atazanvir and saquinavir, using flow cytometry and quantitative RT-PCR. The inhibitory effects of these drugs on ABCB1 were investigated in transfected HEK293T cells. We found no overall changes in ABCB1 or P-gp expression or function after saquinavir-atazanavir treatment in primary lymphocyte samples. However, there was considerable interindividual variability in baseline lymphocyte ABCB1 expression, as well as in the degree of change in ABCB1 expression after saquinavir-atazanavir administration. In cell culture, 5 μM saquinavir increased ABCB1 levels, although it did not affect P-gp expression. Atazanavir inhibited P-gp function at concentrations above therapeutic levels. In conclusion, the observed differences in lymphocyte ABCB1 expression, which may be caused by genetic polymorphisms in *ABCB1* or its regulatory partners, are a likely cause of interindividual

[1] This work has been previously published [1].

variation in the disposition and efficacy of clinically relevant P-gp substrates, including HIV protease inhibitors.

3.2. Introduction

Viral resistance continues to impede the development of effective HIV antiretroviral therapies (ART) [1]. Interindividual variability in ART response presents a real challenge; evaluating the efficacy of multiple antiretroviral drugs for each patient comes at a considerable economic cost [2], as well as a significant cost to the patient's physical and emotional health [3]. In HIV, viral resistance is caused by inherent or acquired viral mutations, which may be influenced by host factors [4]. Xenobiotic transporters which prevent compounds from crossing physiological barriers are thought to be one of the causal host factors in HIV resistance [5]. Many of these xenobiotic transporters, which utilize the energy from ATP hydrolysis to move substrate molecules across cell membranes [6], belong to the ATP-binding cassette (ABC) superfamily [7].

In this superfamily, the most extensively studied xenobiotic transporter is P-glycoprotein (P-gp), which is encoded by *ABCB1*. P-gp was discovered in cells resistant to the anticancer vinca alkaloids and anthracyclines [8], but P-gp also transports a wide variety of structurally unrelated xenobiotics [9]. The effect of P-gp on pharmacokinetics (plasma levels) and pharmacodynamics (efficacy) is routinely investigated during drug development [10]; P-gp can affect pharmacokinetics by limiting bioavailability or increasing clearance [11] and can regulate the drug concentration at the site of action, thereby influencing pharmacodynamics [12].

Several HIV protease inhibitors (PIs) are substrates for P-gp. Saquinavir transport has been demonstrated in ABCB1-overexpressing cell lines [13-16], and increased saquinavir brain accumulation was reported in *mdr1a* knockout mice [17]. Saquinavir also inhibited P-gp function in human lymphocytes [15] and insect cells [18]. P-gp induction [19] and inhibition [19, 20] by atazanavir have also been demonstrated *in vitro*. Because P-gp is expressed on lymphocyte plasma membranes, the cells targeted by HIV antiretrovirals, P-gp may limit the intracellular accumulation of PIs, rendering these drugs ineffectual and providing a sanctuary site for HIV [21].

There is a high degree of interpatient variability in PI pharmacokinetic and pharmacodynamic parameters [22, 23], and the occurrence of drug-related toxicities is difficult to foresee [24]. In order to predict PI efficacy or toxicity, physicians monitor plasma concentrations [25, 26], which are assumed to correspond to pharmacologically relevant intracellular concentrations. Both saquinavir and atazanavir have low levels of plasma protein binding (less than 15%, according to the package inserts), so unbound plasma concentrations range from 1 to 6 μM. A recent study by Colombo *et al.* demonstrated that saquinavir plasma and intralymphocytic concentrations correlated significantly ($r = 0.80$) [27]. However, there was considerable interindividual variability in the ratio between intracellular and plasma concentrations, with a coefficient of variation of 76% [27]. This suggests that there is substantial interindividual variation in the cellular "permeability" of saquinavir, which may be due to expression or functional differences in membrane transporters such as P-gp. Such variability in HIV antiretroviral pharmacokinetic and pharmacodynamic parameters may be influenced by polymorphisms in *ABCB1* or other genes [28].

Although the role of P-gp in PI pharmacokinetics and pharmacodynamics remains ambiguous, the effect of P-gp expression and function on antiretroviral response continues to be investigated. Conversely, few studies have explored the effect of PI exposure on lymphocyte P-gp *in vivo*. Studies have shown that oral administration of the antibiotic rifampicin upregulates intestinal P-gp via the transcription factor pregnane X receptor (PXR), limiting systemic xenobiotic exposure [29]. A similar phenomenon may occur when lymphocytes are exposed to PIs.

In the present study, we examined the effect of coadministration of the HIV PIs saquinavir and atazanavir on lymphocyte P-gp expression and function in healthy individuals. In addition, we determined if exposure to saquinavir or atazanavir *in vitro* resulted in a change in ABCB1 mRNA or P-gp expression in the CD4 cell line CEM, or if these drugs activate or inhibit P-gp function.

3.3. Materials and methods

Chemicals and reagents

Histopaque, cyclosporin A (CsA), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin-streptomycin, and RPMI-1640 cell culture medium were purchased from the UCSF Cell Culture Facility (San Francisco, CA). Calcein acetomethoxyester (calcein AM) was purchased from Molecular Probes (Eugene, OR). Mouse IgG2a-allophycocyanin (APC) secondary antibody was purchased from Caltag Laboratories (Burlingame, CA). The anti-human monoclonal P-gp antibody MRK16 was purchased from Kamiya Biomedical (Seattle, WA). The RNeasy Mini Kit was purchased from

Qiagen (Valencia, CA). TRIzol reagent and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). TaqMan buffer was purchased from the Genome Analysis Core (University of California, San Francisco). $MgCl_2$ and AmpliTaq Gold were purchased from Applied Biosystems (Foster City, CA) and dNTPs and M-MLV reverse transcriptase (RT) were purchased from Promega Corporation (Madison, WI). Saquinavir mesylate and atazanavir sulfate were purchased from the University of California, San Francisco Medical Center pharmacy.

Quantification of protease inhibitor effect on P-gp function

The human embryonic kidney cell line HEK293T was a kind gift from Dr. Warner Greene (Gladstone Institute, San Francisco, CA). Cell medium consisted of minimum essential medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 100 units/mL penicillin and 0.1 mg/mL streptomycin (UCSF Cell Culture Facility, San Francisco, CA). HEK293T cells at 70% confluency were transfected with 10 μg plasmid cDNA (ABCB1-pcDNA5/FRT or vector control) using 25 μL Lipofectamine 2000 (Invitrogen) per T-25 flask. Cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere for 24 hours. Cells were harvested and counted, and $3x10^5$ cells were incubated in 200 nM calcein AM (Invitrogen) ± 5 or 50 μM saquinavir or atazanavir (both from the UCSF Medical Center pharmacy) or 5 μM CsA (Sigma) for 30 minutes at 37ºC. Cells were collected by centrifugation, washed twice with ice-cold PBS, and incubated with 500 μg/mL P-gp antibody MRK16 (Kamiya Biomedical, Seattle, WA) for 30 minutes at 4ºC. Following two additional washes, cells were incubated with an APC-conjugated mouse IgG2a secondary antibody (Caltag, Burlingame, CA) at a concentration of 1.2 μg/mL for 30

minutes at 4ºC. Samples were washed twice and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Forward- and side-scatter signals were detected on a linear scale, and fluorescence was measured in channels 2 and 4 (FL-2, λ_{max} 585 nm and FL-4, λ_{max} 661 nm) on a logarithmic scale. Intracellular calcein fluorescence was evaluated in an $MRK16^+$ P-gp-expressing subpopulation; at least 5000 events were collected per sample.

Incubation of CEM cells with HIV protease inhibitors

The human T-lymphoblast CEM cell line was obtained from ATCC (Manassas, VA). Cell medium consisted of RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin. Cells were seeded at a density of $5x10^4$ cells/mL in the presence of 5 or 10 μM saquinavir or atazanavir, or 0.1% DMSO (vehicle control), and maintained at 37ºC in a humidified chamber in a 5% CO_2 atmosphere. Cell medium was replaced after 48 hours, and after 96 hours cells were harvested for use in P-gp expression assays or for RNA isolation.

Subjects and study procedures

Eighteen HIV-seronegative volunteers (nine male, nine female) between the ages of 18 and 65 were recruited for the ASPIRE II (Atazanavir-Saquinavir Pharmacokinetic Research Endeavor) study coordinated by the Pacific Horizon Medical Group. Four subjects did not complete the study due to protocol noncompliance. The study consisted of three subsequent 11-day arms of ritonavir- or atazanavir-boosted saquinavir, separated by 10-day washout periods. In the first arm, subjects received 1000 mg of saquinavir and

100 mg of ritonavir twice daily; in the second, 1000 mg of saquinavir and 200 mg of atazanavir twice daily; and in the third, 1500 mg saquinavir and 200 mg atazanavir twice daily. For pharmacokinetic purposes, plasma was collected on day 11 of each arm; pharmacokinetic results have been published elsewhere [30]. On day one of the first arm and day 11 of the third arm, blood samples were collected and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque. PBMCs were resuspended in cryopreservation medium (90% FBS, 10% DMSO) and stored in nitrogen vapor. The Pacific Horizon Medical Group Internal Review Board approved ASPIRE II, including the collection of lymphocytes for analysis of P-gp expression and function, and all subjects provided written informed consent prior to participation in this study. Analysis of ASPIRE II samples was approved by the University of California, San Francisco Committee on Human Research.

Quantification of lymphocyte P-gp expression

Cells were washed twice with PBS, then incubated in the P-gp antibody MRK16 at 167 μg/mL for 30 minutes at 4ºC. Following two washes with ice-cold PBS, cells were incubated with an APC-conjugated mouse IgG2a secondary antibody at 0.75 μg/mL for 30 minutes at 4ºC. Samples were then washed twice and analyzed on a FACSCalibur flow cytometer. Forward- and side-scatter signals were detected on a linear scale, and fluorescence was measured in channel 4 on a logarithmic scale. A homogeneous lymphocyte population was selected for data collection based on cellular light-scattering attributes; at least 5000 events were collected per sample.

RNA isolation and quantification of lymphocyte ABCB1 expression

RNA was extracted from cells using the RNeasy Mini Kit (primary lymphocyte samples) or TRIzol reagent (CEM samples) following the manufacturer's instructions (Qiagen, Valencia, CA and Invitrogen, respectively). RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and reverse-transcribed by M-MLV RT according to the manufacturer's instructions, using random hexamers as primers. Gene expression was measured by quantitative real-time PCR (TaqMan) on a PRISM 7700 Sequence Detection System (Applied Biosystems). Each reaction contained 1X TaqMan buffer (UCSF Genome Analysis Core, San Francisco, CA), 5.5 mM $MgCl_2$ (Applied Biosystems), 200 μM dNTP (Promega, Madison, WI), 0.625 U AmpliTaq Gold (Applied Biosystems), 500 nM each primer, and 200 nM probe in a final volume of 25 μL. Primers and probes were designed with the Primer Express 2.0 software (Applied Biosystems); sequences are shown in Table 3.1. Primers and probes were synthesized by Invitrogen and Integrated DNA Technologies (Coralville, IA), respectively. PCR conditions were 12 minutes at 95ºC, followed by 45 cycles of 15 sec at 95ºC and 1 min at 60ºC. All samples were normalized to expression of the human control gene β-glucuronidase (hGUS).

Table 3.1. Taqman primer and probe sequences. Abbreviations used include 6-FAM: 6-carboxyfluorescein; BHQ1: black hole quencher 1.

Gene	Oligonucleotide name	Oligonucleotide sequence (5' to 3')
ABCB1	Forward primer	TGATCATTGAAAAAACCCCTTTG
	Reverse primer	TCCAGGCTCAGTCCCTGAAG
	Probe	(6-FAM)-ACGGAAGGCCTAATGCCGAACACATT-(BHQ1)
hGUS	Forward primer	CTCATTTGGAATTTTGCCGATT
	Reverse primer	CCGAGTGAAGATCCCCTTTTTA
	Probe	(6-FAM)-TGAACAGTCACCGACGAGAGTGCTGG-(BHQ1)

Statistical analysis

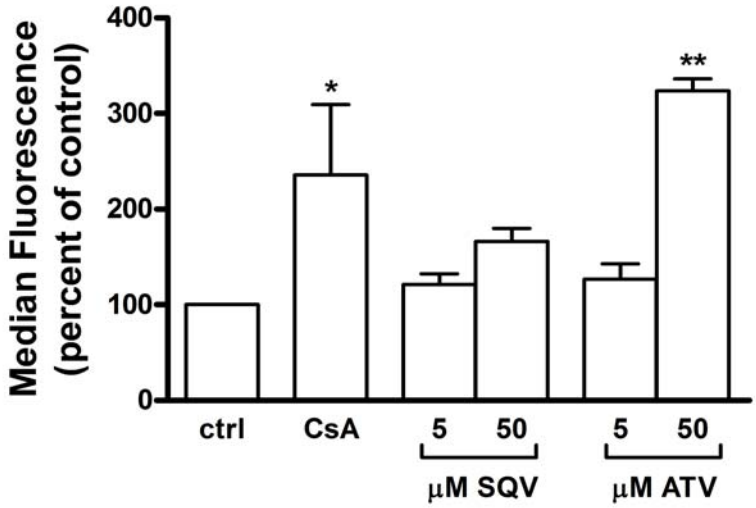
ABCB1 gene expression was normalized to hGUS and compared between baseline (first day of study arm 1) and treated (last day of study arm 3) samples. P-gp expression was compared between baseline and treated samples by considering the median fluorescence in FL-4. P-gp function was represented by the median intracellular fluorescence in FL-2. Statistical analyses of ABCB1 expression and P-gp expression and function in the inhibition and *in vitro* induction studies were performed using the non-parametric Kruskal-Wallis analysis of variance test, followed by the Dunn's multiple comparison test if significance was found in the Kruskal-Wallis test. ABCB1 expression and P-gp expression and function in the ASPIRE II primary lymphocyte samples was statistically analyzed using the Wilcoxon matched-pairs test. The level of significance for all statistical tests was $p = 0.05$. All statistical comparisons were performed using the Prism (GraphPad Software, San Diego, CA) software package.

3.4. Results

Inhibition of P-gp function by saquinavir and atazanavir in vitro

The effects of saquinavir and atazanavir on P-gp function were investigated in HEK293T cells transiently expressing ABCB1. Flow cytometry analysis showed that the addition of a pharmacological concentration (5 μM) of saquinavir or atazanavir slightly increased intracellular calcein AM fluorescence compared to control cells, although this did not reach statistical significance (Fig. 3.1). In the presence of 50 μM atazanavir, there was a significant increase in intracellular fluorescence (324 ± 12.4% of control, $P<0.01$), indicating inhibition of P-gp function. Cyclosporin A, a P-gp inhibitor, also increased intracellular fluorescence (236 ± 73.8% of control, $P<0.05$). A high concentration (50 μM) of saquinavir showed a trend towards increasing intracellular fluorescence, but this did not reach statistical significance. Both saquinavir and atazanavir demonstrated a trend towards inhibition of P-gp function.

Figure 3.1. Inhibition of P-gp function by saquinavir and atazanavir *in vitro*. HEK293T cells transiently expressing ABCB1 were incubated in 200 nM calcein AM ± 5 μM cyclosporin A (positive control) or 5 or 50 μM saquinavir (SQV, n=3) or atazanavir (ATV, n=3) for 30 minutes at 37ºC. Data were normalized to median fluorescence of cells not exposed to inhibitor (ctrl) and are expressed as mean ± S.D. * P<0.05, ** P<0.01 compared to controls.



Effect of protease inhibitor exposure on lymphocyte ABCB1 mRNA and P-gp surface expression in vitro

In order to determine whether saquinavir and atazanavir affected ABCB1 mRNA or P-gp levels *in vitro*, CEM cells were incubated with these drugs for 96 hours. Quantitative RT-PCR showed a 2.0-fold increase (±1.58, P<0.05) in ABCB1 mRNA expression in cells cultured in 5 μM saquinavir compared to vehicle control (Figure 3.2). Exposure to 10 μM atazanavir also increased ABCB1 mRNA expression (1.53 ± 0.54-

fold, $P<0.05$) compared to vehicle control. The small changes in ABCB1 mRNA levels in saquinavir- and atazanavir-treated CEM cells did not translate into changes in P-gp expression. Flow cytometry quantification of bound MRK16 antibody showed that P-gp expression levels were similar across all concentrations (Figure 3.3).

Figure 3.2. Effect of saquinavir and atazanavir on CEM ABCB1 mRNA expression. CEM cells were cultured in 5 or 10 μM saquinavir (SQV) or atazanavir (ATV) or 0.1% (v/v) DMSO vehicle control for 96 hours. ABCB1 mRNA levels were measured by quantitative RT-PCR (n=12). Data were normalized to the control gene hGUS and are expressed as the mean fold change compared to vehicle control (±SD). * P<0.05 compared to control.

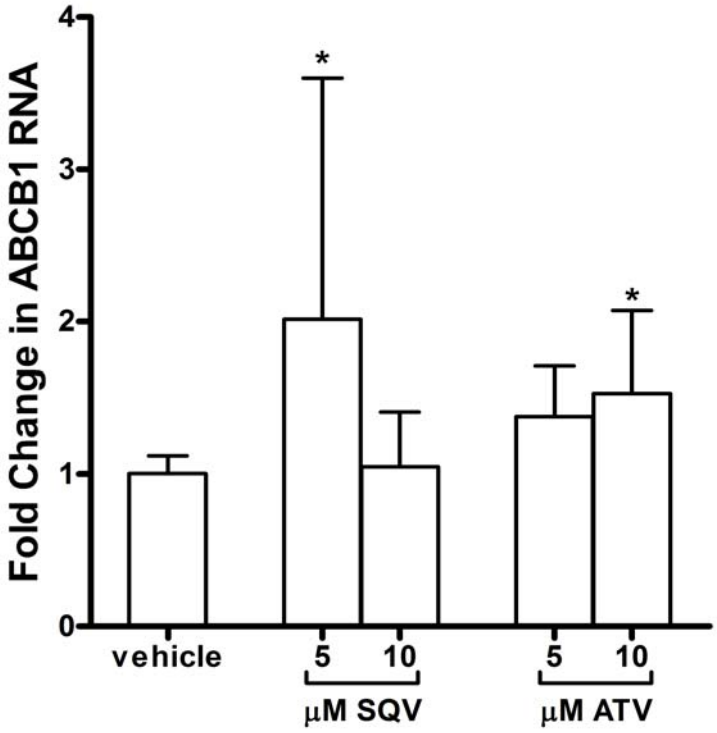
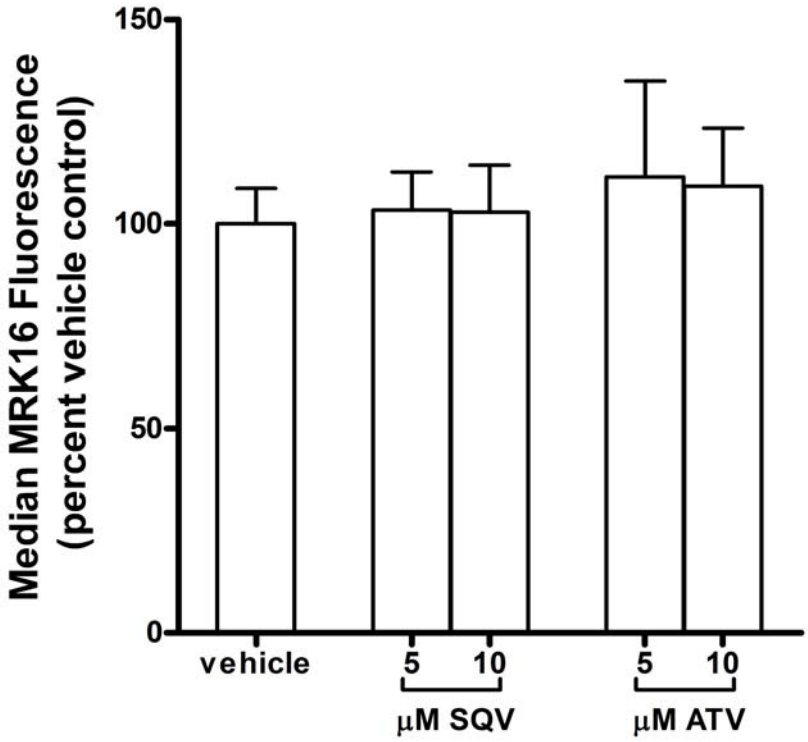


Figure 3.3. Effect of saquinavir and atazanavir on CEM P-gp expression. CEM cells were cultured in 5 or 10 μM saquinavir (SQV) or atazanavir (ATV) or 0.1% (v/v) DMSO vehicle control for 96 hours. P-gp levels were measured by flow cytometry (n=12). Data were normalized to the vehicle control and are expressed as median MRK16 fluorescence normalized to vehicle control (±SD). * P<0.05 compared to control.



Effect of protease inhibitor administration on lymphocyte ABCB1 mRNA and P-gp surface expression in vivo

The demographics of the ASPIRE II population are shown in Table 3.2. All subjects were healthy, HIV-seronegative, had no prior exposure to antiretrovirals and were not currently taking any inducers or inhibitors of P-gp or cytochrome P450

enzymes. The majority of the study population (12 of 14 subjects) was Caucasian. To ascertain whether atazanavir-boosted saquinavir affected ABCB1 mRNA lymphocyte expression in this population, a quantitative RT-PCR analysis was performed. ABCB1 expression was measured in lymphocyte samples obtained before and after treatment; data are expressed as a fold change from the pre-treatment level (Figure 3A). There was a 1.3 ± 0.4-fold increase in ABCB1 mRNA levels after PI exposure. Intersubject variability in response to PI treatment was substantial, with ABCB1 RNA levels increasing from 0.5-1.9-fold over baseline levels.

Table 3.2. ASPIRE II study population demographics.

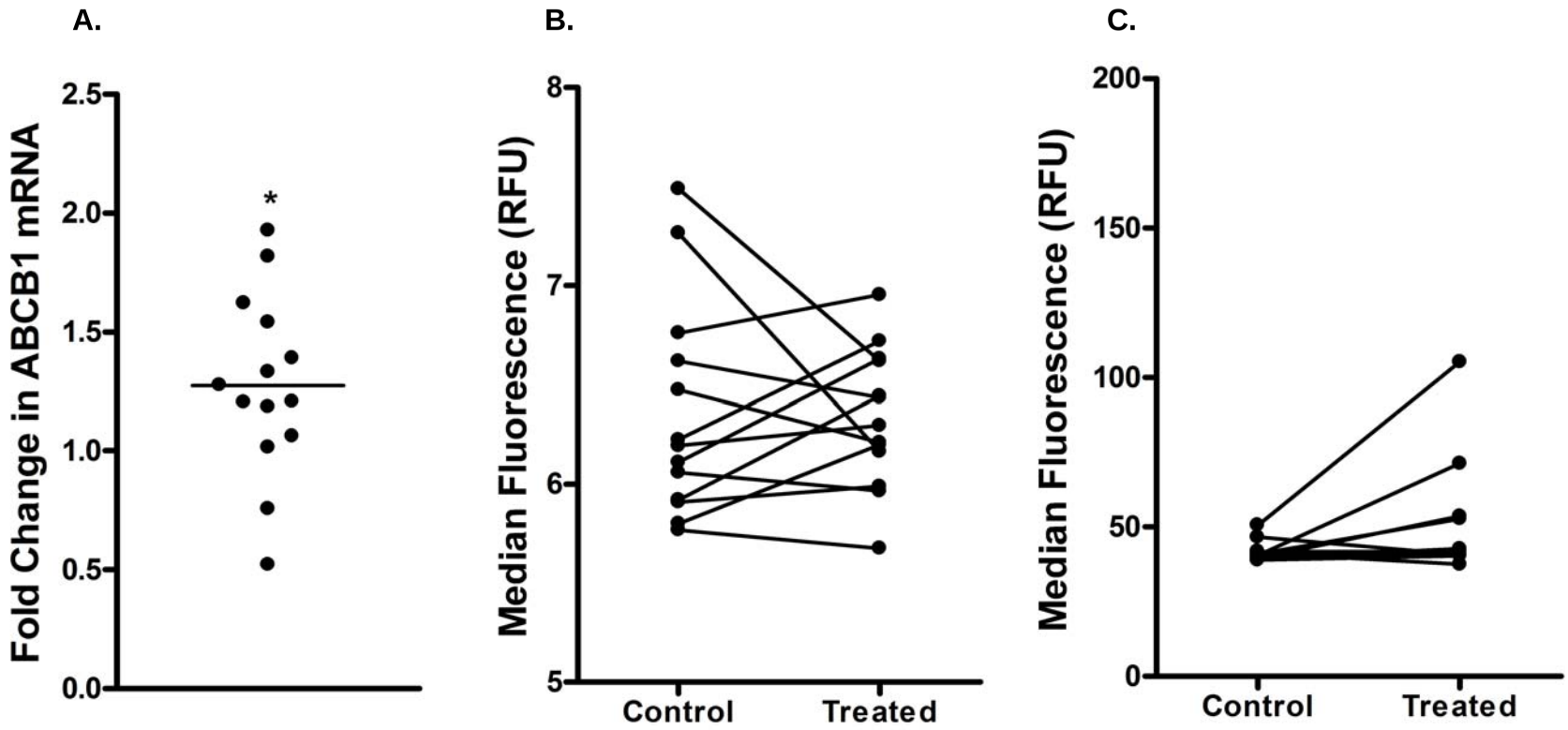
	ASPIRE II subjects (n=14)
Mean age (years), (SD)	36.9 (8.1)
Sex, *n* (%)	
Male	6 (43%)
Female	8 (57%)
Race, *n* (%)	
European-American	12 (86%)
Hispanic	1 (7%)
Other	1 (7%)
Weight (kg), mean (SD)*	
Baseline	74.3 (14)
Treated	75.9 (14)

* p=0.02 for weight before and after treatment (paired t-test)

The effect of PI administration on lymphocyte P-gp expression was examined through flow cytometric quantification of cell-surface protein. Fluorescence of the APC-conjugated P-gp-specific antibody was measured in lymphocyte samples collected before

and after atazanavir-boosted saquinavir administration. The median fluorescence of the total lymphocyte population is shown as relative fluorescence units (RFU) (Figure 3B). No change in mean expression was detected after PI treatment, although P-gp expression increased ~30% in two subjects. The effect of PI administration on the P-gp-positive subpopulation of lymphocytes was also not significant (Figure 3C). The median fluorescence of all subjects increased by an average of 21% (41.5 ± 3.38 RFU before treatment, compared to 50.2 ± 18.9 RFU after treatment), although this change does not reach statistical significance (P=0.058). As with the analysis of the total lymphocyte population, there is a significant intersubject variability in the response to PI treatment, with some subjects showing a nearly 2-fold increase in lymphocyte P-gp levels.

Figure 3.4. Effect of oral atazanavir-saquinavir administration on lymphocyte ABCB1 and P-gp expression *in vivo*. ABCB1 mRNA and protein levels were measured in lymphocytes obtained from healthy subjects (n=14) before and after an 11-day regimen consisting of 1500 mg saquinavir and 200 mg atazanavir taken twice daily. ABCB1 mRNA levels are expressed in a scatterplot as the fold change in ABCB1 mRNA expression after ATV-SQV treatment, where a value of zero indicates no change (A). Each point represents a single subject, and the horizontal line corresponds to the mean. * $P<0.01$ (Wilcoxon signed-rank test). Surface P-gp expression was measured by the MRK16 fluorescence intensity of lymphocytes (B and C). Individual data are shown before and after treatment, with each point representing a single subject. Data are expressed as (B) the median MRK16 fluorescence intensity of the total lymphocyte population of each subject, and (C) the median fluorescence intensity of the P-gp-positive subpopulation for each subject. No statistical significance was found between the control and treated groups in either of these analyses (Wilcoxon matched pairs test).


A.
Fold Change in ABCB1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
*
B.
Median Fluorescence (RFU)
8
7
6
5
Control
Treated
C.
Median Fluorescence (RFU)
200
150
100
50
0
Control
Treated

3.5. Discussion

The results of the present study indicate that the HIV PIs saquinavir and atazanavir interact with and affect expression of the xenobiotic transporter P-gp to a limited extent *in vivo*. P-gp transport of saquinavir has been previously demonstrated, both in cell culture systems [31-33] and in animal studies [34]. Several groups have also reported that saquinavir inhibits P-gp function [15, 18]. Atazanavir has also been shown to have both inhibitory [19, 20] and inductive effects [19]. However, few studies have examined the effect of PIs on ABCB1/P-gp *in vivo*.

In the current study, 5 μM saquinavir and atazanavir exhibited a trend towards P-gp inhibition. These concentrations are similar to the peak plasma concentrations at steady state in patients receiving these drugs (Invirase and Reyataz package inserts; Roche and Bristol-Myers Squibb, respectively). At higher concentrations, saquinavir and atazanavir inhibited P-gp function to a greater extent. These findings may be of clinical importance if these drugs are administered to a patient who is also receiving xenobiotics that alter PI metabolism, as this may lead to increased plasma concentrations of the PIs.

The effect of PIs on ABCB1 expression may also be an important factor in patient response to HIV antiretrovirals, since an increase in lymphocyte P-gp expression may decrease the target cell drug concentration. In 2003, Chandler *et al.* found that a three-day incubation with non-cytotoxic levels of saquinavir did not upregulate lymphocyte P-gp *in vitro* [35], although in a subsequent study Dupuis *et al.* reported that prolonged exposure to saquinavir increased lymphocyte P-gp expression *in vitro* [36], Two studies from Perloff *et al.* demonstrated that a four-day exposure to saquinavir or a three-day exposure to atazanavir increased P-gp levels in an ABCB1-overexpressing cell line [19,

37]. It has been proposed that this P-gp upregulation is caused by the nuclear receptor PXR, for which saquinavir is a weak ligand [38]. PIs may act via this regulatory pathway to generate an increase in lymphocyte P-gp expression, since the presence of PXR has previously been observed in lymphocytes [39-41].

In order to study the inductive properties of saquinavir and atazanavir, we cultured CEM lymphoblast cells in saquinavir and atazanavir for four days. Quantitative RT-PCR results showed that exposure to 5 μM saquinavir and 10 μM atazanavir increased ABCB1 mRNA expression compared to vehicle control by at most two-fold. The relatively small magnitude of the overall change in CEM ABCB1 mRNA expression following exposure to therapeutic concentrations of these PIs suggests that these findings may be of little clinical relevance.

Observed changes in ABCB1 mRNA expression in CEM cells following saquinavir and atazanavir exposure did not translate into P-gp surface expression. This is in accordance with previous findings from Chandler *et al.* [35] P-gp levels may not have changed after incubation in saquinavir and atazanavir because of the comparatively short period of cellular exposure to these drugs. Examination of P-gp expression during continuous exposure to PIs may provide more information about the potential for P-gp upregulation in response to atazanavir or saquinavir treatment. It is also likely that the comparatively small changes in CEM ABCB1 mRNA levels are not large enough to significantly affect P-gp levels.

Lymphocytes obtained from ASPIRE II subjects were used to determine the effects of atazanavir-boosted saquinavir on ABCB1 expression and function in healthy individuals. A large interindividual variability was observed in baseline ABCB1 mRNA

expression, as well as in the change in ABCB1 mRNA expression after atazanavir-saquinavir treatment. Overall, qRT-PCR analysis suggested that ABCB1 expression increased slightly (approximately 1.2-fold) after administration of atazanavir and saquinavir.

In accordance with our *in vitro* results, this slight upregulation did not translate into an increase in lymphocyte P-gp expression, either with respect to the total lymphocyte population or in only that subset which expresses P-gp. However, the overall mean of the median fluorescence intensities of the FL-4^+ lymphocyte subpopulations from each subject showed a trend towards upregulation – in effect, the amount of cell surface P-gp increased slightly in the lymphocytes that were positive for P-gp antibody staining. This small overall increase was primarily due to several outliers who showed significant increases in P-gp expression after saquinavir and atazanavir administration, which may indicate increased sensitivity to regulatory stimuli in a subset of individuals, possibly due to polymorphisms in the *ABCB1* promoter region or in nuclear receptors such as PXR.

It should be noted that the effect of HIV infection on lymphocyte ABCB1 expression was not addressed in this study, since all study participants were HIV-negative. Previous studies have addressed this question, with differing results. Andreana *et al.* demonstrated that the proportion of lymphocytes expressing P-gp was higher in HIV-infected subjects than in uninfected controls, and that this proportion increased with disease progression, although the increase in P-gp expression did not correlate with increased function [42]. However, Meaden *et al*. showed that lymphocyte P-gp expression was reduced in HIV-infected subjects compared to controls [43]. The results

of both studies have been reproduced by several groups [44-46]. Our analyses cannot rule out the possibility that the effects of atazanavir-saquinavir on lymphocyte ABCB1 expression observed in the ASPIRE II population may differ in an HIV-infected population.

The high degree of interindividual variability which we observed in baseline lymphocyte ABCB1 expression can be attributed in large part to genetic and environmental factors. Genetic polymorphisms in *ABCB1* have been identified through comprehensive SNP discovery efforts [47]; studies are ongoing to correlate these polymorphisms with P-gp expression and function. Subjects are excluded from a clinical study if they are exposed to substances known to influence ABCB1/P-gp expression, thus controlling for environmental factors to the greatest practical extent. In a clinical setting, however, it is much more difficult to regulate a patient's environment, leading to variability in drug pharmacokinetics. Genetic factors can also affect drug pharmacokinetics and pharmacodynamics.

Previous studies have demonstrated considerable interpatient variability in saquinavir pharmacokinetics [23, 48-50]; A 2005 report examined ritonavir-boosted saquinavir pharmacokinetics in HIV-seropositive patients in Thailand and the UK in three separate studies and found an average coefficient of variation for saquinavir C_{min} of approximately 86%, with a number of subjects (ranging from 14-50%) having trough concentrations below the minimum effective concentration of 0.1 mg/L [51]. Interestingly, study site was found to significantly correlate with saquinavir AUC, suggesting that ethnic factors influence saquinavir pharmacokinetics. These factors may be environmental or genetic, or a combination of both [51]. It is possible that

polymorphisms in genes in the saquinavir pharmacokinetic or pharmacodynamic pathways, including transporters such as P-gp, influence how a patient responds to saquinavir treatment. The avoidance of excessively low saquinavir plasma levels may increase virologic suppression and immunologic response in certain patients [52], although plasma levels are not the sole determinant of saquinavir efficacy [53], The use of pharmacogenetic markers to predict which patients may require higher dosages may considerably advance HIV treatment.

The findings from the present study indicate that atazanavir-boosted saquinavir exposure may upregulate ABCB1 expression in some individuals. The large interindividual variability in the change in transporter expression after atazanavir-saquinavir treatment may be caused by genetic variation, either in the *ABCB1* gene, its regulatory partners, or in other genes involved in the disposition of these drugs (e.g. uptake and/or other ABC transporters, metabolizing enzymes or plasma binding proteins). Although it is clear that a high degree of interindividual variability exists in both basal transporter expression and in drug response, the source of this variability is at present speculative. The identification of these sources of variability will allow clinicians to more efficiently optimize HIV antiretroviral therapy on an individual basis.

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Chapter 4. Pharmacogenetics of Antiretroviral Therapies

4.1. Abstract

While scientific understanding of the biological and clinical effects of HIV infection has advanced significantly since its identification nearly three decades ago, those infected with HIV continue to experience elevated mortality rates, and response to antiviral drugs varies both among and within populations around the world. In this chapter, we investigated the hypothesis that polymorphisms in genes involved in the pharmacokinetics and pharmacodynamics of these drugs influence HAART response in HIV patients. In particular, we examined polymorphisms in the genes encoding the drug metabolizing enzyme cytochrome P450 2B6 (*CYP2B6*), the drug transporters P-glycoprotein (*ABCB1*) and MRP4 (*ABCC4*), and the cytokine tumor necrosis factor-alpha (*TNF*α) cytokine were investigated in HIV-positive populations in San Francisco (REACH) and Uganda (UARTO). No significant associations were found between HAART response and any of these genotypes. We also characterized the ancestral admixture of the REACH population, which we compare to self-reported ethnicity later in this chapter.

4.2. History of HIV/AIDS and antiretroviral therapy

In 1981, reports describing cases of rare illnesses such as *P. carinii* pneumonia and Kaposi's sarcoma in young homosexual men began to appear in the medical literature [1, 2]. Similar cases were also observed in intravenous drug users and people with hemophilia [3, 4]. These illnesses were associated with profound

immunosuppression, and researchers suspected that this immunodeficiency was caused by a virus which was likely carried in peripheral blood cells. In September of 1982, the CDC released a publication entitled, "Update on acquired immune deficiency syndrome" [5]. In this report, AIDS received both a definition and a name, enabling researchers to begin to study the epidemiology of the disease.

In response to the rapid increase in diagnoses and the mortality rate of those with AIDS, research in the field moved quickly. The virus responsible for AIDS was first isolated in 1983 [6]; the following year, two groups in San Francisco demonstrated that this virus was responsible for AIDS [7, 8]. By 1987, the life cycle of the HIV retrovirus had been established, and various stages of this cycle were being targeted with different compounds in the search for effective antiviral drugs [9].

The first of these, azidothymidine (AZT, zidovudine), had initially been synthesized as an anticancer drug in 1964 [10]. Twenty years later, Burroughs Wellcome entered into a collaboration with the National Cancer Institute to screen anticancer compounds, including AZT, for anti-HIV activity. In 1985, researchers at the NCI discovered that AZT protected human T-cells from infection following *in vitro* exposure to HIV isolates [11]. Following these positive findings, the first AIDS patients received AZT in a randomized trial in February of 1986 [12]. By September of that year the mortality rate in the placebo group was about 14%, while the corresponding rate in the treatment group was less than 1% [12]. In 1987, AZT was the first anti-HIV drug to be approved by the FDA, marketed by Burroughs Wellcome under the trade name Retrovir.

While AZT was efficient at inhibiting replication of the HIV-1 retrovirus *in vitro*, it was most effective *in vivo* when it was given in combination with other antivirals. The

first combination therapies, now referred to as highly active antiretroviral therapy or HAART, became widely available in 1996 [13]. Shortly thereafter, the rate of AIDS-related mortality began to decrease, and then to plateau. It is evident from Figure 4.1 that the growth in the number of HIV antiviral medications available to patients corresponds to the precipitous drop in the AIDS death rate in the United States. Education was also an important factor in controlling the spread of HIV; the number of AIDS diagnoses in the U.S. per year peaked in the early 1990s and steadily decreased for the next few years [14] (Fig. 4.1). Unfortunately, the rate of AIDS-related mortality in sub-Saharan Africa has been slower to decline due to limited access to treatment options and the lack of education about preventing the spread of HIV [15] (Fig. 4.2).

Figure 4.1. The incidence of AIDS diagnoses and deaths in the U.S, in relation to the number of FDA-approved anti-HIV drugs. Data are from the CDC HIV/AIDS Surveillance Reports from 1993 and 2001 [14] and from drug package inserts from various manufacturers.

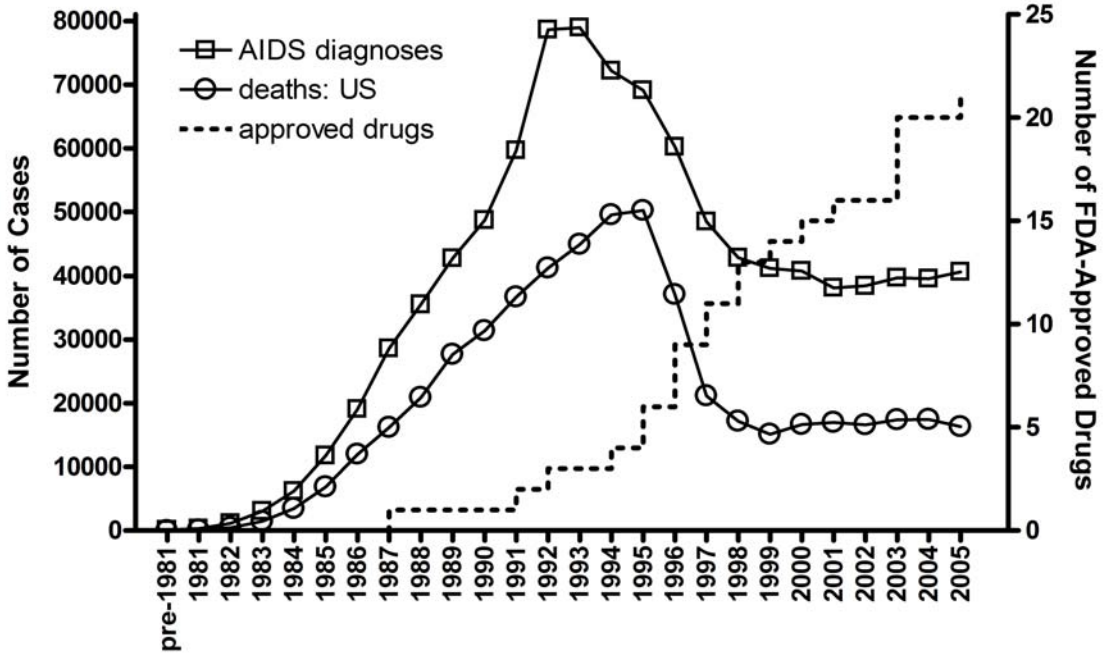
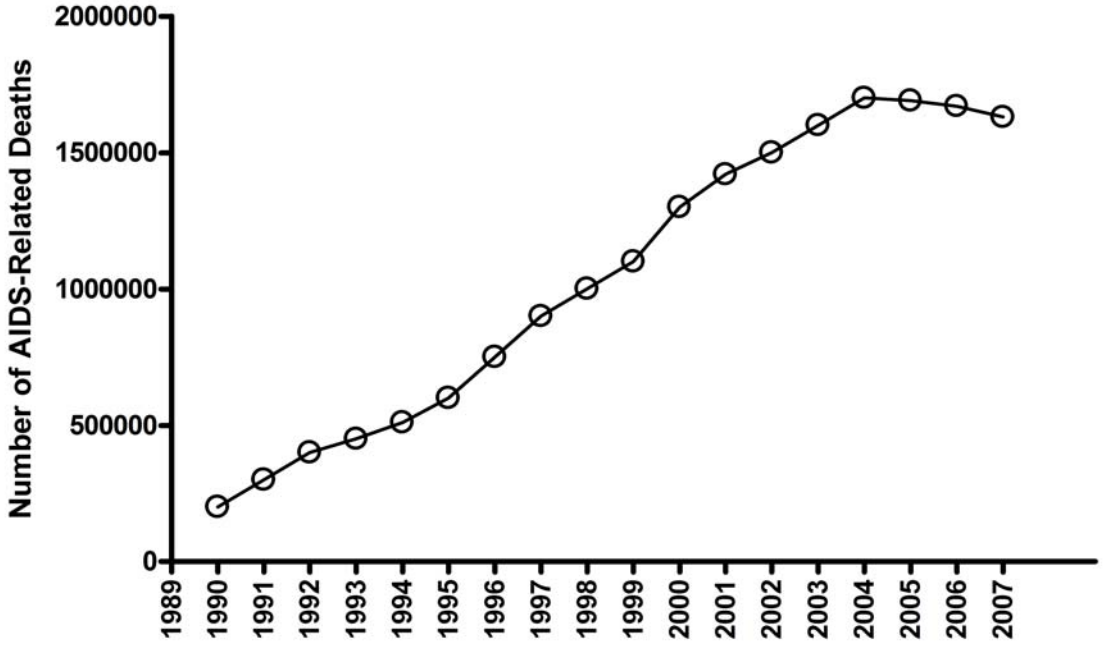


Figure 4.2. **Estimates of the number of AIDS-related deaths in sub-Saharan Africa.** Estimates were taken from the UNAIDS "Global summary of the AIDS epidemic 2007" [15].



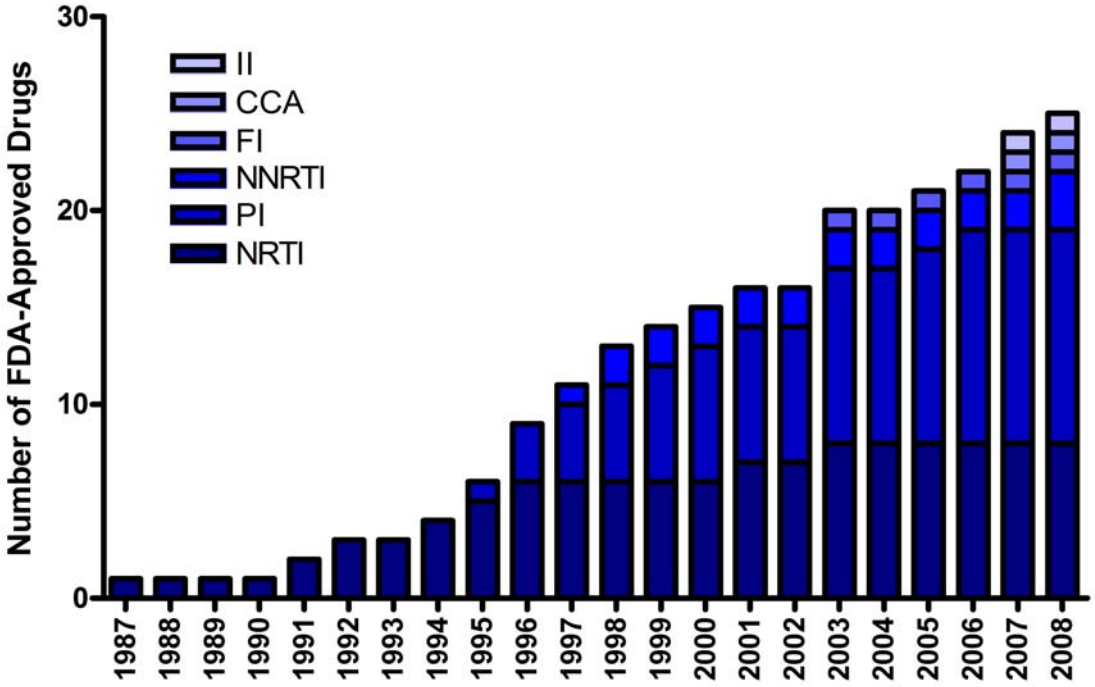
Soon after the advent of HAART, it became apparent that one of the unforeseen issues with combination therapies was adherence [16]. HAART regimens, while possessing potent antiviral activity, also had unpleasant side effects as well as complicating dosing schema [17]. Reduced adherence was shown to be associated with an increased risk of therapy failure due to the development of drug resistance [18], and clinicians began to develop strategies to predict the most effective HAART regimen to which a patient would have a high degree of adherence [19]. Viral genotyping for pre-existing resistance mutations has become a routine part of this process as well [20].

Further complicating the individualized optimization of HAART is the role of natural variation in the host genome. In 1996, researchers demonstrated that individuals with a 32 base pair deletion in the CCR5 (chemokine receptor 5) gene, which encodes for a receptor on the surface of CD4 cells to which HIV-1 binds, protected against infection [21]. With the completion of the sequencing of the human genome, the existence and extent of genetic variation has been determined, and publications about the effects of variants on HAART efficacy and toxicity have become frequent occurrences [22].

In the meantime, the number of FDA-approved anti-HIV therapies has continued to increase, and the range of pharmacological targets against which the drugs act has widened considerably, as seen in Figure 4.2. The traditional components of HAART – protease inhibitors (PIs), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), and non-nucleoside reverse transcriptase inhibitors (NNRTI) – are still used routinely, but other classes of antivirals – fusion inhibitors (FIs), chemokine coreceptor antagonists (CCAs), and integrase inhibitors (IIs) – are in development, and have, in some cases, reached the market (Fig. 4.3) [23]. With each drug that receives FDA approval, a new

group of genes comes under scrutiny for their role in the pharmacokinetics and pharmacodynamics of that drug; accordingly, polymorphisms in those genes are examined for associations with drug efficacy and toxicity in a search for host genotypes which could factor into the design of a personalized HAART regimen.

Figure 4.3. Number of drugs approved by the FDA for the treatment of HIV by year. Drugs are subdivided by category: integrase inhibitor (II), chemokine coreceptor antagonist (CCA), fusion inhibitor (FI), non-nucleoside/nucleotide reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), and nucleoside/nucleotide reverse transcriptase inhibitor (NRTI).



One such example of a clinically relevant pharmacogenetic association is the case of abacavir hypersensitivity. Abacavir, marketed under the brand name Ziagen by GlaxoSmithKline, is a nucleoside inhibitor which was approved by the FDA in 1998. Approximately 5% of patients enrolled in clinical trials experienced hypersensitivity to abacavir, which is often characterized by fever and rash but can become life-threatening [24]. In 2002, researchers discovered that an allele of the major histocompatibility complex (MHC), HLA-B*5701, predicted the incidence of abacavir hypersensitivity [25]. The widespread implementation of a genetic test for this variant has reduced the frequency and cost of treating abcacavir hypersensitivity [26], encouraging the search for other clinically relevant genetic polymorphisms. Pharmacogenetic studies are currently being conducted around the world, since genetic variation may be present in a specific ethnicity, and positive findings could greatly improve the current standard of care even in countries lacking access to advanced technologies. A preliminary examination of several candidate polymorphisms which may influence various HIV phenotypes is described in this chapter, with a focus on polymorphisms in genes which are involved in the pharmacokinetic and pharmacodynamic pathways of HIV antiretroviral therapies.

The NNRTIs nevirapine and efavirenz were approved by the FDA in 1996 and 1998, respectively. Unlike the NRTIs, which inhibit the HIV-1 reverse transcriptase by binding its substrate site, the NNRTIs interact with a non-substrate binding site approximately 10 Å away [27], locking the catalytic site in an inactive conformation [28]. HIV-1 resistance to NNRTIs is often characterized by the development of mutations lining the NNRTI-binding site [29] and is a limitation of long-term NNRTI treatment [30]. However, HAART regimens with NNRTI backbones are becoming increasingly

common as first-line treatment, due to their lower toxicity profile and efficacy in NNRTI-naïve HIV-positive patients [31]. The NNRTIs efavirenz and nevirapine, commonly used in HAART regimens, both undergo metabolism by cytochrome P450 2B6 (CYP2B6) to inactive metabolites. Genetic variants in *CYP2B6*, particularly the *6 haplotype, have been associated with decreased enzymatic expression and function [32], and carriers of the *CYP2B6* 516 G>T variant allele (a marker for the *6 haplotype) exhibit decreased NNRTI plasma levels compared to reference homozygotes [33]. We hypothesize that as a result of decreased metabolism, individuals with the *CYP2B6* 516T allele experience higher NNRTI plasma levels, leading to favorable viral and immunological response outcomes following HAART exposure. We examine this hypothesis in this chapter.

While polymorphisms in *CYP2B6* may influence antiretroviral pharmacokinetics, polymorphisms in ABC transporters are thought to affect antiretroviral pharmacodynamics. The role of ABC transporters in the protection of lymphocytes from potentially toxic xenobiotic substrates has been characterized in a number of reports. Leukemia outcomes (treatment response, remission, survival) have been correlated with transporter expression levels on lymphocytes [34-36]. Other researchers have demonstrated associations between ABC transporter polymorphisms, especially *ABCB1* 2677G>T/A and 3435C>T and transporter expression levels [37-39]. More recently, polymorphisms in *ABCB1* and *ABCC4* have been associated with immunologic and viral HAART response and intralymphocytic NRTI concentrations [40, 41]. As efflux transporters, genetic polymorphisms having deleterious effects on these proteins would correspond with elevated intracellular drug levels, which in turn would lead to improved HAART response and higher occurrence of toxicities. In this chapter, we describe our

findings regarding pharmacogenetic associations between ABC transporters and HIV antiretroviral response.

Like the genetic variants in ABC transporters, polymorphisms in tumor necrosis factor alpha (*TNFα*) may also influence HIV antiretroviral pharmacokinetics. *TNFα* was initially described as an endotoxin-induced serum factor which was able to cause the necrosis of tumors [42]. Further characterization revealed that it is secreted by monocytes in response to inflammatory stimuli such as viruses and immune complexes, and that it has cytotoxic effects on transformed and virus-infected cells and tumor endothelial cells [42]. Researchers have shown that the activation of NFκB by TNFα increases the rate of HIV-1 transcription in infected monocytes and T cells [43]. TNFα is also important during immune response to HIV infection, in which it is involved in the apoptosis of antigen-specific cells following an initial T cell expansion [44]. For both of these reasons, the secretion and function of TNFα could influence the immune response to HAART in HIV patients. Previous studies have investigated the effects of polymorphisms in genes involved in T cell proliferation and apoptosis to determine if they could predict immunologic response or nonresponse and discovered an association with a promoter polymorphism in *TNFα*, -488G>A [44]. In this chapter we describe the results of a tag SNP study which we undertook to determine if *TNFα* polymorphisms are associated with response to HIV antiretroviral therapy.

Besides pharmacogenetic factors, other issues may also influence HIV virologic and clinical outcomes, confounding pharmacogenetic associations. These may be non-genetic influences, such as medication adherence or environmental exposure to certain substances that may regulate the expression of drug metabolizing enzymes or drug

transporters. Another possible confounding factor is population substructure, or the existence of unidentified subpopulations (often ethnic) which may have distinct genetic characteristics which seem to be associated with the phenotype in question. In this chapter, we also investigate the population admixture in a group of HIV-positive individuals in San Francisco who are involved in the REACH study. Characterization of admixture is accomplished through the genotyping and analysis of a panel of ancestry informative markers (AIMs). These data will provide more information about population substructure in REACH, which can be taken into account in future pharmacogenetic studies.

Our examinations of the effects of polymorphisms in genes encoding proteins which influence HIV antiretroviral pharmacokinetics and pharmacodynamics, including the drug metabolizing enzyme CYP2B6, the ABC transporters P-glycoprotein and MRP4, and the cytokine TNFα, will shed light on whether or not these genetic variants affect the response of HIV patients to antiretroviral medications.

4.3. Methods

Study populations

For these studies, two populations were used, representing different genetic, socioeconomic, and environmental factors; both of these populations were assembled by David Bangsberg, M.D. and his associates. The first, REACH (REsearch in Access to Care in the Homeless), is a group of indigent HIV-positive patients who are receiving medical treatment in San Francisco. At any one time, there are approximately 500 subjects enrolled in REACH who are evaluated quarterly for various data, including viral

load and CD4 count, adherence, and incidence of side effects. The second population, UARTO (Uganda AntiRetroviral Treatment Outcomes), is based in Mbarara and Kampala, Uganda and represents a population of HIV-positive drug naïve individuals who are receiving generic HAART regimens. At the time of the pharmacogenetic analyses described below, there were approximately 300 subjects for whom we had available DNA and phenotypic data.

DNA extraction

While DNA had previously been extracted from blood and stored for REACH subjects, DNA was isolated from saliva samples for the UARTO cohort. Samples were collected in Oragene discs (DNA Genotek, Ottawa, Ontario, Canada) and DNA was extracted from 0.5 ml saliva/stabilizer solution according to the manufacturer's protocol. Briefly, the Oragene disc was incubated overnight in a water bath at 50˚C, then cooled to room temperature. An aliquot (0.5 ml) was transferred to a 1.5 ml microcentrifuge tube, and 20 µl of Oragene DNA purifier was added to the sample. The tube was vortexed and incubated on ice for 10 min, then centrifuged at 15,000x*g* for 10 minutes. Supernatant was transferred into a clean 1.5 ml microcentrifuge tube. Ethanol (0.5 ml) was added and the sample was inverted 10 times, then incubated at room temperature for 10 min to allow for nucleic acid precipitation. The sample was centrifuged for 5 min at 15,000x*g* and the pellet was resuspended in TE buffer (Mediatech, Manassas, VA) and incubated overnight at room temperature. DNA was quantified on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and diluted to a concentration of 10 ng/µl in TE buffer.

Tag SNP selection for TNFα

Tagger, a haplotype-based method which increases tagging efficiency and coverage compared to genotype-based tests [45], was used to select tag SNPs that would cover the extent of genetic variation in the *TNFα* region [46]. Tagger conditions were designed to select tag SNPs which would cover polymorphisms in the HapMap Yoruban population with greater than 1% minor allele frequency with a power of 0.8 in the genomic region 31,650,800-31,655,000 on chromosome 6, surrounding the *TNFα* gene (GoldenPath May 2004 build [47]).

Genotyping

Genotyping was performed in 430 REACH and 277 UARTO subjects using commercially available TaqMan assays (Applied Biosystems, Foster City, CA) for *CYP2B6* 516G>T (rs3745274), *ABCC4* 4131T>G (rs3742106), *ABCB1* 1236C>T (rs1128503) and 3435C>T (rs1045642), and four polymorphisms in *TNFα* (rs1800750, rs1800629, rs3093662, and rs3093669). Additionally, two primer and probe sets designed by Travis Taylor (Genomics Core Facility, UCSF) were used to genotype the triallelic 2677 G>T/A polymorphism in the *ABCB1* gene (rs2032582). Ten ng of DNA were used per 5 μl reaction with 0.5X primer/probe and 1X TaqMan Universal PCR Master Mix, No AmpErase: UNG buffer (Applied Biosystems). Reaction conditions consisted of a 10 min hold at 95˚C, followed by 40 cycles of 15 sec at 92˚C followed by 1 min at 60˚C. Amplification was performed in 384-well plates and fluorescence was measured on a 7900HT system (Applied Biosystems). Genotypes were automatically

called with 95% confidence or higher by SDS version 2 (Applied Biosystems). Negative controls and random sample duplicates were included on each plate for quality control purposes. All SNPs were tested for deviation from Hardy-Weinberg equilibrium using the Chi-squared test.

Statistical analysis

Both contingency tables and multivariate logistic regressions were employed to measure associations between genotypes and clinical phenotypes. Analyses were performed only on subjects for whom all data were available, including baseline viral load and CD4+ cell count, genotype, and adherence measurements. Genotypes were analyzed both as an ordinal variable (i.e. number of copies of the variant allele) and as a nominal variable. The level of statistical significance was set at $p=0.05$.

Ancestry Informative Marker genotyping and analysis

To infer the population substructure in REACH, we used a program called Structure, which uses a model-based clustering method on multi-locus genotype data [48]. The input data for our model included genotypes for 112 SNPs, selected as ancestry informative markers (AIMs) because their minor allele frequencies differed by at least 0.5 between one or more designated ethnic groups [49]. These SNPs were genotyped in 335 REACH subjects who reported at least one grandparent of African, Hispanic, or Native American ancestry, or who did not report their ethnicity. Genotyping was carried out by the Functional Genomics Core at the Children's Hospital Oakland Research Institute, on the Illumina Golden Gate platform [50]. Three groups of DNA from

ethnically identified individuals (African American, European American, and Native American [49]) were used to identify subpopulations which were detected by Structure.

4.4. Results

4.4.1. Genotyping results

Representative data generated from the *CYP2B6* 516G>T genotyping assay are shown in Figure 4.4. Observed minor allele frequencies are graphed in Figure 4.5 alongside expected minor allele frequencies predicted by the Hardy-Weinberg principle. The minor allele frequencies in both REACH and UARTO were in Hardy-Weinberg equilibrium. The minor allele frequency in the UARTO population was similar to a report from Zanzibar, also in East Africa [51], but differed from minor allele frequencies in populations from West Africa [52-54] (Fig. 4.6).

The effects of admixture in the REACH population on *CYP2B6* 516G>T minor allele frequencies were evident when data were sorted by self-reported ethnicity: REACH subjects who reported at least three grandparents of African ancestry had a minor allele frequency similar to the HapMap African American population and to the HapMap Yoruban population from Nigeria (Fig. 4.6), in accordance with historical accounts and genetic studies demonstrating that the majority of African American lineages have West African ancestry [55].

Figure 4.4. Representative output from the *CYP2B6* 516G>T genotyping assay. Individuals with the *CYP2B6* 516TT variant genotype are in red, heterozygotes are in yellow, and reference homozygotes are shown in green. The two axes represent the relative fluorescence of two fluorophores, each representing a homozygous genotype, in an assayed DNA sample.

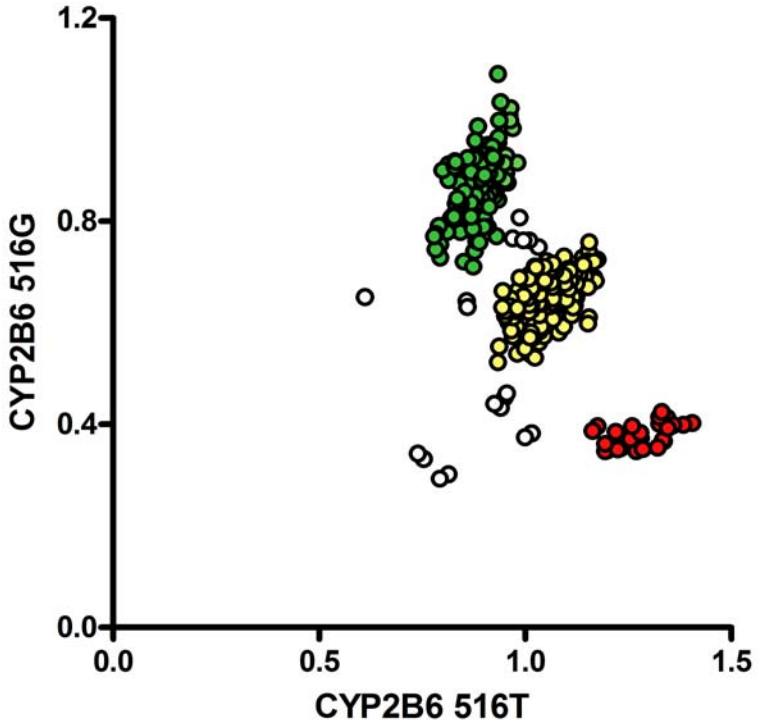


Figure 4.5. *CYP2B6* 516G>T genotype frequencies in REACH and UARTO. The numbers of subjects with GG, GT, or TT genotypes are shown below (observed). The genotype frequencies for both populations were in accordance with the Hardy-Weinberg principle (expected) as calculated by Chi-squared tests.

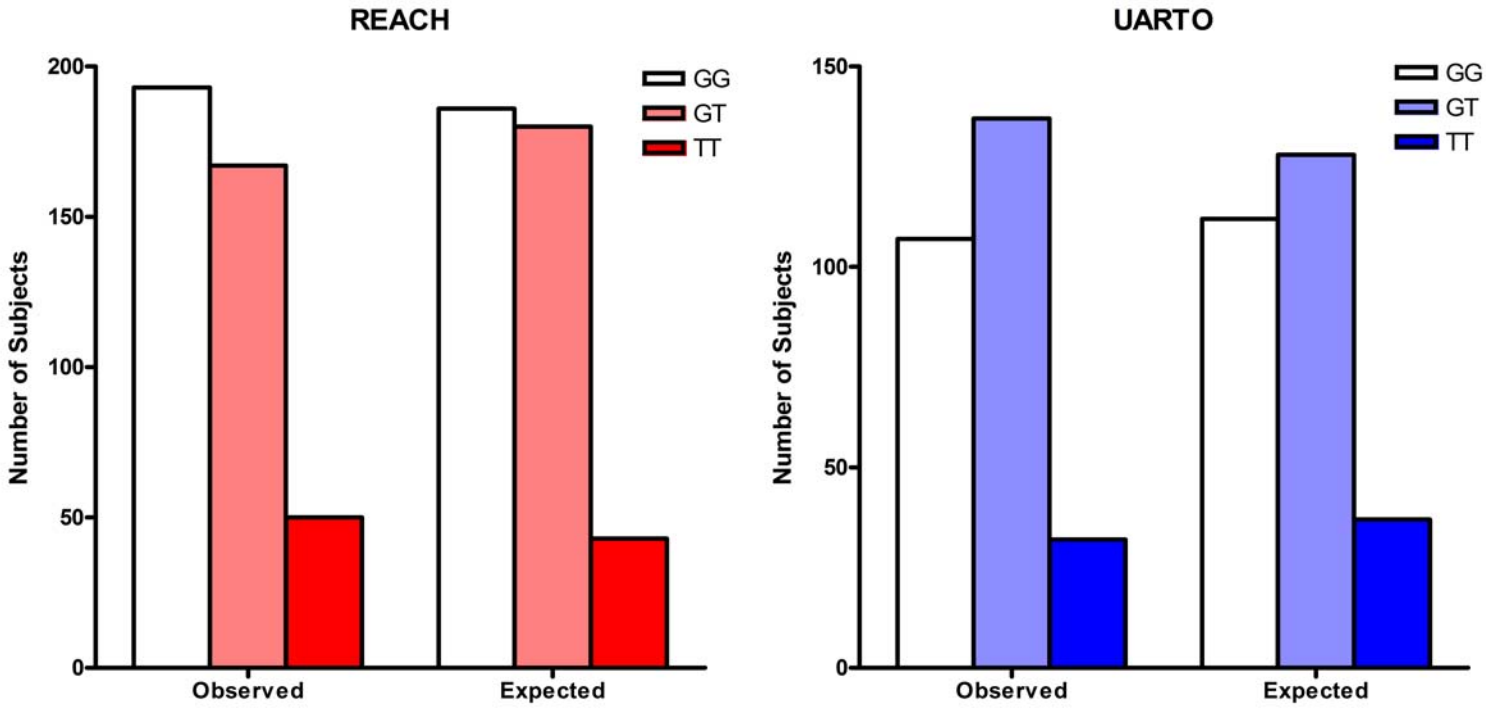
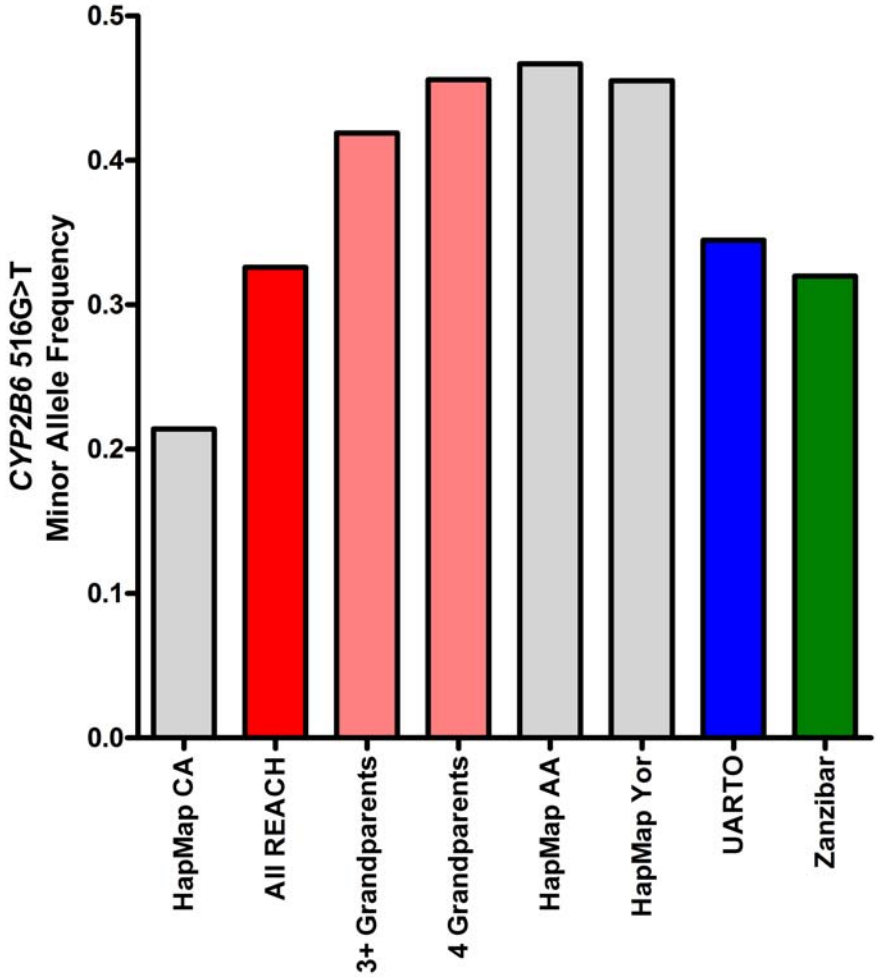


Figure 4.6. *CYP2B6* 516T allele frequency by population. The calculated minor allele frequencies for the *CYP2B6* 516G>T polymorphism in the REACH (red) and UARTO (blue) populations are displayed, along with published minor allele frequencies from the HapMap project shown in gray (CA: Caucasian, AA: African American, Yor: Yoruban/Nigerian) and from a population in Zanzibar (Tanzania) in green [51]. REACH subjects are further categorized by self-reported ethnicity, with lighter red bars representing subjects with at least three or four grandparents of African or African American descent.



The HAART response phenotype examined in this analysis was viral suppression, with suppression defined as a viral load less than or equal to 400 copies per ml. This phenotype was selected to increase the number of REACH subjects who could be included, due to missing nadir CD4+ count data for many REACH subjects. Variables investigated for significance included patient adherence to HAART, nadir CD4+ cell count, and *CYP2B6* 516G>T genotype. Ninety REACH and 185 UARTO subjects receiving an NNRTI-based regimen had complete data for all of these parameters and were included in this analysis. Neither the GT or TT genotypes nor presence of the variant T allele were significantly associated with viral suppression in the UARTO cohort following Chi-square testing (Table 4.1). Because the REACH population is more heterogeneous, a linear regression was performed to allow the inclusion of more variables. None of the variables were significantly associated with viral suppression in logistic regression analyses, although adherence neared the level of statistical significance.

Table 4.1. *CYP2B6* 516G>T genotype frequency in UARTO viral responders and non-responders.

Genotype		VL<400 copies/ml	VL>400 copies/ml	p-value
516G>T				>0.05
	GG	143	15	
	GT	21	3	
	TT	0	0	

ABC transporter polymorphisms were also genotyped and examined for associations with HIV antiretroviral response. Shown in Figure 4.7 are the observed

minor allele frequencies. For comparison, the reported minor allele frequencies from four available HapMap populations are graphed alongside our data. The same data are shown in Figure 4.8 for *ABCB1* 2677G>T/A, Figure 4.9 for *ABCB1* 3435C>T, and Figure 4.10 for *ABCC4* 4131T>G. With the exception of the *ABCB1* 2677A allele, the variant alleles of these four SNPs are fairly common, with frequencies ranging from approximately 10% to 65% in HapMap, REACH, and UARTO. (The frequency of the ABCB1 2677A allele was less than 1% in all populations, including HapMap populations, and is not included in Figure 4.8B.) Interestingly, of the HapMap populations, the minor allele frequencies for all four of these SNPs are lowest in the Yoruban population. The REACH minor allele frequencies reported here are compiled from all REACH subjects of varying, and in some cases multiple, ethnicities, and fall in between the HapMap Caucasian (CEU) and Yoruban (YRI) populations. However, upon subdividing REACH subjects by self-identified ethnicity (with four grandparents being described as Caucasian (CA, n=76) or African American (AA, n=84)), the minor allele frequencies of those of African descent still differ from the UARTO population. In UARTO, the minor allele frequencies tend to be most similar to the HapMap Yoruban population, but are still noticeably different in some cases, suggesting that the geographical region of a population in Africa – or a North American population derived from a certain region of African (e.g. East vs. West Africa) – also affects the allele frequencies of these SNPs.

Figure 4.7. Minor allele frequencies of *ABCB1* 1236C>T. Minor allele frequencies for REACH (red), REACH subpopulations grouped by self-identified ethnicity (four grandparents identified as of Caucasian (CA) or African American (AA) descent), and UARTO (blue) are graphed, along with four HapMap populations (gray). Abbreviations used include CEU: Caucasian, AS: Asian, HISP: Hispanic, YRI: Yoruban.

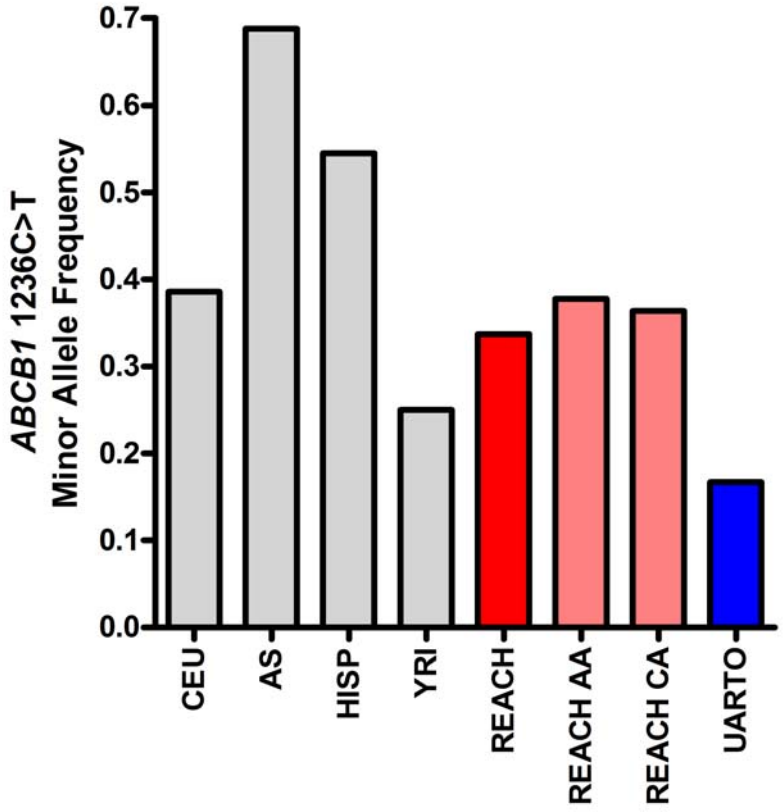
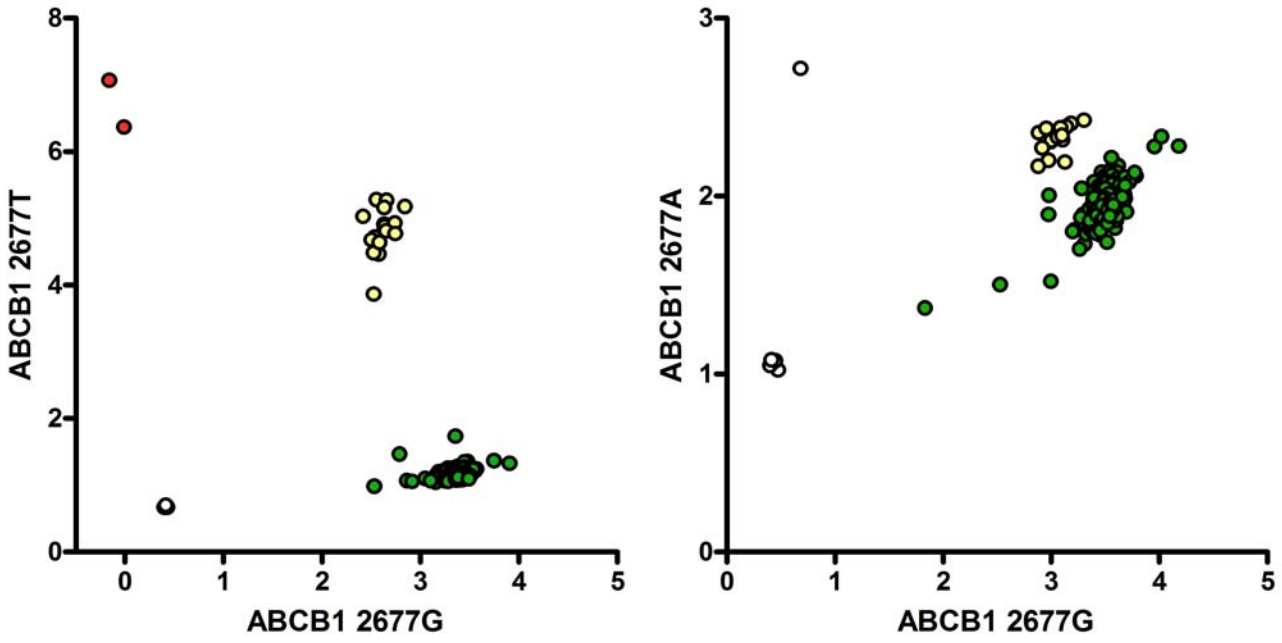


Figure 4.8. Representative genotyping output and minor allele frequencies of *ABCB1* 2677G>T/A (Ala893Ser/Thr). The triallelic nature of this SNP necessitates two allelic discrimination assays, one for each variant allele. Genotypes are displayed in groups defined by the fluorescence of one allele (x-axis) vs. the second (y-axis) (A). Reference homozygotes are in green, heterozygotes are in yellow, variant homozygotes are in red, and failed or uncalled samples are in white. Note that no AA variant homozygotes were found in the samples shown here (right panel). Minor allele frequencies for REACH (red) and UARTO (blue) are graphed, along with four HapMap populations (gray) (B). Abbreviations used include CEU: Caucasian, AS: Asian, HISP: Hispanic, YRI: Yoruban. The frequency of the A allele was less than 1% in the REACH, UARTO, and all four HapMap populations and is not reported here.

A.



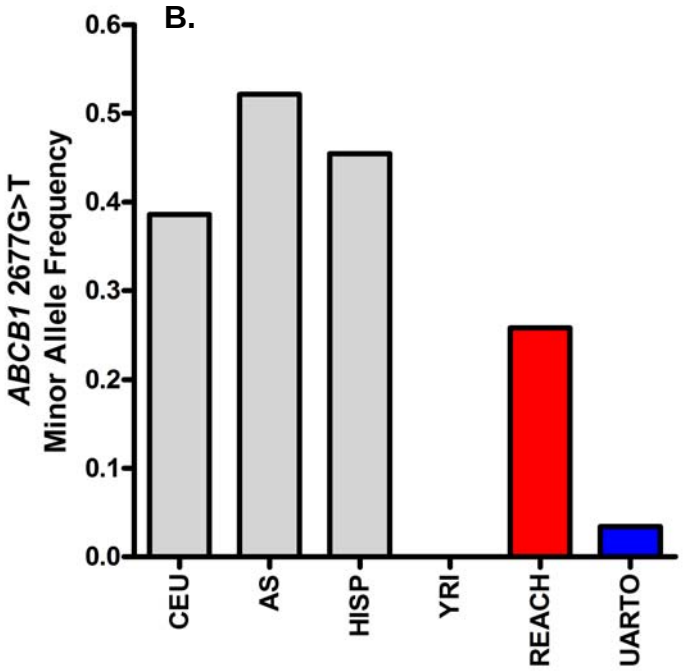

B.
ABCB1 2677G>T
Minor Allele Frequency
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CEU
AS
HISP
YRI
REACH
UARTO

Figure 4.9. Minor allele frequencies of *ABCB1* 3435C>T. Minor allele frequencies for REACH (red), REACH subpopulations grouped by self-identified ethnicity (four grandparents identified as of Caucasian (CA) or African American (AA) descent), and UARTO (blue) are graphed, along with four HapMap populations (gray). Abbreviations used include CEU: Caucasian, AS: Asian, HISP: Hispanic, YRI: Yoruban.

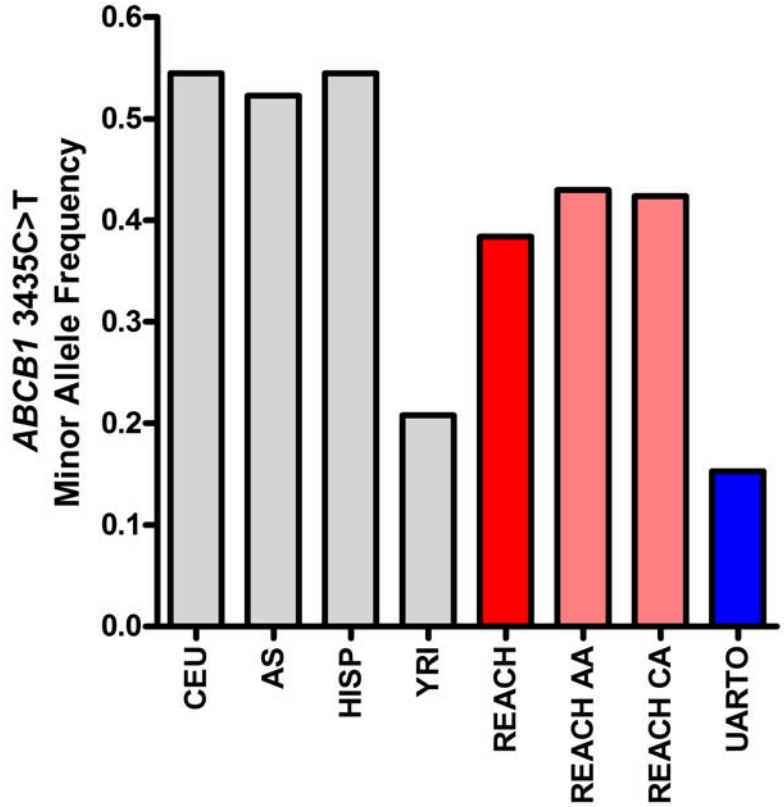
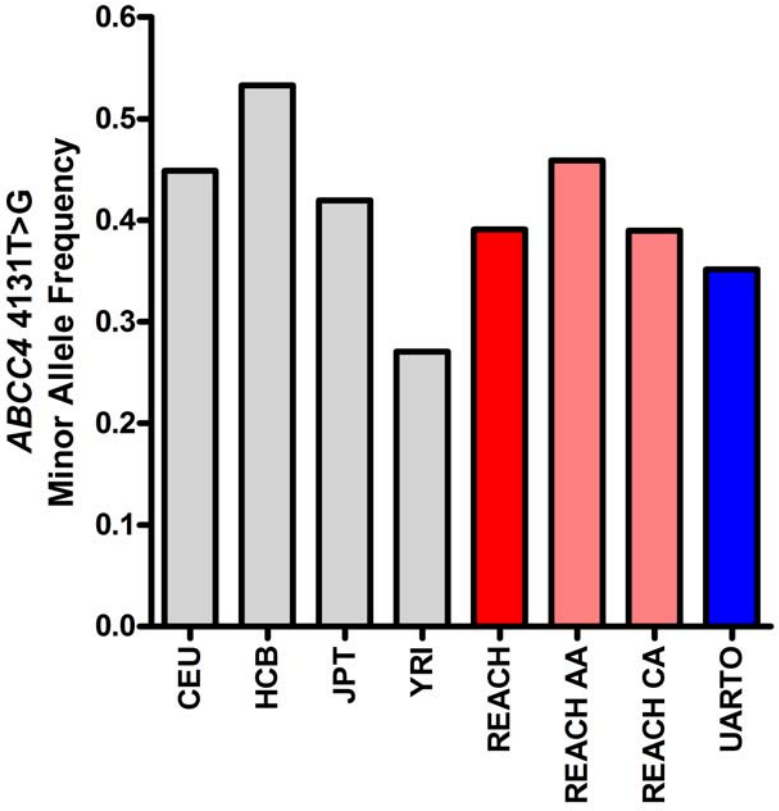


Figure 4.10. Minor allele frequencies of *ABCC4* 4131T>G. Minor allele frequencies for REACH (red), REACH subpopulations grouped by self-identified ethnicity (four grandparents identified as of Caucasian (CA) or African American (AA) descent), and UARTO (blue) are graphed, along with four HapMap populations (gray). Abbreviations used include CEU: Caucasian, HISP: Hispanic, YRI: Yoruban, HCB: Han Chinese, JPT: Japanese.



The HAART response phenotype examined in this analysis was the CD4+ cell change per month for six months following treatment initation (nadir CD4+ count was available for all UARTO subjects, but was missing for many REACH subjects; therefore, this analysis included UARTO subjects only). The *ABCB1* 1236C>T and 3435C>T and *ABCC4* 4131T>G genotypes were investigated for association with CD4+ cell change per month for the first six months of HAART treatment (*ABCB1* 2677G>T/A genotypes were not available at the time of statistical analysis). None of the genotypes was significantly associated with the rate of CD4+ cell change (Table 4.2).

Table 4.2. UARTO transporter genotype frequencies and rate of CD4+ cell change in the first six months of HAART therapy.

Gene	Genotype		ΔCD4/mo>10 cells/μl	ΔCD4/mo<10 cells/μl	p-value
ABCB1	1236C>T				>0.05
		CC	88	39	
		CT	39	11	
		TT	3	1	
	2677G>T				>0.05
		GG	119	49	
		GT	9	2	
		TT	1	0	
	3435C>T				>0.05
		CC	90	39	
		CT	32	10	
		TT	5	1	
ABCC4	4131T>G				>0.05
		TT	57	20	
		GT	56	20	
		GG	15	10	

To investigate the influence of polymorphisms in *TNFα* on HIV antiretroviral response, we used four SNPs in the *TNFα* region which were selected by Tagger:

rs1800750, rs1800629, rs3093662, and rs3093669. Figure 4.11 shows output from the GoldenPath browser with these four SNPs highlighted in red, and also displays the phased haplotypes in this region, as generated by the HapMap browser [56]. Shown in Figures 4.12-4.15 are the observed minor allele frequencies for each SNP. For comparison, the reported minor allele frequencies from four available HapMap populations are graphed alongside our data. With the exception of the *TNF*α rs1800629 SNP, the minor allele frequencies in UARTO were higher than all of the other reported populations, including REACH; in fact, the rs3093669 variant appears to be African-specific (Fig. 4.15). The rs1800629 variant allele occurs more frequently in persons of European descent, so it is most common in the CEU and REACH populations, as seen in Figure 4.13. This SNP corresponds to the -488G>A polymorphism which has been significantly associated with immunologic response to HIV antiretroviral therapy [44]. The differences observed between the minor allele frequencies in the UARTO population compared to the Yoruban and REACH populations may be due to the relatively low minor allele frequencies, as well as the fact that the Ugandan population has a genetic background reflecting its East African geographic location, as opposed to the Yoruban population from West Africa, while the REACH population contains individuals of Caucasian and African American ancestry; the latter are descended largely from West African ancestry.

Figure 4.11. Output from the UCSC Genome and HapMap browsers. The *TNFα* gene is displayed in blue (NCBI reference sequence NM_000594) and the four SNPs selected by Tagger to cover this region are shown in red. Below, the phased haplotype display, which includes SNPs with minor allele frequencies less than 1%, for the HapMap Yoruban population is shown. The tag SNPs cover these haplotype blocks with a power of 0.8.

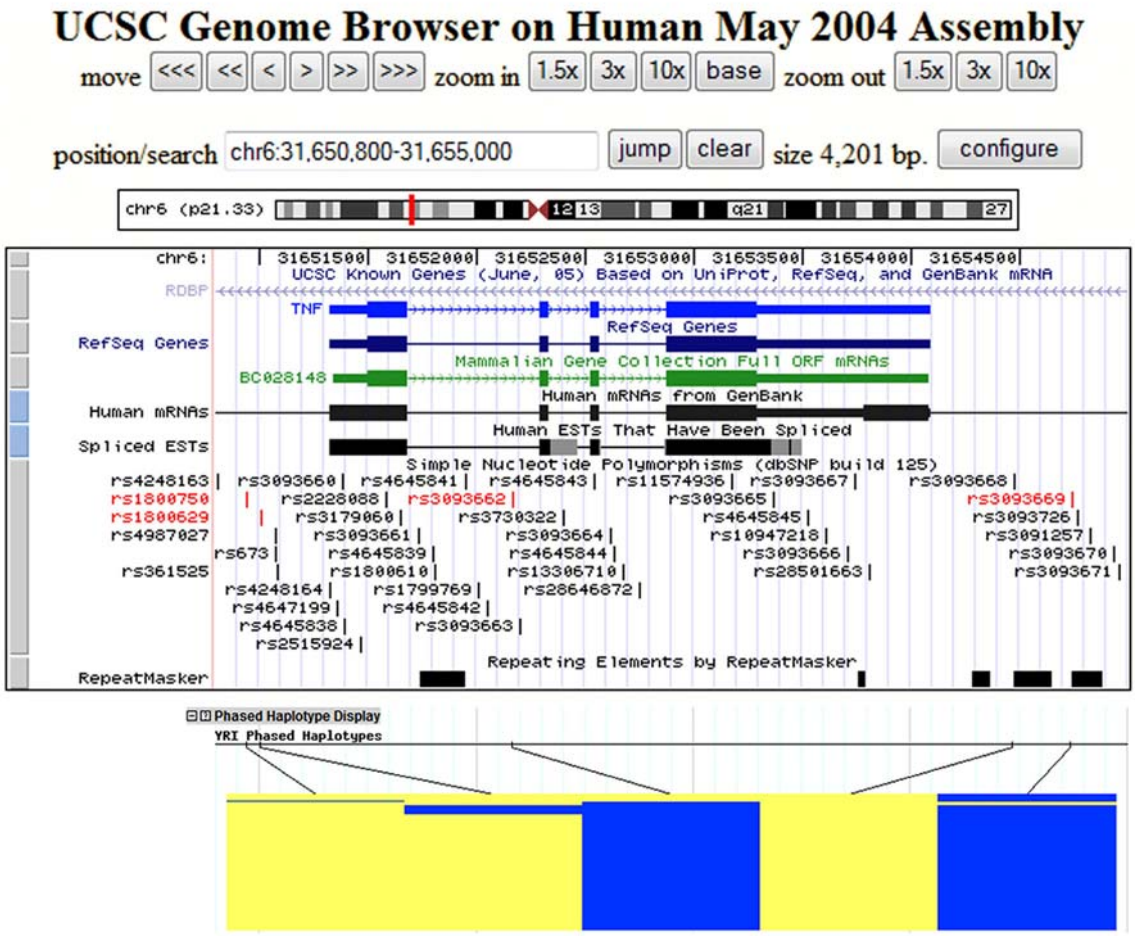


Figure 4.12. Minor allele frequencies of *TNFα* rs1800750G>A. Minor allele frequencies for REACH (red), REACH subpopulations grouped by self-identified ethnicity (four grandparents identified as of Caucasian (CA) or African American (AA) descent), and UARTO (blue) are graphed, along with four HapMap populations (gray). Abbreviations used include CEU: Caucasian, AS: Asian, HISP: Hispanic, YRI: Yoruban, HCB: Han Chinese, JPT: Japanese.

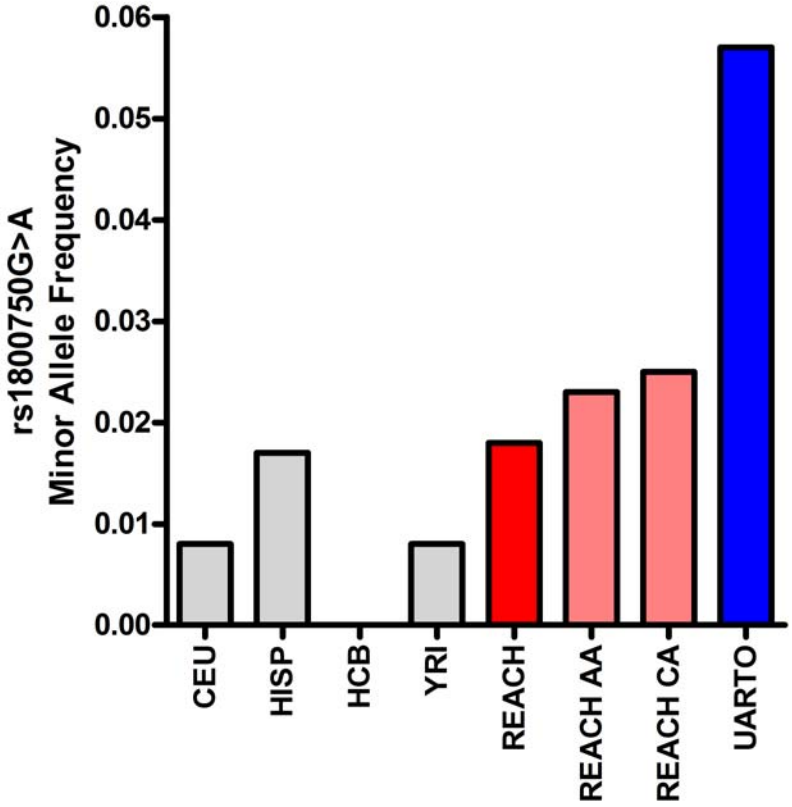
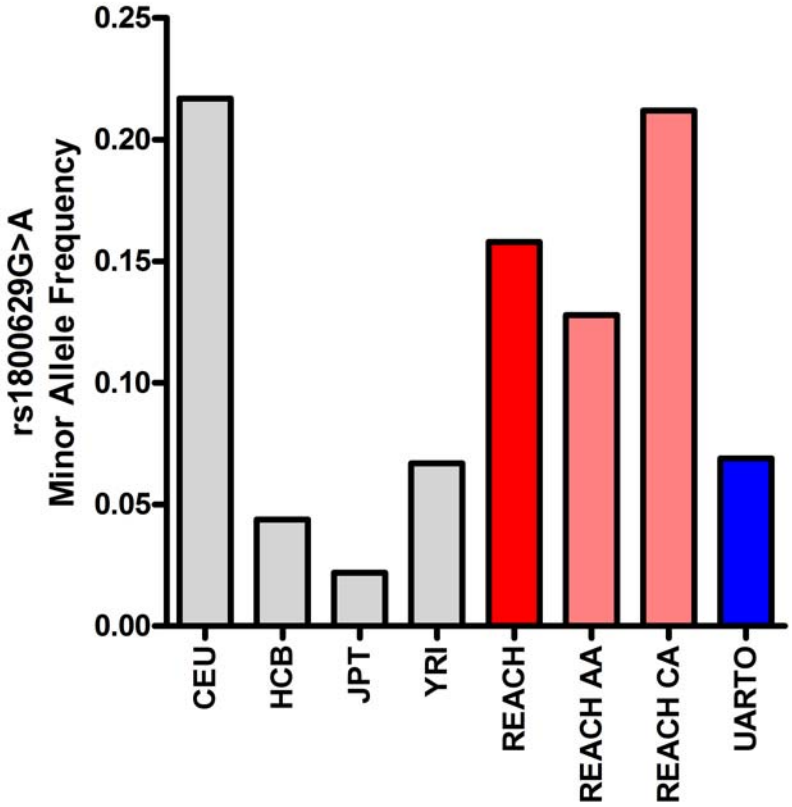


Figure 4.13. Minor allele frequencies of *TNFα* rs1800629G>A. Minor allele frequencies for REACH (red), REACH subpopulations grouped by self-identified ethnicity (four grandparents identified as of Caucasian (CA) or African American (AA) descent), and UARTO (blue) are graphed, along with four HapMap populations (gray). Abbreviations used include CEU: Caucasian, YRI: Yoruban, HCB: Han Chinese, JPT: Japanese.



igure 4.14. Minor allele frequencies of *TNFα* rs3093662A>G. Minor allele frequencies for REACH (red), REACH subpopulations grouped by self-identified ethnicity (four grandparents identified as of Caucasian (CA) or African American (AA) descent), and UARTO (blue) are graphed, along with four HapMap populations (gray). Abbreviations used include CEU: Caucasian, HISP: Hispanic, YRI: Yoruban, HCB: Han Chinese.

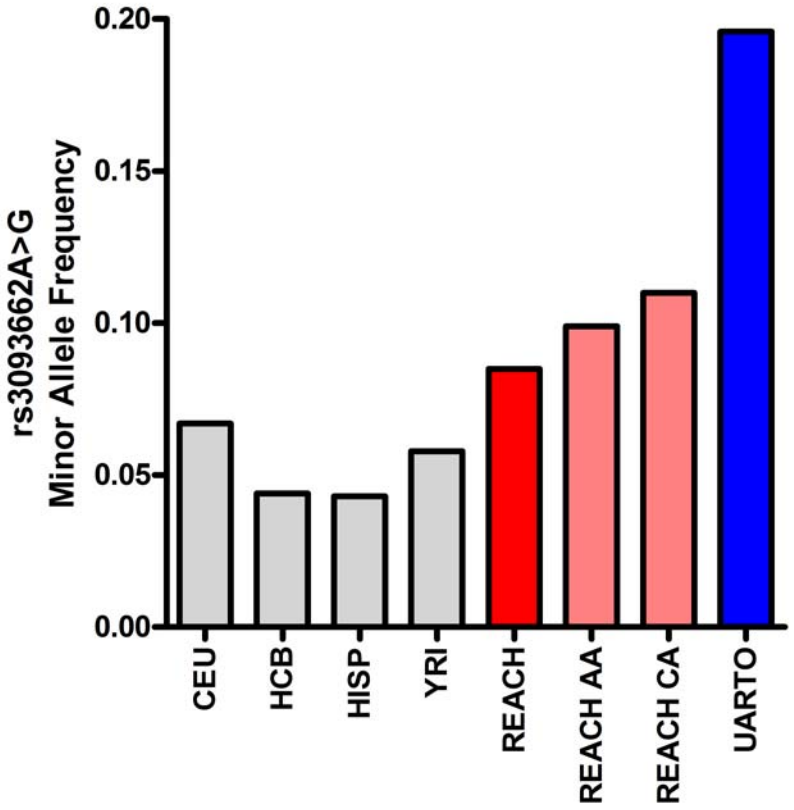
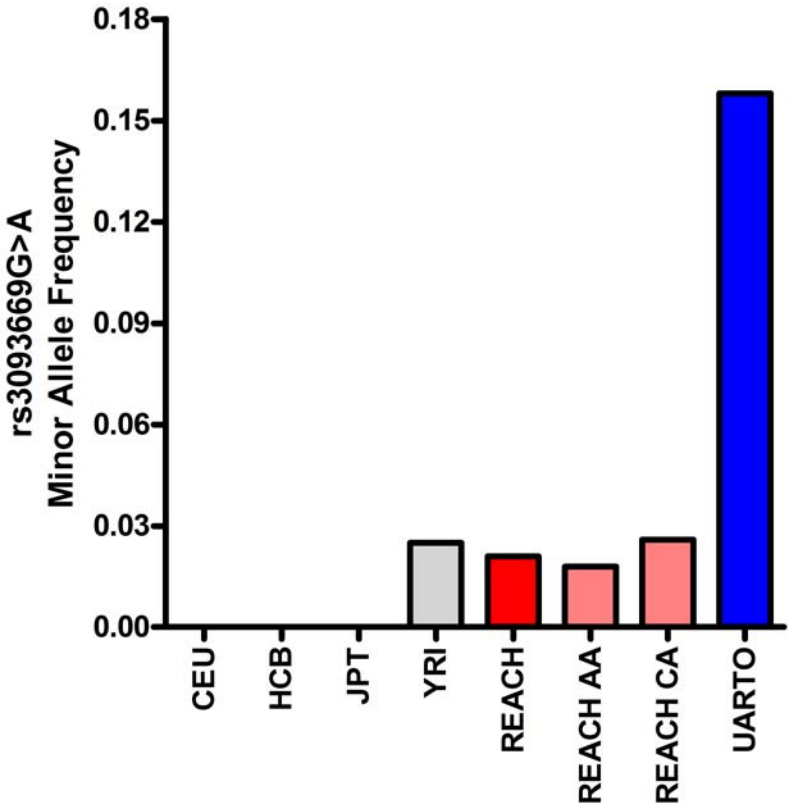


Figure 4.15. Minor allele frequencies of *TNFα* rs3093669G>A. Minor allele frequencies for REACH (red), REACH subpopulations grouped by self-identified ethnicity (four grandparents identified as of Caucasian (CA) or African American (AA) descent), and UARTO (blue) are graphed, along with four HapMap populations (gray). Abbreviations used include CEU: Caucasian, YRI: Yoruban, HCB: Han Chinese, JPT: Japanese.



The HAART response phenotype examined in this analysis was the CD4+ cell change per month for six months following treatment initation. Analyses were performed for associations between *TNFα* rs1800750, 1800629, 3093662, and 3093669 genotypes and the rate of CD4+ cell count change in the first six months following HAART initiation. A total of 180 UARTO subjects had complete data for these parameters and were included in these analyses, which consisted of multivariate logistic regressions. None of the genotypes was significantly associated with the rate of CD4+ cell change (Table 4.3). However, this may be due, at least in part, to the lack of phenotypic spread in the data; r^2 values evaluating the goodness of fit of linear regression models to the data were poor, being less than 0.02 in all cases.

Table 4.3. UARTO *TNFα* genotype frequencies and rate of CD4+ cell change in the first six months of HAART therapy.

Genotype		ΔCD4/mo>10 cells/μl	ΔCD4/mo<10 cells/μl	p-value
rs1800629				>0.05
	GG	110	48	
	GA	20	4	
	AA	0	0	
rs1800750				>0.05
	GG	112	46	
	GA	17	6	
	AA	1	0	
rs3093662				>0.05
	AA	85	35	
	AG	38	13	
	GG	6	4	
rs3093669				>0.05
	GG	94	35	
	GA	33	15	
	AA	2	2	

4.4.2. Population substructure analysis

The failure to reproduce previous pharmacogenetic associations may be due to confounding factors such as phenotypic ambiguity, environmental effects, or genetic background. One method of decreasing the influence of genetic background in pharmacogenetic analyses is to use unlinked markers to detect and eliminate confounding caused by genetic admixture [57]. This is especially important in pharmacogenetic studies implemented in highly admixed populations, such as in the United States, where the population is a heterogeneous mixture with Amerindian, European, and African genetic components [58]. Consequently, genetic admixture should be considered in the REACH population, although it is not relevant in the UARTO cohort.

The assumption underlying the population substructure theory is that there are two or more significant subpopulations, designated by values of k (i.e. k>1) [48]. To determine the relevant value of k for our REACH population, we modeled k=1 through 5 in Structure, using 10,000 iterations per value of k. Figure 4.15 displays the posterior probability ln(P)D plotted against k. From this graph, it is evident that the posterior probability plateaus at a k value of 3, indicating that there are 3 significant subpopulations in the REACH groups that we analyzed; using a k>3 is not any more informative than using a k=3. The population substructure results for k=1 through 5 for three groups of ethnically identified controls are shown in Figure 4.16. Again, it is clear that a k of 3 most efficiently and accurately groups these three populations by ethnicity. Note that when k=2, the Caucasian and Native American controls are grouped together, indicating that the genetic distance between these two populations is less than that between African Americans and either Caucasians or Native Americans. Consequently, a

k value of 3 was used to calculate the genetic substructure for REACH subjects. A triangle plot is shown in Figure 4.17, with the contribution from each subpopulation (which sums to 1) plotted for REACH subjects and ethnically identified controls.

Figure 4.16. Selection of k. A panel of 112 markers was used to determine population substructure by the program Structure in a subset of REACH subjects using k values (the number of subpopulations) ranging from 1 (no subpopulations) to 5. The posterior probability (ln(P)D) for each value of k is plotted here.

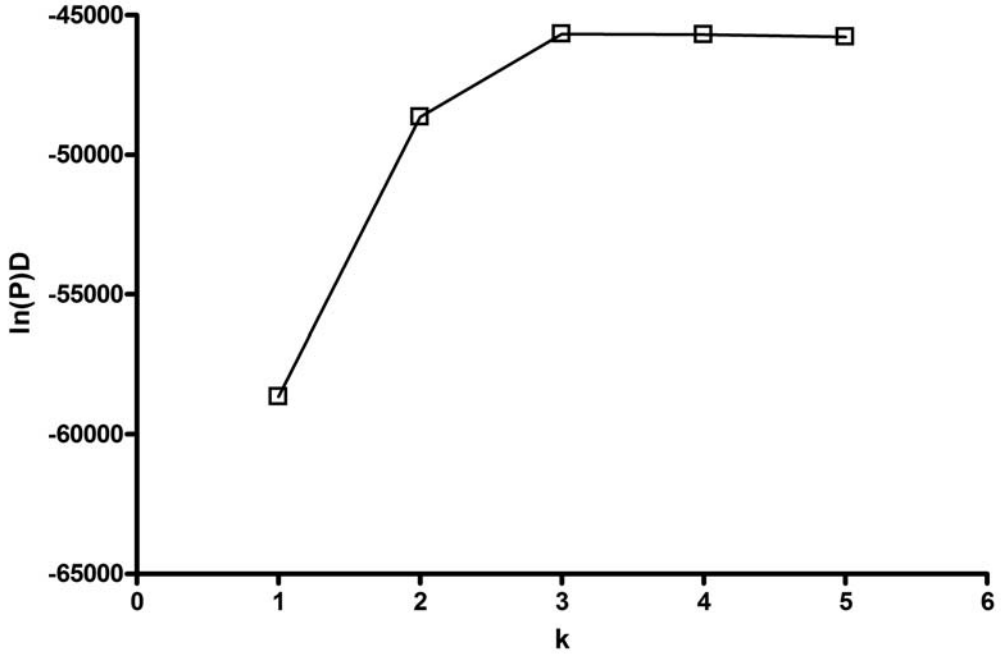


Figure 4.17. Structure evaluation of subpopulations in ethnically identified controls. Genotypes from African American (AA, n=37), European/Caucasian American (CA, n=42), and Native American (NA, n=30) individuals were input into Structure using values of k ranging from 1 to 5. Colors indicate subpopulation groups as calculated by Structure, and do not necessarily correspond to the designated ethnic group (AA, CA, or NA).

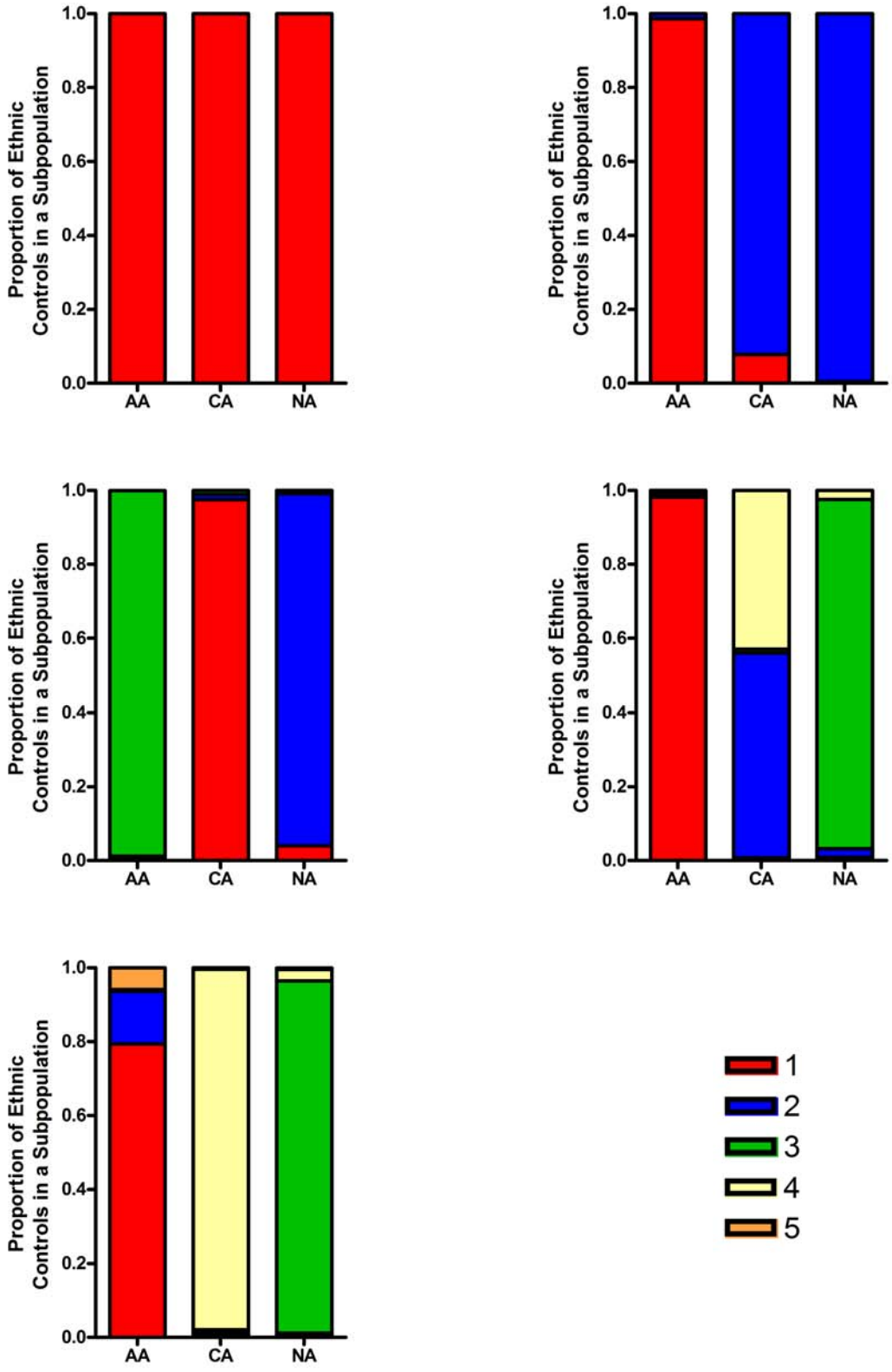
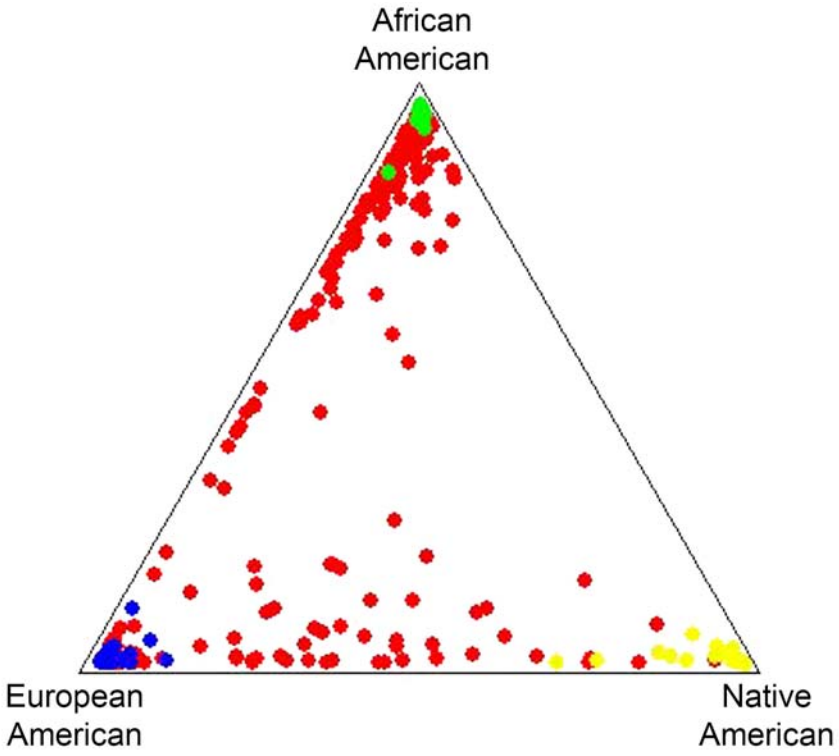
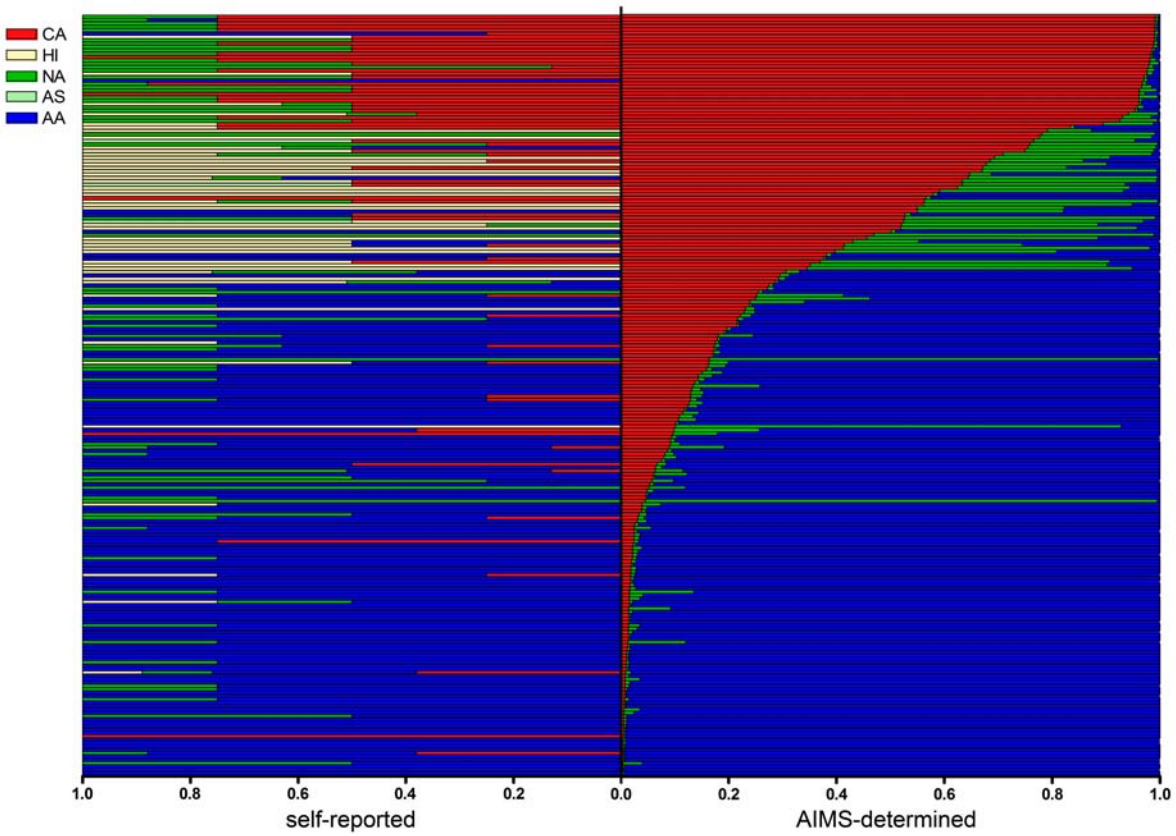

Proportion of Ethnic Controls in a Subpopulation
1.0
0.8
0.6
0.4
0.2
0.0
AA
CA
NA
1
2
3
4
5

Figure 4.18. Structure determination of ethnic subpopulations in REACH (k=3). REACH subjects who reported at least one grandparent of African, Hispanic, or Native American descent, or who did not report ethnicity, were included in this analysis (red). Ethnically identified controls were also included and were used to identify the groups which were detected by Structure (green: African American, yellow: Native American, blue: European/Caucasian American).



From the Structure results, it appears that many of the REACH subjects who were included in this analysis are either of mixed African/European or European/Native American ancestry, while a significant genetic contribution from the third subgroup was found less often. To evaluate the accuracy of self-reported ethnicity in the REACH population, Structure-computed ethnicity is compared to self-reported ethnicity in Figure 4.18. The ethnicity surveys administered to REACH subjects request the designation of grandparents as Caucasian, African, Hispanic, Asian, or Native American (N. delaCruz, personal communication), all of which are included in the self-reported ethnicity plots. The Structure-determined ethnicity estimations were based on three subpopulations – Caucasian, African, and Native American – which are included in the AIMS-determined ethnicity plots (Fig. 4.18). From this figure, it appears that REACH subjects are largely accurate in reporting ethnicity, although there are several subjects for whom there were inconsistencies with the Structure determinations. Persons with Hispanic ancestry were not included as controls, but it seems that the markers associated with Hispanic ancestry in REACH subjects are being grouped with Caucasian or Native American markers because of their similarity in minor allele frequencies.

Figure 4.19. Self-reported vs. Structure-determined ethnicity of REACH subjects. Each subject is plotted as a horizontal bar, with self-reported ethnicity shown on the left half of the figure, and Structure-calculated ethnicity shown on the right. Self-reported ethnicity is binned by eighths (e.g. if a grandparent was half Caucasian and half African American, then each of those contributed 1/8 of the total) while Structure determines ethnicity as a continuous variable composed of three components: Caucasian (CA, red), African American (AA, blue), or Native American (NA, green) ancestry. Self-reported ethnicities also included Hispanic (HI, yellow) and Asian (AS, light green) ancestry.



4.5. Discussion

In the present study we evaluated the effects of polymorphisms in genes which have been shown to be involved in the transport, metabolism, and pharmacological action of HIV antiretroviral drugs. Specifically, we considered the 516G>T polymorphism in the drug metabolizing enzyme *CYP2B6*, which plays a role in the pharmacokinetics of the NNRTIs efavirenz and nevirapine. We also investigated the effects of the *ABCB1* 1236C>T, 2677G>T/A, and 3435C>T polymorphisms and the 4131T>G polymorphism in *ABCC4*. SNPs in both of these ABC transporters have been shown to modulate intracellular drug concentrations. The last gene we examined was *TNFα*, polymorphisms in which have been associated with T cell proliferation and apotosis, and therefore may be important in immunological response to HIV antiretroviral drugs.

CYP2B6 is primarily responsible for efavirenz metabolism, and plays a large role in the metabolism of nevirapine. Efavirenz undergoes primary oxidative hydroxylation to its major metabolite 9-hydroxyefavirenz via CYP2B6 [59]. Nearly all nevirapine undergoes oxidative metabolism to 2-, 3-, 8-, and 12-hydroxynevirapine, with the major routes of metabolism occurring through CYP3A4 and CYP2B6 [60]. Interestingly, both efavirenz and nevirapine induce CYP2B6 through activation of the constitutive androstane receptor (CAR) [61]. Because of the importance of CYP2B6 in the metabolism of efavirenz and nevirapine, researchers in the field of HIV pharmacogenetics initially focused on the effects of *CYP2B6* genetic variation on the pharmacokinetics and pharmacodynamics of these two drugs [62].

To date, there have been 28 polymorphisms reported in *CYP2B6*, which are arranged in 53 haplotypes [62]. A schematic of the most common haplotypes can be

found in Figure 4.19. One of these haplotypes, *CYP2B6*6*, is comprised of two non-synonymous polymorphisms which result in two amino acid changes (Q172H and K262R) and has been correlated with decreased protein expression in human liver microsomes [32]. *In vivo*, lower levels of hepatic CYP2B6 protein would correspond to a decrease in drug metabolism and an increase in drug exposure. Indeed, the *CYP2B6**6 haplotype has been associated with altered plasma efavirenz concentrations and increased incidence of neurological side effects (Table 4.1) and has also been linked to altered nevirapine pharmacokinetic parameters (Table 4.2). The *CYP2B6* 516G>T polymorphism (rs3745274) is frequently used to genotype for *CYP2B6**6, since it commonly occurs in combination with 785A>G in this haplotype, although it is also rarely found by itself (*CYP2B6*9*) [62].

Figure 4.20. Haplotype structure of *CYP2B6* (NCBI refseq NM_000767). Several common *CYP2B6* haplotypes are displayed, along with pie charts depicting the haplotype frequencies in Caucasian (CA), African American (AA), African (AF), and Asian (AS) populations. (Adapted from Zanger *et al.* [62]). NM_000767 is designated as the reference sequence in the NCBI database.

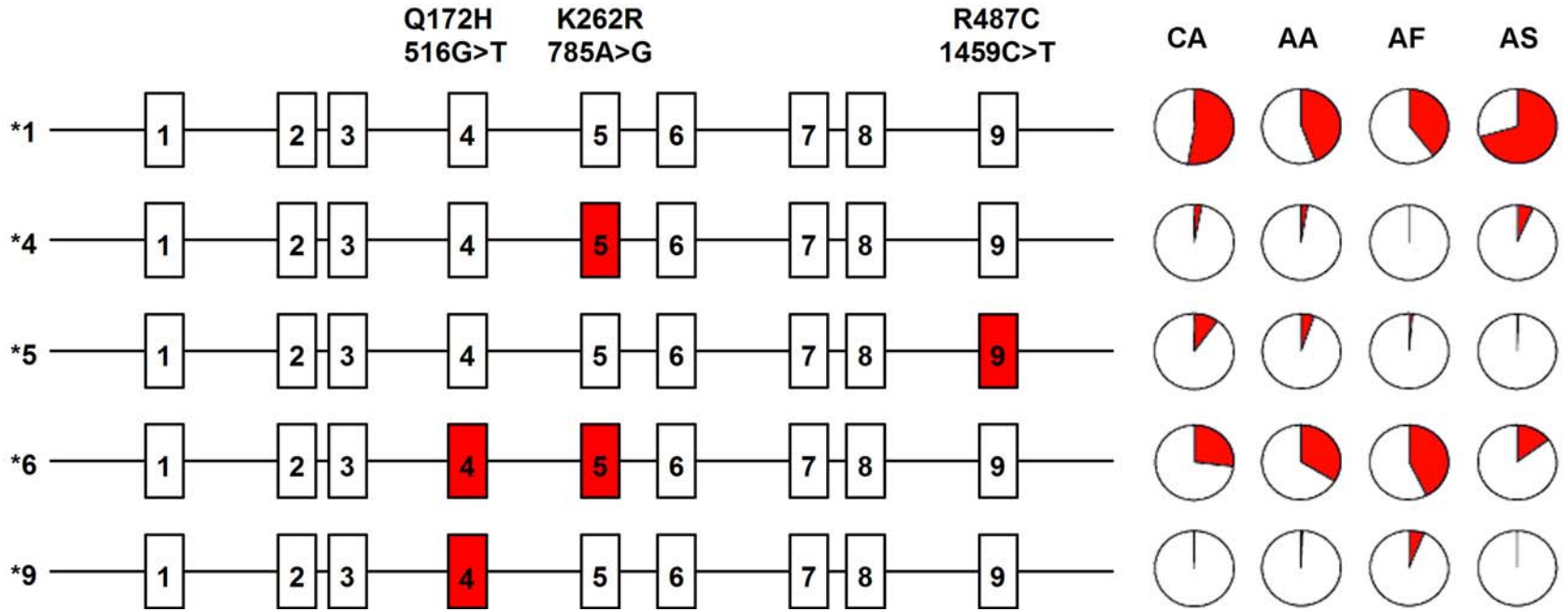


Table 4.4. *CYP2B6* polymorphisms associated with altered efavirenz pharmacokinetics and pharmacodynamics (adapted from Zanger *et al.* [62]).

CYP2B6 SNP/allele	Phenotype	Results	Ref
*2 through *8	C_p	increased in *6/*6	[63]
516G>T, 1459C>T	C_{max}, C_{p24h}, AUC, CNS toxicity	TT>GT>GG	[64]
1459C>T	C_{p24h}, AUC, viral load	not significant	[65]
516G>T	AUC, PBMC intracellular concentration, CNS toxicity	TT>GT,GG	[66]
516G>T	C_p	TT>GT>GG	[67]
516G>T, 1459C>T	AUC, viral load	TT>GT,GG (AUC)	[33]
1459C>T	C_p	not significant	[68]
516G>T	C_p, $t_{1/2}$	TT>GT>GG	[69]
516G>T, 785A>G	C_p	TT>GT>GG	[70]
516G>T, 1459C>T	AUC, viral load	516G>T best predictor in model	[71]
15 SNPs	AUC	TT>GT>GG	[72]
516G>T	CL, toxicity, resistance	TT>GT>GG	[73]
516G>T, 983T>C, 1459T>C	C_p	TT>GT>GG (516), CC>TC>TT (983)	[74]

Abbreviations used: PBMC (peripheral blood mononuclear cell).

Table 4.5. *CYP2B6* polymorphisms associated with altered nevirapine pharmacokinetics and pharmacodynamics.

CYP2B6 SNP	Phenotype	Results	Ref
516G>T	CL, immunologic response	GG>GT>TT (CL), TT>GT>GG (response)	[75]
516G>T	C_{min}	TT>GT>GG	[76]
516G>T	hepatotoxicity	not significant	[77]
516G>T	C_p	TT>GT>GG	[66]

We studied the effect of the *CYP2B6* 516 allele as a marker of the *6 haplotype on viral suppression following HAART. Our findings indicate that in the population studied, the *CYP2B6* 516G>T polymorphism has less of an effect on the time to viral suppression than does patient adherence. Although other studies have reported associations between this SNP and efavirenz and nevirapine pharmacokinetics and response (Tables 4.1 and 4.2), it should be noted that this study was observational, and therefore was less controlled than previous studies. We also did not evaluate pharmacokinetic data; it is possible that despite the lack of genotype effect on NNRTI pharmacodynamics, the pharmacokinetics of these drugs may have been significantly impacted. Future studies can consider using plasma drug levels to make the connection between genotype, pharmacokinetics, and pharmacodynamics.

We also investigated whether or not polymorphisms in ABC transporters affected response to HIV antiretroviral drugs, hypothesizing that SNPs which alter transporter expression or function at the site of action (i.e. on lymphocyte plasma membranes) would therefore have an effect on intralymphocytic drug concentrations, resulting in altered drug response. The first report to examine the effects of ABC transporter polymorphisms and lymphocyte expression levels with regard to anti-HIV therapy was published in 2002 by Fellay *et al.* [41]. The authors described an association between the *ABCB1* 3435TT homozygous variant genotype and decreased lymphocyte ABCB1 RNA levels, compared to lymphocytes from individuals with the 3435CT and CC genotypes [41]. They also found that the *ABCB1* 3435 polymorphism was associated with both pharmacokinetic and pharmacodynamic parameters, with the CC genotype associated with higher plasma nelfinavir (a P-gp substrate) and efavirenz (not an established P-gp substrate) levels,

while the TT genotype predicted a more favorable immunologic response [41]. These two findings appear contradictory; however, given the correlation between TT genotype and decreased ABCB1 expression in lymphocytes, it follows that the drug levels in lymphocytes (the pharmacological site of action) increase correspondingly, and antiviral ABCB1 substrate drugs will be more efficacious in patients with the TT genotype. One interpretation of the results of this study is that plasma drug levels have less of an effect on HIV antiviral response than do intralymphocytic drug levels. A recent study explored this concept by evaluating the influence of polymorphisms in *ABCC4* (MRP4) on intracellular drug levels in HIV-positive patients and found a statistically significant correlation between *ABCC4* 4131T>G genotype and lamivudine-triphosphate concentrations in lymphocytes [40].

It should be noted that a number of researchers have attempted to replicate previously reported pharmacogenetic associations between transporter polymorphisms and response to HIV antiretrovirals and have failed (see Table 4.3). This lack of reproducibility is likely due, at least in part, to differences between study populations, including genetic and environmental factors, HAART regimen, and which outcome measures were examined. Fellay *et al*. studied a group of HIV-positive people who were treatment-experienced, while a number of other studies only considered treatment-naïve patients [64, 78-81]. Regardless of the conflicting data on the association between *ABCB1* 3435C>T and HIV antiretroviral response, many researchers now include this SNP routinely in HIV pharmacogenetics studies.

Table 4.6. ABC transporter polymorphisms associated with HIV outcomes.

Transporter	SNP	Phenotype	Results	Ref
ABCB1	3435C>T	Efavirenz and nelfinavir C_p	CC>CT>TT	[41]
		Change in CD4+ cell count at 1, 3, 6 mos	TT>CC/CT	
		Change in viral load at 1, 3, 6 mos	Not significant	
ABCB1	3435C>T	Change in CD4+ cell count at 3 and 6 mos	Not significant	[80]
		Change in viral load at 3 and 5 mos	Not significant	
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Phase 1 or 2 viral decay	Not significant	[79]
		Percent CD4+ cells	Not significant	
		Ritonavir C_{min}	Not significant	
ABCB1	3435C>T	Lopinavir C_{min} and efavirenz $C_{p,12h}$	Not significant	[82]
ABCB1	2677G>T (Ala893Ser), 3435C>T	Disease progression before HAART	Not significant	[78]
ABCB1	3435C>T	Efavirenz CNS side effects	Not significant	[64]
		Efavirenz AUC	Not significant	
ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	Change in CD4+ count	1236 genotype associated with CD4+ recovery	[83]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Change in CD4+ count	Not significant	[81]
		Change in viral load	Not significant	
ABCB1	3435C>T	Decrease in viral load	CT>TT at 8 wks	[65]
		Nelfinavir C_p and AUC and efavirenz C_p	CT>CC/TT (NFV C_p), CC/TT>CT (NFV CL)	

ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Virologic response	TT>CC/CT (3435)	[33]
		Change in CD4+ count	Not significant	
		Efavirenz and nelfinavir AUC	Not significant	
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Indinavir C_{max}, C_{min}, and CL	Not significant	[84]
		Change in CD4+ count	Not significant	
		Change in viral load	Not significant	
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Saquinavir CL	Not significant	[85]
ABCB1	3435C>T	Atazanavir C_p	CC>CT/TT	[86]
		Bilirubin levels	CC>CT/TT	
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Nevirapine hepatotoxicity	3435C allele associated with hepatotoxicity	[77]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Atazanavir and lopinavir C_{min}	Not significant	[87]
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Indinavir CL, V_d, and k_a	Not significant	[88]
ABCA1	969G>A, 2962A>G	HAART-associated dyslipidemia	2962G allele associated with increased plasma triglyceride levels	[89]
ABCB1	3435C>T		Not significant	
ABCB1	3435C>T	HIV-seropositivity	C allele associated with seropositivity	[90]
		Change in CD4+ count	Not significant	
ABCB1	2677G>T/A (Ala893Ser/Thr), 3435C>T	Nelfinavir metabolism	Not significant	[91]
ABCB1	1236C>T, 2677G>T/A (Ala893Ser/Thr), 3435C>T	Tenofovir-induced renal proximal tubulopathy (rPT)	Not significant	[92]
ABCC2	SNP discovery		1249A associated with increased risk; 3563A and 4544A associated with decreased risk	
ABCC4	SNP discovery		Not significant	

ABCB1	39 SNPs	Intralymphocytic nelfinavir exposure	*ABCB1* 3435TT>CT>CC; intron 26 +80T>C CC>CT>TT	[93]
ABCC1 *ABCC2* *ABCG2*	7 SNPs 27 SNPs 16 SNPs			
ABCC2 *ABCG2* *ABCB1* *ABCC4*	1 SNP 2 SNPs 2677G>T (Ala893Ser) 1612C>T, 3463G>A, 3724G>A, 4131T>G	Indinavir CL; intralymphocytic lamivudine- and zidovudine-triphosphate levels; change in viral load	*ABCB1* 2677GT/TT>GG (decrease in viral load) *ABCC4* 4131GG>GT>TT (intracellular 3TC-triphosphate)	[40]

The results of our analysis are consistent with previous pharmacogenetics studies which found no association between *ABCB1* polymorphisms and HIV pharmacokinetic and pharmacodynamic outcomes (see Table 4.3). The *ABCC4* 4131T>G polymorphism, while linked to intracellular lamivudine- and zidovudine-triphosphate levels, was not associated with HAART response [40], similar to the findings in this study.

We also investigated the involvement of polymorphisms in *TNFα* in immunological response to HAART. Currently, the prevailing theory is that T cell depletion is due to viral destruction of CD4+ cells resulting in an unsustainable rate of cell turnover, but it has been suggested that the decline in T cells may actually be caused by chronic immune activation triggered by viral infection [94]. Sousa *et al*. examined the latter hypothesis by comparing the clinical course of HIV-1 infection with that of HIV-2 infection, a less virulent strain of HIV which nevertheless results in immunodeficiency, and found that in patients with HIV-2, the rate of CD4+ turnover was similar to that of HIV-1 infected patients with similar levels of CD4+ cell depletion, despite the fact that viral load was substantially higher in the HIV-1 group [95]. These findings support the hypothesis that immune activation has a larger role in CD4+ cell depletion (and clinical outcome) than does viral load. Others have shown that biomarkers of immune activation, including chemokines and their receptors, are predictive of AIDS occurrence [96]. The *TNFα* promoter polymorphism -488G>A has been significantly associated with immunological response to HAART [44]. However, we failed to find significant associations between any of the polymorphisms in *TNFα* and the rate of CD4+ cell change in the current study.

There are several other interesting clinical phenotypes that could be considered in future *TNFα* pharmacogenetic studies. For example, interindividual variability in HAART response is not limited to response vs. non-response. In most HIV patients to whom HAART is administered, plasma viral load becomes undetectable and the number of CD4+ T cells increases significantly. However, the decrease in viral load does not always result in a corresponding CD4+ cell increase; there are patients who experience viral suppression, but not CD4+ cell recovery (immunologic nonresponders), or vice versa (virologic nonresponders) [97]. It has been suggested that levels of TNFα could play a role in this phenotype [97]. TNFα has also been implicated in HIV-associated dementia, neuropathy, and lipodystrophy, as well as disease progression [98-100], and *TNFα* polymorphisms have been associated with lipodystrophy [101] and disease progression [102, 103]. The dementia, neuropathy, and lipodystrophy phenotypes, while more difficult to quantify than disease progression or HAART response, are intriguing, and investigations into *TNFα* SNP associations with these clinically important disorders may shed light on the involvement of *TNFα* in their etiologies.

4.6. Conclusions

The pharmacogenetic studies described in this chapter represent preliminary findings that should be further evaluated in larger populations. The establishment of the techniques used in these studies (DNA extraction, genotyping) will facilitate the implementation of future studies in these populations. Methods to incorporate population substructure, a possible confounding factor in REACH, have been discussed in this chapter, but a systematic way to incorporate measures of genetic admixture into pharmacogenetic analyses should be instituted in order to avoid both false positive and false negative associations. The incorporation of the collection of other outcome measures (lipodystrophy, neuropathy, etc.) into regular patient visits would provide interesting and robust phenotypes for future pharmacogenetics studies. The REACH and UARTO populations are valuable tools in evaluating the effects of genetic polymorphisms on HIV outcomes; the projects discussed in this chapter may be considered cursory examinations of pharmacogenetic associations that should be expanded upon in the future in these and other HIV-positive populations.

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Chapter 5. The Influence of Transporters on the Mitochondrial Toxicity of Antiviral Nucleoside Analogue Drugs

5.1. Abstract

With the significant advances in anti-HIV drug development over the past two decades, the life expectancy of those infected with HIV has increased considerably. However, long-term exposure to antivirals can also result in unforeseen toxicities, including a wide array of mitochondrial toxicities, which are caused by nucleoside analogues. One of these toxicities is lipodystrophy, a broad term for a range of disorders which have both physical and emotional effects on patients. Lipodystrophy is thought to result from inhibition of the mitochondrial DNA polymerase, rather than the HIV reverse transcriptase for which it is intended. In this chapter, we characterize transporter levels (specifically the SLC transporter ENT1 and the ABC transporter MRP4) in cultured hepatocytes and primary adipose tissue, and we investigate the mechanism of nucleoside analogue-associated mitochondrial toxicity. We also explore the hypothesis that transporters expressed in adipocytes influence the intracellular and mitochondrial accumulation of these drugs, which may lead to susceptibility to lipodystrophy. We investigate the effects of *ABCC4* polymorphisms on adipose ABCC4 expression and on the occurrence of stavudine-associated lipodystrophy in HIV-positive individuals.

5.2. Introduction

In 1975, the Nobel Prize in Medicine was awarded to Baltimore, Temin, and Dulbecco for “their discoveries concerning the interaction between tumor viruses and the genetic material of the cell” [1]. Dulbecco’s contribution to this work was the

demonstration that viral genetic material was incorporated into the host genome following viral transformation. But while Dulbecco used DNA viruses in his experiments, Baltimore and Temin focused on the incorporation of genetic material from RNA viruses (retroviruses) into the host cell genome, a concept widely challenged at the time because of its violation of the central dogma of molecular biology (i.e. DNA provides a template for RNA, which in turn serves as a guide for protein synthesis) [2-4]. Baltimore and Temin provided incontrovertible evidence for the mechanism of retroviral replication with their simultaneous discoveries of reverse transcriptase, an enzyme which assembles DNA using RNA as a template [5, 6].

The awarding of the 1975 Nobel Prize for the discovery and characterization of retroviruses by Baltimore and Tenin proved prescient, given the emergence of AIDS in the 1970s. In May of 1983, two research groups, led by Montagnier and Gallo, isolated a novel retrovirus in lymphocytes from AIDS patients [6, 7]. This retrovirus, designated HTLV-III (human T-cell leukemia virus III) by Gallo and LAV (for lymphadenopathy associated virus) by Montagnier, displayed reverse transcriptase activity [7, 8], and the HTLV-III/LAV reverse transcriptase enzyme was purified and characterized in 1986 [9]. Because of its necessity in retroviral replication, and because it is not expressed in normal human cells, reverse transcriptase became a high-priority pharmacological target for anti-AIDS therapies [10]. A program to develop drugs against HTLV-III/LAV was implemented at the National Cancer Institute, in coordination with the National Institute of Allergy and Infectious Diseases, in 1985, with the stated goal of "bringing candidate compounds from all over the world through preclinical development, using NCI's drug development process, into clinical trials… supported by the NIAID" [10]. One of the

earliest and most promising hits was a decades-old drug, 3'-azido-3'-deoxythymidine (AZT), which, following phosphorylation to the active triphosphate, blocked replication of the HTLV-III/LAV retrovirus via inhibition of the reverse transcriptase enzyme [11, 12]. AZT was subsequently approved by the FDA for the treatment of HIV in 1987 [13].

Nucleoside/nucleotide reverse transcriptase inhibitors, or NRTIs, are competitive inhibitors of the HIV reverse transcriptase enzyme, replacing naturally occurring dNTPs in the nucleotide binding site of HIV RT [14] and resulting in chain termination due to the absence of a 3'-hydroxyl group on the ribose moiety, which prevents 3' to 5' phosphodiester bond formation [13] (see Figure 5.1). On the other hand, NNRTIs are allosteric non-competitive inhibitors [15]. Following FDA approval of AZT, a number of other nucleoside/nucleotide analogue drugs were approved for use as anti-HIV agents, including didanosine [16], zalcitabine [17], stavudine [18], abacavir, lamivudine [19], emtricitabine [20], and the nucleotide analogue tenofovir [21]. The structures and dates of FDA approval of anti-HIV nucleoside and nucleotide analogues are shown in Table 5.1. Table 5.2 displays a list of other clinically available nucleoside analogue antiviral and anticancer compounds, which exhibit similar mechanisms of action via inhibition of viral reverse transcriptase, or DNA polymerase in cancer cells. These drugs are administered in their unphosphorylated forms; di- [22] or tri-phosphorylation [23] occurs intracellularly and is required prior to reverse transcriptase inhibition. Generally, the first phosphorylation step is the slowest of the three [24]. It should be noted, however, that this metabolic activation does not occur until the nucleoside analogues have been transported into the cell, likely by nucleoside transporters such as those in the SLC superfamily [25].

Figure 5.1. Mechanism of HIV nucleoside/nucleotide reverse transcriptase inhibitor action. NRTIs (red) are administered in their unphosphorylated forms (with the exception of AZT, which is given as a monophosphate) and are transported into cells by SLC nucleoside transporters. NRTIs are subsequently phosphorylated (indicated by the "P" symbol) by intracellular kinases to their active triphosphates. Incorporation of triphosphorylated NRTIs by HIV reverse transcriptase (RT) into a DNA chain will inhibit HIV RT because of its inability to form 3' to 5' phosphodiester bonds (bottom). NRTI-triphosphates may also be transported out of the cell by ABC transporters.

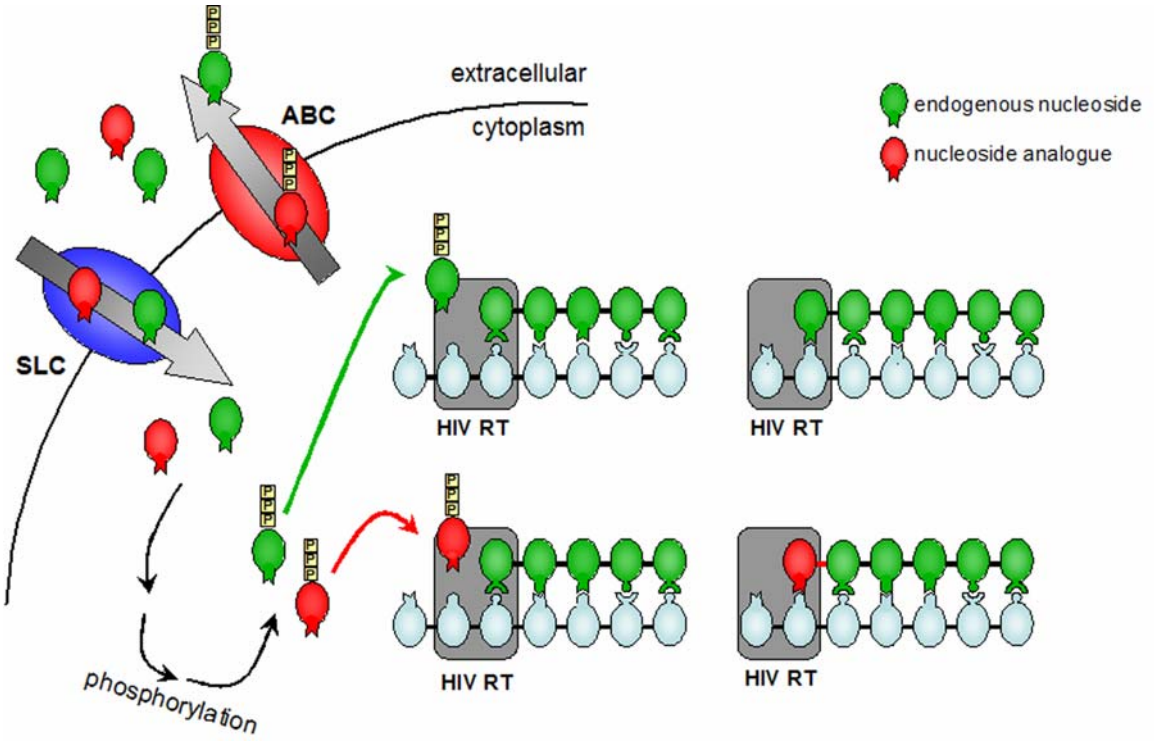


Table 5.1. HIV nucleoside- and nucleotide-analogue reverse transcriptase inhibitors.

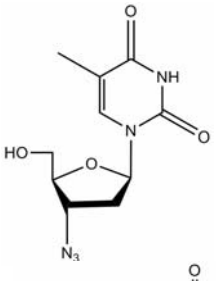
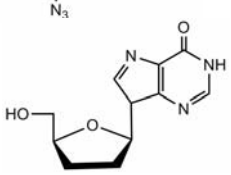
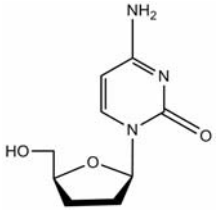
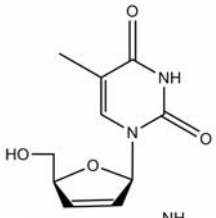
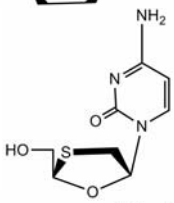
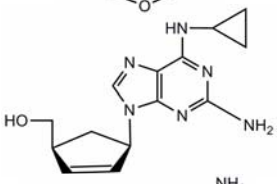
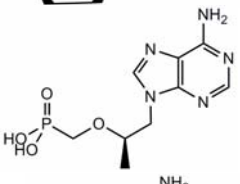
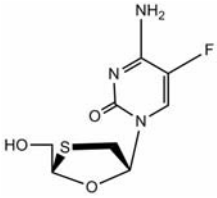
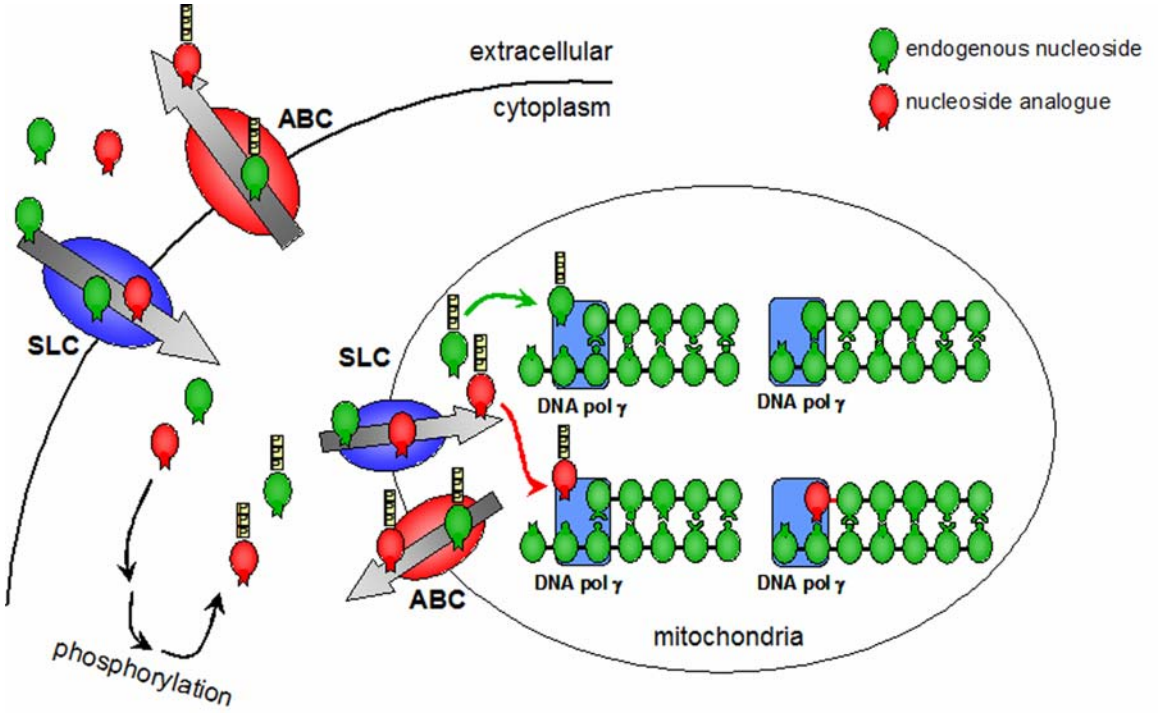
Drug name	Common name	Analogue of	Structure	Approved in
AZT	zidovudine	thymidine		1987
ddI	didanosine	adenosine		1991
ddC	zalcitabine	cytidine		1992
d4T	stavudine	thymidine		1994
3TC	lamivudine	cytidine		1995
ABC	abacavir	guanosine		1998
PMPA	tenofovir	adenosine		2001
FTC	emtricitabine	cytidine		2003

Table 5.2. Nucleoside analogues used in antiviral and anticancer therapy (adapted from [26, 27]).

Nucleoside	Analogue drug	Clinical use	Derivatives	Type of derivative
adenosine	fludarabine	leukemia		
	cladribine	leukemia	clofarabine	fluorinated analogue
	vidarabine	herpes		
cytidine	cytarabine	leukemia	DMDC (2-deoxy-2-methylidenecytidine)	methylated analogue
	gemcitabine	solid tumors		
	troxacitabine	leukemia, solid tumors		
	cidofovir	cytomegalovirus		
uridine	idoxuridine	herpes		
	trifluridine	herpes		
uracil	5-fluorouracil	solid tumors	capecitabine	prodrug
			floxuridine	fluorinated analogue
thymidine	telbivudine	hepatitis B		
guanosine	acyclovir	herpes	valacyclovir	prodrug
	ganciclovir	cytomegalovirus	valganciclovir	prodrug
	penciclovir	herpes	vamciclovir	prodrug
	adefovir (bis-POM PMEA)	hepatitis B		
	ribavirin	hepatitis C		
	entecavir	hepatitis B		

The widespread availability of nucleoside analogues and other anti-HIV drugs has changed the classification of AIDS from an acute, life-threatening illness to a chronic condition [28]. As such, HIV patients are now taking antiretroviral regimens for years, even decades. With this long-term exposure comes a host of uncharacterized side effects, including several which seem to be caused by nucleoside analogues, such as lactic acidosis, peripheral neuropathy, and lipodystrophy [29]. These disorders are thought to be caused by the depletion of mitochondrial DNA which occurs through NRTI inhibition of DNA polymerase gamma, the enzyme responsible for replication of the mitochondrial genome [30] (see Figure 5.2). It has been proposed that the subsequent depletion of mitochondrial RNA and mitochondrial proteins involved in oxidative phosphorylation leads to mitochondrial dysfunction [31], which has been demonstrated in a number of cell culture systems including human HepG2 cells [32]. This appears to be a similar mechanism to the accumulation of mutations in and oxidative damage to mitochondrial DNA which occurs during the normal aging process [33]; indeed, age is a predictor of disease progression in HIV patients [34], indicating that nucleoside analogue exposure may compound the effects of aging on mitochondrial DNA.

Figure 5.2. Mechanism of HIV nucleoside/nucleotide reverse transcriptase inhibitor mitochondrial toxicity. NRTIs (red) are administered in their unphosphorylated forms (with the exception of AZT, which is given as a monophosphate) and are transported into cells and intracellular organelles such as mitochondria by SLC nucleoside transporters. NRTIs are subsequently phosphorylated (indicated by the "P" symbol) by intracellular kinases in the cytoplasm or in mitochondria to their active triphosphates. Incorporation of triphosphorylated NRTIs by DNA polymerase gamma (DNA pol γ) into a mitochondrial DNA chain will inhibit DNA pol γ because of its inability to form 3' to 5' phosphodiester bonds (bottom). NRTI-triphosphates may also be transported out of the mitochondria or cell by ABC transporters.



The lipodystrophy syndrome experienced by HIV patients with extended exposure to nucleoside analogue drugs is a broadly-defined disorder which may include lipoatrophy (fat loss) of the face and extremities, lipohypertrophy (fat accumulation) in the neck, trunk, and abdomen, and insulin resistance and hyperlipidemia [35]. The methodologies for quantifying lipoatrophy and lipohypertrophy vary between studies and can be largely subjective, but estimates of the incidence of NRTI-associated lipodystrophy range from 30-62% [36], with lipoatrophy occurring more frequently in patients receiving stavudine and didanosine [35]. Lipoatrophy itself has been associated with mitochondrial DNA depletion and decreased mitochondrial mass in subcutaneous adipocytes collected from HIV patients on HAART regimens which included stavudine, and to a lesser extent zidovudine [37].

The mitochondrial toxicities of the anti-HIV nucleoside analogues seem minor compared to those experienced by hepatitis B patients who received the investigational nucleoside analogue fialuridine (FIAU) in 1995. Out of 15 patients, seven experienced severe hepatotoxicity with lactic acidosis; five of these patients died, and two survived following liver transplantation [38]. Electron microscopy images revealed abnormal mitochondria [38], and it was subsequently shown in HepG2 cells that FIAU-triphosphate inhibits DNA polymerase gamma by preventing chain elongation, thereby depleting the pool of mitochondrial DNA and causing the clinically observed toxicities [39]. It was further demonstrated that mitochondrial expression of the equilibrative nucleoside transporter 1 (ENT1, encoded by *SLC29A1*) resulted in increased FIAU uptake into mitochondria, enhancing its mitochondrial toxicity *in vitro* [40]. In mice, ENT1 lacks the signal which localizes the protein to the mitochondria, explaining why FIAU toxicities

were not observed in animal studies [41]. The authors of the ENT1 studies suggest that this transporter may also play a role in the mitochondrial toxicities of anti-HIV nucleoside analogue drugs [40].

The efflux transporter MRP4 (encoded by *ABCC4*) has also been implicated in the transport of nucleosides and nucleotides. *In vitro*, it has been demonstrated that zidovudine [42, 43], indinavir [44], and PMEA [42, 45] are MRP4 substrates, and *Mrp4*-/- mice exposed to the nucleoside analogue PMEA were protected from tissue-specific toxicities [45]. A polymorphism in the 3'-UTR of *ABCC4* has also been associated with increased intralymphocytic concentrations of lamivudine-triphosphate in HIV patients [46]. We have also recently characterized a non-synonymous polymorphism in *ABCC4* which decreases MRP4 expression and function *in vitro* [42]. In the present study, we investigate the mechanism of the mitochondrial toxicity of nucleoside analogue drugs fialuridine and stavudine and determine the effects of ENT1 and ABCC4 expression on stavudine mitochondrial toxicity. We also examine the relationships between *ABCC4* polymorphisms, adipose MRP4 RNA expression, and the occurrence of lipodystrophy in HIV-positive subjects receiving stavudine-containing HAART regimens. The findings of this study will allow us to evaluate the effects of polymorphisms on transporter expression and function in adipocytes, and the role of adipocyte transporters in nucleoside analogue-associated mitochondrial toxicities such as lipodystrophy.

5.3. Materials and methods

Mitochondrial isolation

HepG2 cells (UCSF Cell Culture Facility, San Francisco, CA) were cultured to confluency, then trypsinized and counted. If cells were transfected, Lipofectamine 2000 (Invitrogen) was used following the manufacturer's directions, and cells were allowed to express the transporter for 24 hr before mitochondria were isolated. Reagents used to isolate mitochondria were included in the MitoIso Kit (Sigma-Aldrich, St. Louis, MO) in accordance with the manufacturer's directions. Briefly, cells were washed in PBS, then resuspended in 1X extraction buffer with protease inhibitors at a concentration of 1 ml per $2x10^7$ cells and incubated on ice for 15 min. Cells were homogenized in a 1 ml Dounce homogenizer and tight pestle for 25 strokes on ice, then centrifuged at 600x*g* for 10 min at 4˚C. The resulting supernatant was centrifuged at 3500x*g* for 10 minutes at 4˚C, and the mitochondrial pellet was resuspended in 1X storage buffer, in one-tenth the volume of extraction buffer used. Yield was evaluated by BCA protein quantification assay (Pierce/ThermoFisher, Rockford, IL). Mitochondria were stored at 4˚C and were used for functional assays within three days of isolation.

Western blotting

HepG2 cells or isolated mitochondria were lysed in 150 mM NaCl, 1% Igepal CA-630 (Sigma-Aldrich) for 30 min on ice. Lysates were centrifuged at 12,000x*g* at 4˚C for 20 min and supernatants were collected. Proteins were separated by gel electrophoresis using 4-12% Bis-Tris gels (NuPage, Invitrogen, Carlsbad, CA) and transferred to 0.2 μm nitrocellulose membranes (BioRad, Hercules, CA). Blots were probed with 1:50 $M_4$1-10

MRP4 antibody (Covance/Signet, Princeton, NJ) or 1:100 ENT1 antibody (Proteintech Group, Chicago, IL), followed by 1:5000 anti-rat or 1:10,000 anti-mouse HRP-conjugated secondary, respectively. Immunoreactive proteins were visualized using an ECL chemiluminescent system (Millipore, Billerica, MA).

JC-1 assay

The JC-1 assay was used to determine the purity and yield of intact mitochondria from the MitoIso Kit by measuring the mitochondrial membrane potential. Reagents were from the Isolated Mitochondria Staining Kit (Sigma-Aldrich) and were used according to the manufacturer's instructions. Briefly, 1 μg of mitochondria in 1X storage buffer were centrifuged at 3500x*g* for five min, then resuspended in 10 μl 1X storage buffer. Ninety microliters of 1X JC-1 assay buffer, with or without 100 μM quercetin, were added per well, and fluorescence (excitation: 490 nm, emission: 590 nm) was measured over the course of 10 min, with readings taken approximately every 15 sec. The fluorescence of a blank (no protein) sample was subtracted from each data point, and the fluorescence of the t_0 data point was subtracted from the fluorescence of every data point thereafter for each sample.

DNA polymerase assay

The procedure developed for the quantitation of DNA polymerase activity was adapted from Banki and Anders [47] and Kapsa *et al.* [48]. A protein aliquot (20 μg) was centrifuged at 3500x*g* for five min, and resuspended in 100 μl of 1X polymerase assay buffer (50 mM Tris, 4 mM KCl, 7.5 mM Tris succinate, 5 mM potassium phosphate, 7

mM magnesium chloride, 7 mM sodium pyruvate, 0.45 mM sodium maleate, 22.5 nM each dATP, dCTP, dGTP) and in some cases, 50 μM fialuridine or stavudine. Mitochondria or whole cell homogenate were incubated at 37˚C for 10 min. ^{3}H-dTTP was added to a final concentration of 2 μCi/ml and the reaction was allowed to proceed at 37˚C. The polymerase reaction was stopped by the addition of 20 μg glycogen (Roche) and 400 μl ice-cold TCA-NaPP (20% w/v trichloroacetic acid, 40 mM sodium pyrophosphate) to each 100 μl sample. DNA was precipitated during a five min incubation on ice, and pelleted by centrifugation at 14,500x*g* for 30 min at 4˚C. The pellet was washed twice in 300 μl ice-cold TCA-NaPP, followed by centrifugation at 14,500x*g* for 10 min at 4˚C. The resulting pellet was resuspended in 150 μl water and added to 2 ml Ecolite scintillation fluid (MP Biomedicals, Solon, OH) for evaluation of radioactivity.

Adipose tissue collection and storage

Adipose tissue was collected either as surgical waste from HIV-negative individuals (yield: several grams, snap-frozen in liquid nitrogen and stored at -80˚C), or as 3 cm punch biopsies from HIV-positive BHIVE subjects (lower trunk area; yield: 50 mg, stored at -80˚C in RNALater, Invitrogen). The BHIVE study was approved by the appropriate human research committees at both California Pacific Medical Center, where tissue collection was carried out, as well as at UCSF; approval was not needed for the adipose tissue collection from HIV-negative individuals, since samples would otherwise have been discarded as surgical waste.

RNA extraction from adipose tissue

Approximately 150 mg of adipose tissue from HIV-negative subjects were homogenized using a motorized homogenizer and Omni Tip soft tissue probes (Thermo Fisher Scientific, Waltham, MA) in 1 ml Trizol reagent (Invitrogen). The punch biopsy samples from BHIVE subjects were pulverized using five passes of the handheld BioPulverizer apparatus (Biospec Products, Bartlesville, OK) on dry ice, then vortexed vigorously in 0.4 ml Trizol reagent. Samples were incubated at room temperature for 15 min. Chloroform (0.2 volumes) was added and samples were shaken vigorously for 15 sec, followed by a three min incubation at room temperature. Samples were centrifuged at 12,000x*g* for 15 min at 4˚C. The upper lipid layer was discarded, then the aqueous phase was transferred to a new tube and 0.5 volumes Trizol was added for a second extraction (necessary because of the high lipid content of adipose tissue). After vortexing, 0.1 volumes chloroform were added, followed by shaking, incubation, and centrifugation as above. Following collection of the aqueous phase, 20 μg glycogen (Roche Applied Sciences, Indianapolis, IN) was added to the BHIVE sample only (because of small tissue sample size). RNA was precipitated with 0.5 volumes isopropanol, and collected by centrifugation at 12,000x*g* for 10 min at 4˚C following a 10 min incubation at room temperature. RNA pellets were washed in 1 volume 75% ethanol and collected by centrifugation at 7500x*g* for five min at 4˚C. Pellets were briefly air-dried, then resuspended in water, normalized to 100 ng/μl, and stored at -80˚C.

DNA extraction from adipose tissue

The remaining aqueous phase from the first Trizol extraction above was discarded and the interface was collected. Back Extraction Buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1 M Tris base, 0.5 volumes) was added to the interface and mixed by inversion. Samples were incubated for 10 min at room temperature, then centrifuged at 12,000x*g* for 15 min at 4˚C. The upper phase was collected and 1 μg glycogen (Roche) was added. DNA was precipitated by addition of 0.4 volumes isopropanol. After five min incubation at room temperature, DNA was pelleted by centrifugation at 12,000x*g* for 10 min at 4˚C. Pellet was washed three times with 1 volume 75% ethanol for 15 min, vortexing every five min, and collected by centrifugation at 12,000x*g* for five min at 4˚C. Pellet was air-dried briefly, then resuspended in 0.05 volumes of 8 mM NaOH and heated at 55˚C for two min to dissolve. The solution was centrifuged at 12,000x*g* for 10 min at 4˚C, and the supernatant was transferred to a new tube and neutralized with 1 M HEPES and 100 mM EDTA. Samples were normalized to 10 ng/μl with water and stored at -80˚C.

Quantification of RNA expression

RNA was reverse-transcribed by M-MLV RT (Promega Corporation, Madison, WI) according to the manufacturer's instructions, using random hexamers as primers. Gene expression was measured by quantitative real-time PCR (TaqMan) on a PRISM 7700 or 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each reaction contained 1X TaqMan buffer, 5.5 mM $MgCl_2$, 200 μM dNTP, 0.625 U AmpliTaq Gold, 500 nM each primer, and 200 nM probe in a final volume of 25 μL (7700) or 10 μl (7900 HT). PCR conditions were 12 minutes at 95ºC, followed by 45

cycles of 15 sec at 95ºC and 1 min at 60ºC. All samples were normalized to expression of the human control gene β-glucuronidase (hGUS).

Genotyping

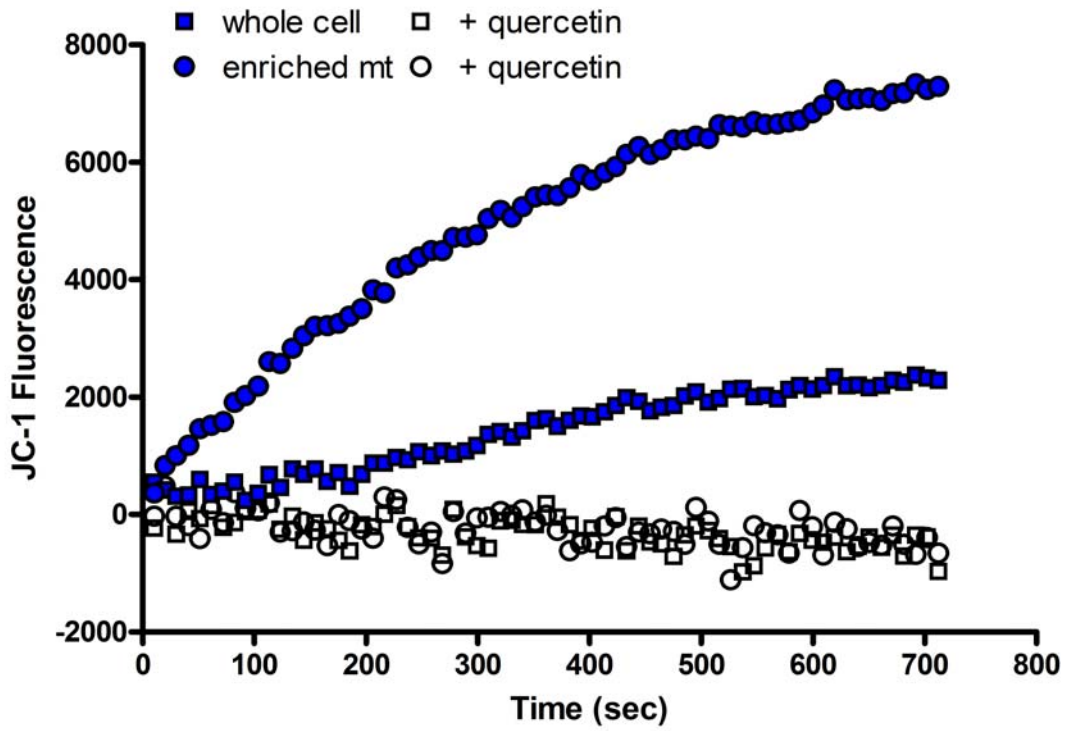
Genotyping was performed using commercially available TaqMan assays (Applied Biosystems, Foster City, CA) for the 559G>T (G187W) and 4131G>T polymorphisms in *ABCC4*. Ten nanograms of DNA were used per 5 µl reaction with 0.5X primer/probe and 1X TaqMan Universal PCR Master Mix, No AmpErase: UNG buffer (Applied Biosystems). Reaction conditions consisted of a 10 min hold at 95˚C, followed by 40 cycles of 15 sec at 92˚C followed by 1 min at 60˚C. Amplification was performed in 384-well plates and fluorescence was measured on a 7900 HT system (Applied Biosystems). Genotypes were automatically called with 95% confidence or higher by SDS version 2 (Applied Biosystems). Negative controls and random sample duplicates were included on each plate for quality control purposes.

5.4. Results and Discussion

The HepG2 cell line, derived from human hepatoma, is widely used as a model to investigate mitochondrial toxicity because of their enrichment in mitochondria compared to other available cell lines [49]. For this reason, as well as the rate of growth in culture and the human origin of this cell line, we used HepG2 cells for this study. Mitochondrial isolation was carried out using differential centrifugation steps which yielded purified mitochondria. The purity of the preparation and the intactness of the mitochondrial membranes (a reflection of mitochondrial health) were evaluated using the JC-1 assay.

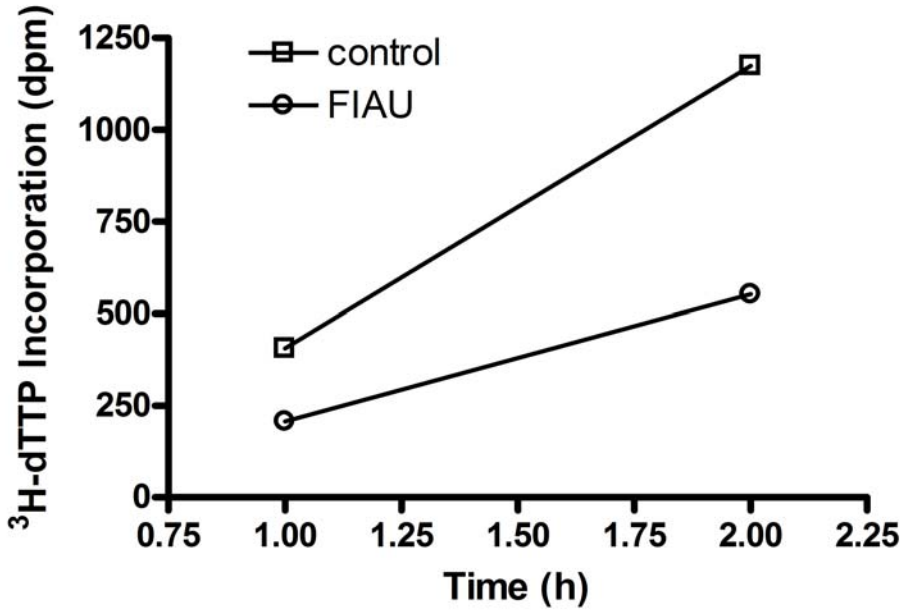
JC-1 is a cationic carbocyanine dye which accumulates in mitochondria in the presence of positive membrane potential, exhibiting a red fluorescence which can be quantified [50]. As shown in Figure 5.3, the fluorescence of the enriched mitochondrial preparation from HepG2 cells is substantially greater than that of the HepG2 cell homogenate. Quercetin, a compound which abrogates mitochondrial membrane potential, decreases the JC-1 fluorescence in both the whole cell homogenate and enriched mitochondria samples (Fig. 5.3).

Figure 5.3. Evaluation of mitochondrial membrane potential by JC-1 fluorescence. The cationic dye JC-1 emits red fluorescence in the presence of positive mitochondrial membrane potential, which is higher in the enriched mitochondrial sample (circles) compared to the whole cell HepG2 homogenate (squares). Quercetin (open symbols) abolishes mitochondrial membrane potential, resulting in low JC-1 fluorescence. Each point in this time course represents the average JC-1 fluorescence of a sample (whole cell homogenate or purified mitochondria, with or without quercetin) collected from a single mitochondrial isolation.



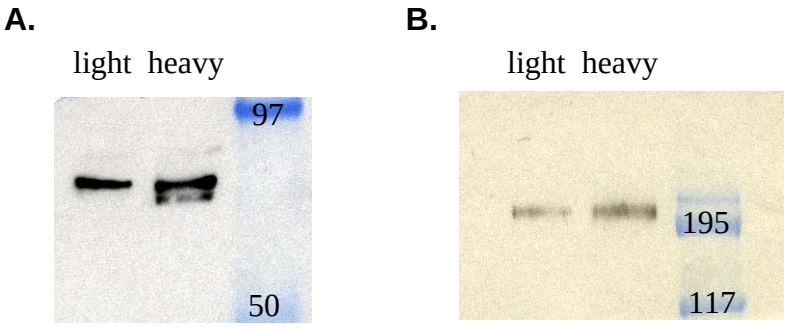
The nucleoside analogue drugs exert their pharmacological activity via inhibition of viral reverse transcriptases or endogenous DNA polymerases [30]. The toxicities (lipodystrophy, peripheral neuropathy, lactic acidosis) observed with fialuridine and stavudine exposure are thought to be a result of inhibition of DNA polymerase gamma, which is responsible for replication of the mitochondrial genome [30]. To test this theory, an assay to measure the activity of DNA polymerase gamma was carried out. Mitochondria isolated from HepG2 cells were incubated with the reagents needed for DNA synthesis, including radiolabeled dTTP. Polymerase function was examined in the presence or absence of fialuridine, an established inhibitor of DNA polymerase gamma. Figure 5.4 demonstrates the effects of fialuridine exposure on DNA polymerase gamma activity over two hours. The level and rate of ^{3}H-dTTP incorporation into newly synthesized mitochondrial DNA is decreased in the presence of fialuridine (Fig. 5.4).

Figure 5.4. DNA polymerase gamma activity in HepG2 mitochondria. Purified mitochondria from HepG2 cells were allowed to synthesize DNA *in vitro*. Each point on the graph below represents the ^{3}H-TTP decay measured in a single HepG2 mitochondrial sample after 1 or 2 hours in the presence (FIAU) or absence (control) of fialuridine, a nucleoside analogue which inhibits DNA polymerase gamma [39]. These results are representative of three experiments



The nucleoside transporter ENT1 has been shown to play a crucial role in fialuridine uptake into mitochondria *in vitro* [40]. However, the role of efflux transporters at the mitochondrial membrane in the development of mitochondrial toxicities has not been examined. To determine if the uptake transporter ENT1 and the efflux transporter MRP4 (which has previously been implicated in nucleoside analogue transport [42, 44-46]) are localized to the mitochondrial membrane in HepG2 cells, Western blotting was used to examine protein expression. As shown in Figure 5.5, both transporters are detected in the light (plasma membrane) and heavy (mitochondria) fractions of a HepG2 homogenate, indicating expression at both locations.

Figure 5.5. Transporter expression in HepG2 mitochondria. HepG2 homogenate was centrifuged at 1000x*g* to give a "light" fraction which includes plasma membranes. The resulting supernatant was centrifuged at 3500x*g* to give a "heavy" fraction, including mitochondria. Western blotting was performed on the subcellular fractions to examine expression of endogenous ENT1 (A) and MRP4 (B) transporter proteins. The expected molecular weight of ENT1 is 72 kDa, and the expected molecular weight of MRP4 is 190 kDa.



Although both ENT1 and MRP4 are endogenously expressed on the plasma and mitochondria membranes of HepG2 cells, a higher expression level was desired in order to ensure that the effects of these transporters on mitochondrial toxicity would be large enough to be observed. Therefore, HepG2 cells were transiently transfected with ENT1 or MRP4 in the pcDNA3.1 mammalian expression vector. To make certain the transfection reagents or transporter expression did not affect mitochondrial membrane potential, a JC-1 assay was performed. As shown in Figure 5.6, transient transfection of ENT1 and MRP4 did not affect HepG2 mitochondrial membrane potential. Preliminary

results from a DNA polymerase activity assay using the mitochondrial preparations examined in Fig. 5.6 showed that stavudine decreased polymerase activity (similar to fialuridine), but the presence of transiently-transfected ENT1 and MRP4 had no effect on polymerase activity (Fig. 5.7).

Figure 5.6. Evaluation of mitochondrial membrane potential following transient transporter transfection by JC-1 fluorescence. The cationic dye JC-1 emits red fluorescence in the presence of positive mitochondrial membrane potential, which is higher in the enriched mitochondrial samples (mt) compared to the whole cell HepG2 homogenate. Quercetin (open symbols) abrogates mitochondrial membrane potential, resulting in low JC-1 fluorescence. Each point in this time course represents the average JC-1 fluorescence of a sample (whole cell homogenate or purified mitochondria, with or without quercetin) obtained from transfected or untransfected HepG2 cells during a single mitochondrial isolation and is representative of two experiments.

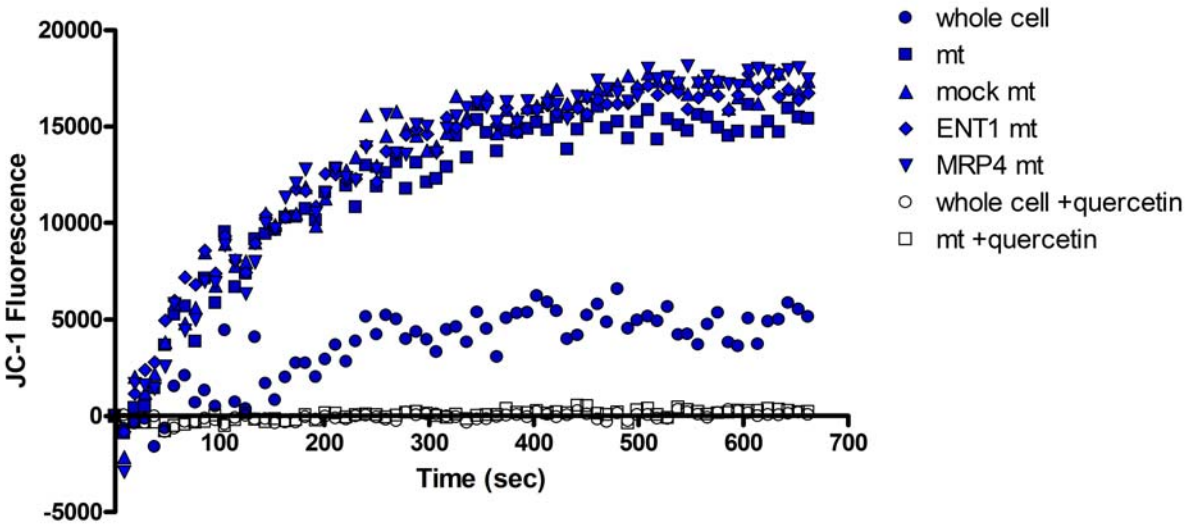
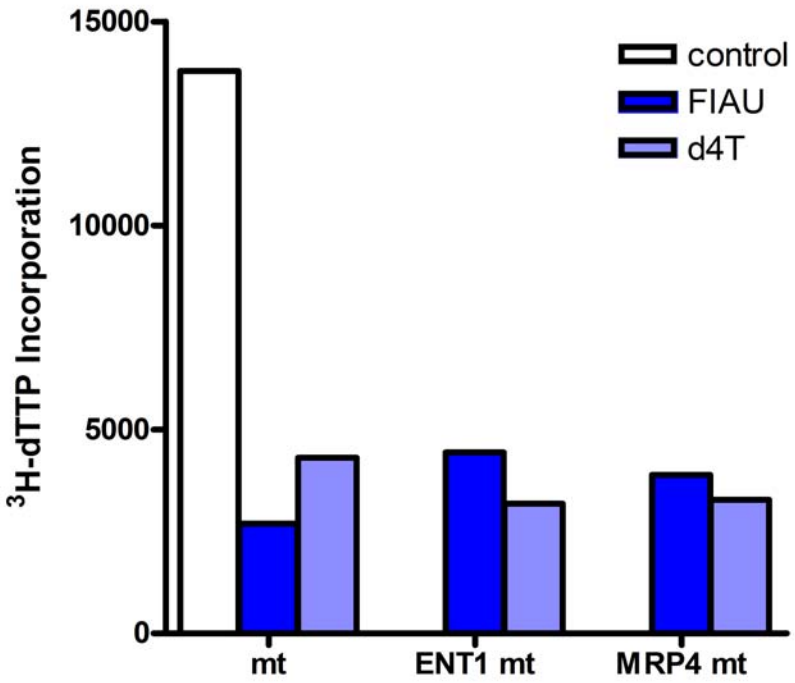


Figure 5.7. Effect of transporters on DNA polymerase gamma activity and inhibition. DNA polymerase activity of HepG2 mitochondria transfected with ENT1 or MRP4 was evaluated in the presence and absence of the nucleoside analogues fialuridine (FIAU) and stavudine (d4T). Each bar represents the ^{3}H-TTP decay measured in mitochondria from untransfected, ENT1-transfected, or MRP4-transfected HepG2 cells after 3 hr in the presence (FIAU, d4T) or absence (control) of nucleoside analogues. The amount of ^{3}H-dTTP incorporated into newly synthesized mitochondrial DNA was decreased upon exposure to fialuridine and stavudine, and the presence of ENT1 and MRP4 did not affect the inhibitory properties of these drugs on DNA polymerase gamma.



The results of the initial experiments examining the effects of transporter expression on nucleoside analogue inhibition of DNA polymerase gamma should be considered preliminary for several reasons. First, although HepG2 cells are useful for observing mitochondrial toxicity, the yield of mitochondrial isolations is low enough that the number of replicates that can be taken is severely limited; therefore, we are unable to ascertain the experimental variability in this system. This may be avoided by using stably transfected cell lines, so that mitochondria may be isolated from a greater number of cells. The generation of HepG2 cell lines stably transfected with ENT1 and MRP4 is currently in progress. Secondly, the use of ENT1 and MRP4 inhibitors (e.g. NBMPR and MK-571, respectively) would provide crucial information as to the functional effects of these transporters in this experimental system; these inhibitors should be included in future experiments in order to make conclusive statements about the influence of ENT1 and MRP4 on nucleoside analogue-induced mitochondrial toxicity.

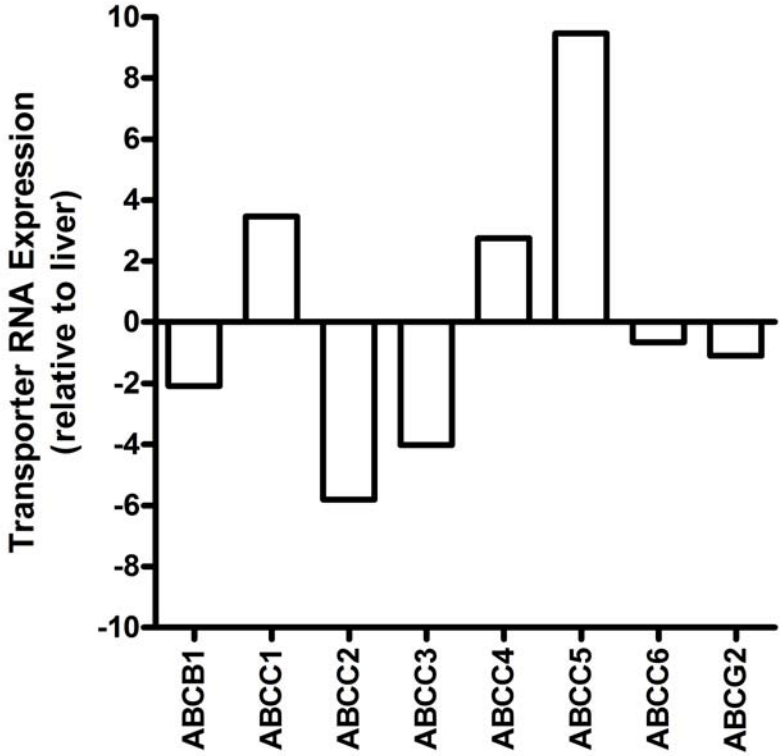
Third, the clinical relevance of the HepG2 studies has not been established. For example, although the molecular mechanism of nucleoside analogue toxicity (as ascertained by the *in vitro* HepG2 work) appears to be inhibition of DNA polymerase gamma, the overall cellular effects may differ between hepatocytes and adipocytes. To address this concern, we attempted to perform similar experiments in NIH3T3-L1 cells. This cell line is derived from murine fibroblasts which may be differentiated into adipocytes following hormone treatment. However, the differentiation process takes 10-14 days, and the consequences of using a lipid-based transfection reagent post-differentiation are unknown. NIH3T3-L1 cells have the added disadvantages of having

fewer mitochondria than HepG2 cells, and being of non-human origin. For these reasons, we used HepG2 cells to study the mitochondrial toxicity of nucleoside analogues *in vitro*.

The translation of experimental findings in HepG2 cells into the clinical phenotype of nucleoside analogue-induced lipoatrophy is difficult to assess, due to confounding factors (e.g. patient age, ethnicity, adherence) which are inherent to a clinical population. We assessed the relationships between transporter genotype, expression, and occurrence of lipodystrophy using adipose samples from two populations. The first, an HIV-negative population, was collected by Dr. Hobart Harris in the Department of Surgery at UCSF. These samples were gathered during surgery from the abdominal region. The second consisted of HIV-positive patients in the Biobank for HIV Evaluation (BHIVE) cohort, enrolled by Dr. Stephen Becker of the Pacific Horizon Medical Group. These samples were punch biopsies, taken during the baseline visit of the observational BHIVE study.

To establish the presence of transporters in adipose tissue, we measured the RNA expression of a panel of ABC transporters – ABCB1, ABCC1-6, and ABCG2 – in pooled adipose RNA samples and compared adipose expression to the expression in RNA from healthy liver tissue. Figure 5.8 shows the results of RNA expression as determined by quantitative RT-PCR. ABCC1, ABCC4, and ABCC5 appear to be more highly expressed in adipose tissue compared to liver samples (Fig. 5.8). As expected, ABCC2 expression is much higher in liver tissue than in adipose, as it plays an important role in hepatobiliary excretion [51].

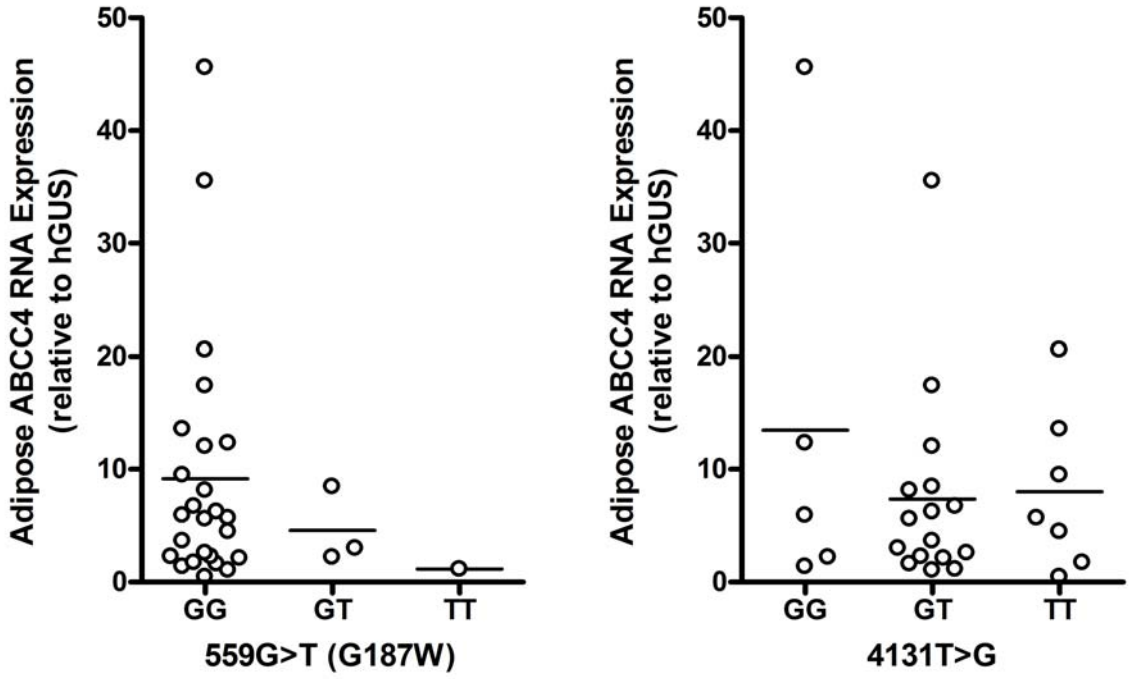
Figure 5.8. RNA expression of ABC transporters in adipose tissue. The expression of each transporter in adipose was normalized to a housekeeping gene (hGUS) to control for inaccurate loading, and is expressed relative to liver expression. Each bar represents a ratio of normalized transporter expression in pooled adipose RNA (n=5) to normalized liver transporter expression (n=1). Negative values indicate expression is higher in liver tissue, and positive values indicate the opposite.



We also investigated the effects of genetic polymorphisms in *ABCC4* which have previously been associated with altered expression (559G>T) or function (559G>T, 4131T>G) [42, 43] on adipose ABCC4 RNA expression. As shown in Figure 5.9, there is a trend between the 559T allele and decreased ABCC4 RNA expression in adipose tissue, although it did not reach statistical significance in either the HIV-negative or HIV-positive populations because of the large interindividual variability in ABCC4 expression. The 4131 polymorphism did not appear to influence ABCC4 adipose expression (Fig. 5.9). We also evaluated the effect of HIV infection on adipose ABCC4 expression (Fig. 5.10), further subdividing those with HIV into two groups – those who were receiving antiretroviral therapy at the time of biopsy, and those who were not. There were no statistical associations between HIV infection or antiretroviral therapy and ABCC4 expression, although the three subjects with the highest ABCC4 expression were all HIV-positive and receiving HAART (Fig. 5.10).

Figure 5.9. Effects of *ABCC4* polymorphisms on adipose ABCC4 RNA expression. ABCC4 was quantified in cDNA synthesized from adipose RNA. Genotypes are plotted against ABCC4 expression, which has been normalized to the housekeeping gene hGUS. Neither of these genotypes is significantly associated with ABCC4 adipose expression, although the 559G>T polymorphism shows a trend for an association with decreased RNA levels. Adipose samples were analyzed from 30 HIV-negative subjects (A) and 20 HIV-positive subjects (B). Samples were analyzed from HIV-positive patients with (open symbol) and without (closed symbol) lipodystrophy. Horizontal lines represent the mean of each group.

A.



B.

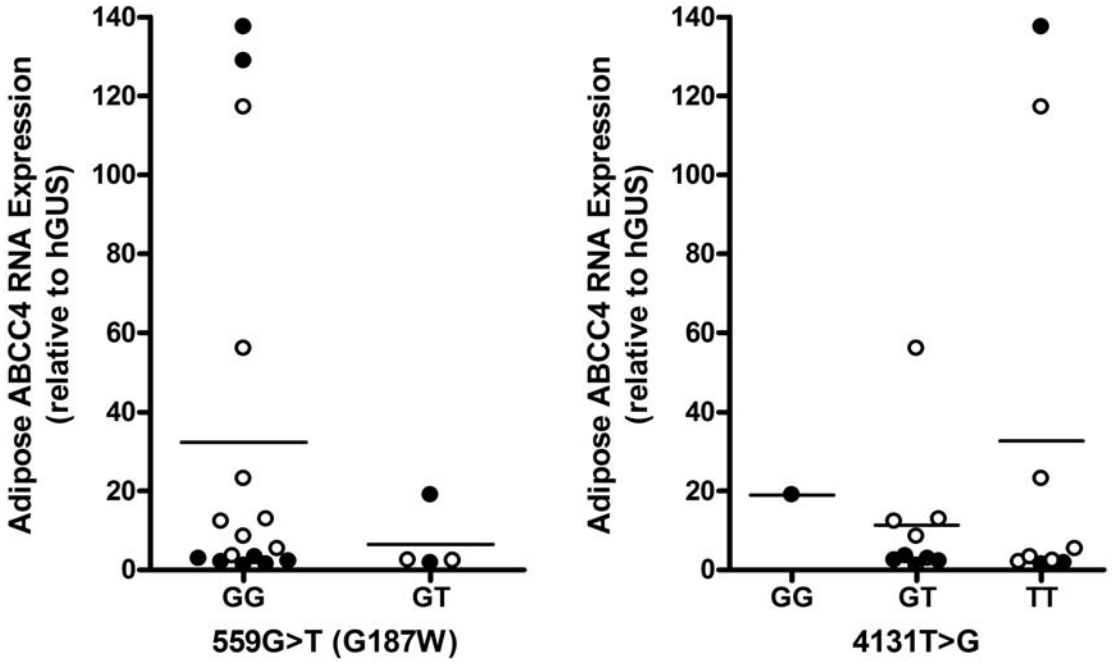
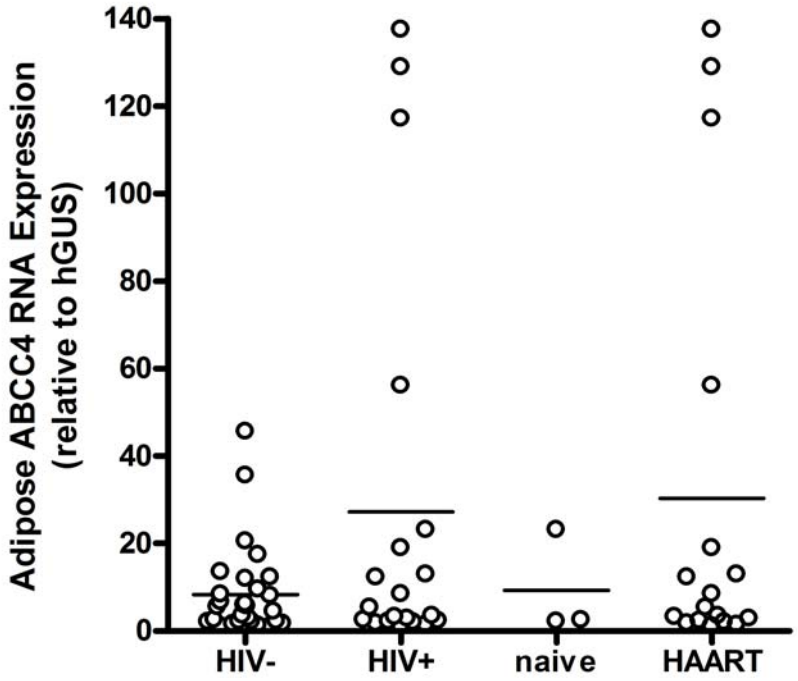

Adipose ABCC4 RNA Expression
(relative to hGUS)
140
120
100
80
60
40
20
0
GG
GT
559G>T (G187W)
Adipose ABCC4 RNA Expression
(relative to hGUS)
140
120
100
80
60
40
20
0
GG
GT
TT
4131T>G

Figure 5.10. The effect of HIV infection and antiretroviral therapy on adipose ABCC4 expression. ABCC4 expression in adipose samples from HIV-negative (n=30) and HIV-positive (n=20) subjects is shown below. HIV-positive individuals are further categorized into those receiving highly active antiretroviral therapy (HAART) or those who were not (naïve) at the time the biopsy was collected. No statistically significant associations were observed between HIV infection or exposure to antiretroviral drugs and ABCC4 expression. Horizontal lines represent the mean of each group.

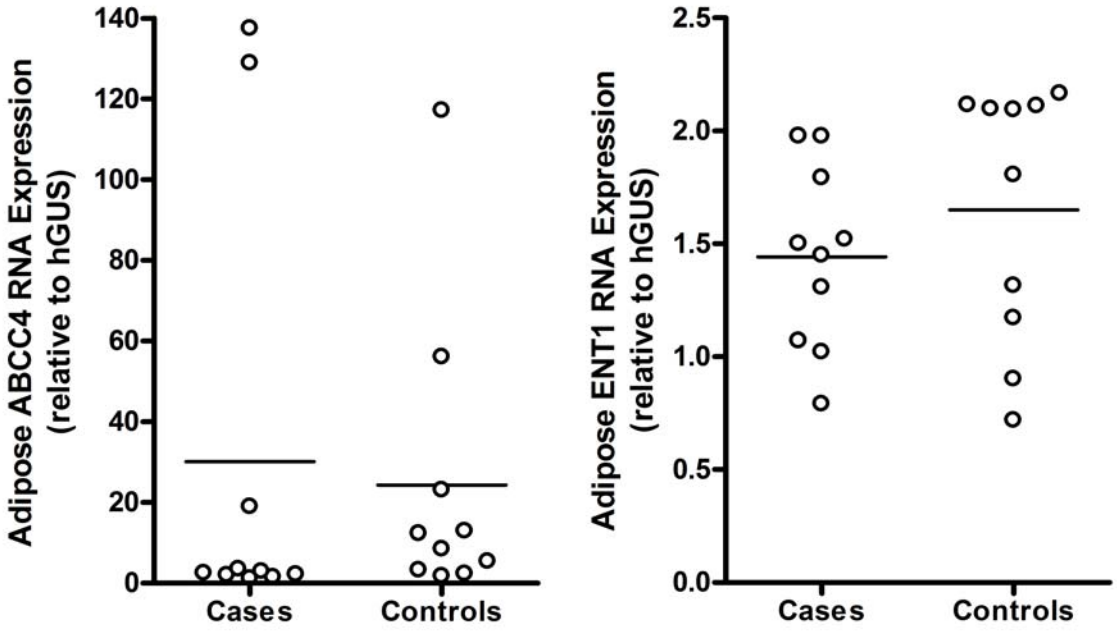


Previous studies have demonstrated the importance of ENT1 in the mitochondrial uptake of the nucleoside analogue fialuridine and its subsequent toxic effects [40]. It was suggested that this transporter, as well as ABC efflux transporters, may influence the amount of nucleoside analogue drugs which accumulate in mitochondria [40], which in turn might translate into susceptibility to mitochondrial toxicities such as lipodystrophy upon exposure to anti-HIV nucleoside analogues. It follows that individuals who express high levels of the uptake transporter ENT1 and/or low levels of the efflux transporter MRP4 would experience lipodystrophy more frequently, due to elevated nucleoside analogue concentrations in adipocytes. We investigated this hypothesis using adipose samples from the BHIVE cohort.

Ten subjects who had experienced clinician-reported lipodystrophy while on a stavudine-containing regimen (it has been widely reported that stavudine has more severe lipodystrophic effects than other nucleoside analogues) were classified as cases. Subjects were not necessarily experiencing lipodystrophy or on a stavudine-containing regimen at the time of biopsy. Ten additional subjects who were regimen-matched to the cases, but did not experience lipodystrophy, were classified as controls. The expression of ENT1 and ABCC4 in adipose tissue is plotted for cases and controls in Figure 5.11. There is no statistical difference between ENT1 or ABCC4 expression in cases or controls. No information about viral or immunologic response or HAART regimen at the time of biopsy is taken into account in this analysis, which therefore assumes that none of these factors affect adipose ENT1 or ABCC4 expression. The coefficients of variation for adipose ENT1 RNA expression were 28% and 34%, and for MRP4 expression, 181% and 150%, for cases and controls, respectively. To detect a 25% difference in ENT1

expression with a power of 0.8, 17 cases and 17 controls would be needed. However, because of the degree of interindividual variability in adipose ABCC4 RNA expression in these samples, substantially larger populations will be necessary to detect an expression difference of 25%, on the order of several thousand individuals per group. If the variance in expression decreases inversely with sample size, the statistical power will be greater than predicted in these power calculations.

Figure 5.11. Correlation between adipose expression of ENT1 and ABCC4 RNA and occurrence of lipodystrophy. Ten subjects who experienced lipodystrophy while on a stavudine-containing regimen (though not necessarily at the time of adipose biopsy) were studied as cases, while ten regimen-matched controls were used as controls. Horizontal lines represent the mean of each group. No correlation between ENT1 or ABCC4 adipose expression and lipodystrophy occurrence was observed.



Although human *ENT1* is well-conserved, *ABCC4* contains a number of synonymous and non-synonymous polymorphisms, several of which have been reported to influence transporter expression or function [42, 43]. We therefore did a prelimary analysis of associations between the *ABCC4* 559G>T (G187W) and 4131T>G SNPs and the occurrence of lipodystrophy in the BHIVE cases and controls described earlier. Table 5.3 displays the frequencies of genotypes for both SNPs in the cases and controls. No significant association was observed between the 559G>T polymorphism and lipodystrophy. However, the 4131 variant allele was found more frequently in those who experienced lipodystrophy compared to controls ($p<0.05$). This polymorphism was also associated with elevated intracellular concentrations of the nucleoside analogue lamivudine in HIV-positive patients [43], indicating that it may have a functional effect on MRP4. It should be noted that the current sample sizes of 10 cases and 10 controls, we have only 40% power to detect a difference in minor allele frequencies of 0.5. In order to reach 80% power, 19 cases and 19 controls are needed.

Table 5.3. *ABCC4* genotype frequency in lipodystrophy cases and controls.

ABCC4 genotype	Cases	Controls	p-value
559G>T			>0.5
GG	8	8	
GT	2	2	
TT	0	0	
4131T>G			0.036
TT	1	6	
GT	5	4	
GG	3	0	

5.5. Conclusions

The present study evaluated the expression and function of transporters in adipose tissue, and the effects of these transporters on mitochondrial toxicity caused by nucleoside analogues, specifically the antivirals fialuridine and stavudine. We demonstrated the presence of ENT1 and MRP4, both of which have been shown to transport nucleosides and nucleoside analogues, at both the plasma and mitochondrial membranes of the HepG2 cell line. Isolation of mitochondria from these cells allowed for an examination of the mechanism of mitochondrial toxicity that results from exposure to fialuridine and stavudine – namely, the inhibition of DNA polymerase gamma, and the subsequent depletion of mitochondrial DNA. Both of these nucleoside analogues decreased mitochondrial DNA synthesis by DNA polymerase gamma, but the modulation by ENT1 and MRP4 on nucleoside analogue mitochondrial toxicity requires further investigation before conclusions may be drawn.

We also report the expression of ABCC4 in adipose tissue in both HIV-positive and HIV-negative individuals, and a trend towards an association between the *ABCC4* 559G>T (G187W) polymorphism and decreased ABCC4 expression in adipose, supporting previous *in vitro* findings [42]. Although ABCC4 adipose expression was not statistically associated with the occurrence of lipodystrophy in HIV patients receiving stavudine, the *ABCC4* 4131T>G minor allele was more common in patients who experienced lipodystrophy compared with controls. This polymorphism has also been associated with intralymphocytic nucleoside analogue concentrations in HIV patients [43]. The current analysis, however, is limited by the number of BHIVE subjects who meet the inclusion criteria (stavudine-containing regimen, physician-reported

lipodystrophy). Future studies might utilize quantitative measures of lipodystrophy (e.g. anthropometric measurements) rather than adipose biopsies, which would increase the sample size and provide greater statistical power for similar analyses. The findings reported here suggest that polymorphisms in *ABCC4* may be correlated with both adipose ABCC4 expression and with the incidence of HIV nucleoside analogue-associated lipodystrophy.

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Chapter 6. Summary

A wide range of organisms, from bacteria to yeast to humans, share similar mechanisms through which biological processes necessary for survival are performed [1]. One of these is the protection of an organism against exposure to environmental toxins, which is accomplished in part through the activity of membrane transporter proteins [2]. The first of these proteins to be characterized was P-glycoprotein (P-gp), encoded by *ABCB1*. Overexpression of P-gp, a xenobiotic efflux transporter, was detected in drug-resistant cells and was shown to be a factor in the development of multidrug resistance – the expression of cell-surface P-gp limits the amount of drug which accumulates intracellularly, thereby modulating its pharmacological activity [3].

Such drug resistance is an extension of the natural chemoprotective role of P-gp. Although they are certainly effective at limiting physiological exposure to toxins, P-gp and other efflux transporters in the ABC superfamily also impede the pharmacological action of drugs which are designed to be beneficial. For example, the expression of P-gp has been shown to restrict drug exposure by limiting absorption (gut) and enhancing excretion (liver, kidney) [4], again demonstrating the robustness of this chemoprotective mechanism. In these cases, transporters influence drug pharmacokinetics.

ABC transporters have also been shown to affect drug pharmacodynamics. These effects are due to P-gp expression at the site of pharmacological action [5]. Drug pharmacodynamics may be intended (efficacy) or unintended (toxicity); both efficacy and toxicity are crucial elements of how well a patient tolerates and responds to a medication. The work described in this dissertation focuses on the characterization of ABC

transporter expression and function in peripheral tissues where transporters may have pharmacodynamic effects. In particular, we focus on lymphocytes, where drugs used to treat leukemia and HIV infection act, and adipocytes, where antiviral nucleoside analogue drugs exhibit toxic effects which present as lipodystrophy. The overall goal of this dissertation was to expand upon the current limited body of knowledge concerning the effects of ABC transporters on drug pharmacodynamics, with the hope that our results will inform the design of future investigations into transporter-drug interactions.

In Chapters 2 and 3, we examined P-gp/ABCB1 expression and P-gp function in lymphocytes. As mentioned previously, lymphocytes are targets of anti-cancer and anti-HIV drugs, some of which are known to be substrates of P-gp. Lymphocyte P-gp expression and function have also been used to reflect P-gp in less accessible tissues such as the gut [6, 7] under the assumption that P-gp in all tissues is identical. However, we observed the expression of a half-size P-gp in human primary lymphocytes, which we describe in Chapter 2. This mini P-gp has altered immunoreactivity but normal transport function of several classic P-gp substrates. We also uncovered evidence of a half-sized ABCB1 RNA transcript, and we cloned several putative ABCB1 splice variants from primary human lymphocytes. However, none of the cloned putative splice variants displayed efflux transport function when expressed in a cell culture system. Additional studies are required to determine which, if any, of the cloned transcripts encode the mini P-gp detected in lymphocytes. Nevertheless, our results demonstrate that lymphocyte P-gp may not be identical to the classic P-gp expressed in other tissues.

In Chapter 3, we investigated the effects of the HIV protease inhibitors atazanavir and saquinavir on lymphocyte P-gp/ABCB1 expression and P-gp function as a substudy

of a clinical trial called ASPIRE. The substudy was based on reports of inhibitory effects of HIV protease inhibitors on P-gp function [8] which can affect the pharmacokinetics of concomitant drugs [9]. A number of HIV protease inhibitors have also been shown to be transported by P-gp [10, 11] or to induce P-gp expression [12]. We found no overall changes in lymphocyte ABCB1 expression or P-gp expression or function, although several study subjects experienced an increase in the level of lymphocyte P-gp expression [13]. The interindividual variability in baseline lymphocyte ABCB1/P-gp expression was considerable and may be a result of genetic polymorphisms or environmental factors. Samples are being collected from both healthy volunteers and HIV patients to assess this potential association.

We subsequently undertook a pharmacogenetic study to look for associations between genetic polymorphisms in several candidate genes and HIV outcomes such as viral suppression and immunological recovery following the initiation of antiretroviral treatment (Chapter 4). The candidate genes we considered were involved in HIV antiretroviral drug pharmacokinetics, such as *CYP2B6*, which metabolizes efavirenz and nevirapine [14], or pharmacodynamics, including *ABCB1* and *ABCC4*, which encode two efflux transporters which may modulate intralymphocytic concentrations of antiviral drugs [15-17], and TNFα, which has been implicated in immunologic response to HIV therapies [18]. These pharmacogenetic analyses were carried out in two HIV-positive populations, one in San Francisco (REACH) and one in Uganda (UARTO). We also characterized the population substructure of REACH using ancestry informative markers. We found no positive associations between any of the SNPs and response to antiretroviral therapy, although our statistical power was low due to the relatively small sample sizes.

The ancestral data obtained through subpopulation analysis may be used in future pharmacogenetic studies to investigate the relationship between ethnicity and antiretroviral response in REACH. Toxicity data are currently being collected in these two cohorts and will be focus of additional studies.

While the previous chapter investigated the effects of genetic polymorphisms on drug pharmacodynamics in the context of efficacy, Chapter 5 details a preliminary examination of the expression and functional effects of transporters in adipose tissue, where antiviral nucleoside analogue toxicities occur. Lipodystrophies are experienced by HIV patients who have had extensive exposure to nucleoside analogue drugs, especially stavudine; the toxicity is thought to result from the inhibition of DNA polymerase gamma and subsequent depletion of mitochondrial DNA [19]. We employed *in vitro* assays to investigate the mechanism of nucleoside analogue toxicity in mitochondria isolated from HepG2 cells expressing the nucleoside transporters ENT1 (uptake) and MRP4 (efflux). We also examined the adipose RNA expression of these transporters but found no association with the occurrence of lipodystrophy in patients receiving stavudine. However, we were only able to examine adipose samples from ten lipodystrophic cases, so the work presented here should be considered preliminary. Nevertheless, we uncovered a significant association between an *ABCC4* polymorphism and lipodystrophy, and another polymorphism in *ABCC4* was correlated with adipose ABCC4 expression. Both of these findings warrant further investigation in larger populations, as they could have clinical implications.

The work presented in this dissertation characterized P-gp and other ABC transporters in tissues which are often overlooked, but which may have important

pharmacodynamic roles. In the past, the focus has been on the effects of ABC transporters on drug pharmacokinetics, since this aspect of drug pharmacology is quantifiable and may be easily resolved through dosing changes. Pharmacodynamic studies are often problematic to perform because of the complexities involved in collecting clinical phenotypes.

In particular, the pharmacodynamics of antiviral drugs are complicated by difficulties in distinguishing between host and virus effects. One host factor which may affect antiviral drug pharmacodynamics is the expression and/or function of xenobiotic transporter proteins, including members of the ABC superfamility. Investigations into the pharmacodynamics of antiviral drugs can provide valuable insight into the mechanism through which an antiviral drug reaches its site of action and exerts its effects. The relationships between ABC transporters and antiviral drug pharmacodynamics are complex yet intriguing, and they deserve extensive evaluation, both experimentally and with regard to the clinical applications of experimental findings.

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