Published in final edited form as:

Pharmacogenet Genomics. 2009 February ; 19(2): 129–138. doi:10.1097/FPC.0b013e32831bd98c.

Absorption of Montelukast is Transporter Mediated: a Common Variant of OATP2B1 is Associated with Reduced Plasma Concentrations and Poor Response

Edward B. Mougey, Ph.D.^a, Hua Feng, M.D., M.S.^b, Mario Castro, M.D., M.P.H.^{c,d}, Charles G. Irvin, Ph.D.^{c,e}, and John J. Lima, Pharm.D.^{a,c}

^aNemours Children's Clinic, Pharmacogenetics Center, Nemours Biomedical Research, 807 Children's Way, Jacksonville, FL, 32207-8426, USA

^bPharmacy Practice, University of Florida, Gainesville, FL 32610, USA

^cAmerican Lung Association Asthma Clinical Research Centers, USA

^dDepartment of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA

^eVermont Lung Center, University of Vermont College of Medicine, Burlington, VT 05405-0075, USA.

Abstract

Objectives—(i) determine if montelukast undergoes carrier mediated uptake; (ii) classify the carrier protein(s) responsible for uptake; (iii) identify specific transporters that mediate transport of montelukast; (iv) evaluate whether variation in the gene encoding the transport protein(s) influences the pharmacokinetics and pharmacodynamics of montelukast.

Methods—In vitro permeability studies of montelukast are conducted using Caco-2 cell culture, a standard model of human intestinal drug transport. In vivo plasma concentrations of montelukast in an asthmatic population are determined by HPLC, and genotyping of transport proteins is by LightTyper analysis.

Results—Permeability of montelukast has an activation energy of 13.7 ± 0.7 kcal/mol, consistent with carrier mediated transport. Permeability is saturable at high concentrations of montelukast and follows Michaelis-Menten kinetics. Permeability is subject to competition by sulfobromophthalein, estrone 3-sulfate, pravastatin, taurocholic acid, and cholic acid ($p < 0.05$, % of control; $72 \pm 7 - 86 \pm 7$) and is inhibited by 5–10% citrus juice ($p < 0.05$, maximal inhibition % of control; 31 ± 2). An MDCKII cell line expressing OATP2B1 (coded for by the *SLCO2B1* gene) displays significantly increased permeability of montelukast ($p < 0.05$, % of control; 140 ± 20). A nonsynonymous polymorphism in *SLCO2B1*, (rs12422149; *SLCO2B1*{NM_007256.2}:c.935G>A) associates with significantly reduced plasma concentration in subjects measured on the morning after an evening dose ($p < 0.025$, square root mean transformed plasma concentration $\pm$ SE; c.[935G>A]+[935G]= 3 ± 1 , c.[935G]+[935G]= 7.0 ± 0.9) and differential response as assessed by change in baseline Asthma Symptom Utility Index scores following one month of therapy (delta mean Asthma Symptom Utility Index; c.[935G>A]+[935G] = 0.02 ± 0.01 , $p = 1.0$; c.[935G]+[935G] = 1.0 ± 0.3 , $p < 0.0001$).

Conclusions—Together these observations suggest that the genetics of *SLCO2B1* may be an important variable in determining the pharmacokinetics and the pharmacodynamics of montelukast.

Keywords

Asthma; human OATP2B1 protein; intestinal absorption; leukotriene antagonists; membrane transport proteins; montelukast; pharmacodynamics; pharmacokinetics; single nucleotide polymorphism

Introduction

Montelukast (Singulair™, Merck & Co., Inc., Whitehouse Station, NJ) is a leukotriene receptor antagonist (LTRA) that is available as a once-daily oral formulation used to control symptoms associated with asthma in children and adults. Montelukast selectively inhibits binding of Cys-LTD₄, a potent mediator of airway inflammation and bronchoconstriction, to the cysteinyl leukotriene 1 (Cys-LT₁) receptor [1]. Cys-LTs are synthesized from arachidonic acid released from membrane-phospholipids by cytosolic phospholipase A₂ (cPLA₂) through the action of 5-lipoxygenase and FLAP in response to stimulation [2,3].

Numerous clinical trials have established the efficacy and safety of montelukast [4–6], however the high inter-individual variability in response, both in children and adults, limits the drug's effectiveness [7,8]. It has been estimated that as much as 60 to 80% of the variability in response to asthma drugs may be due to genetic variation [9]. We and others have reported associations between response to leukotriene receptor antagonists and polymorphisms in leukotriene pathway genes [10–14], suggesting that these genetic variants are important contributors to the variability in pharmacodynamic responsiveness to leukotriene receptor antagonists (LTRAs).

The physical and chemical properties of montelukast disfavor absorption by passive diffusion [15]. For example, montelukast is a weak acid with a pK_a of 5.7 [16] indicating that the drug is partially ionized at intestinal pH. Montelukast has a high molecular weight (MW = 586.2 g/mol), and is strongly lipophilic (calculated logarithm of the octanol:water partition coefficient = 8.98) suggesting that once partitioned into the cell membrane its release would be disfavored [17]. Montelukast is highly bound to plasma albumin and is 64% bioavailable [18]--characteristics shared by oral drugs whose transport is mediated at least in part by membrane transport proteins [19].

The aims of the present study were to: (i) determine if montelukast undergoes carrier mediated uptake; (ii) classify the carrier protein(s) that may be responsible for uptake of the drug; and (iii) identify specific transporters that contribute to transport of montelukast. Finally, to determine the clinical relevance of carrier mediated transport; (iv) evaluate whether variation in the gene(s) encoding the transport protein(s) influence the pharmacokinetics and pharmacodynamics of montelukast.

We report that montelukast undergoes carrier mediated transport by both OATP2B1 and OATP1A2 (coded for by the *SLCO2B1* and *SLCO1A2* genes, respectively) and that genetic variation in *SLCO2B1* contributes to variability in its pharmacokinetics and therapeutic response in subjects with asthma.

Methods

Chemicals

Montelukast, sodium salt was purchased from LKT Laboratories, Inc., St. Paul Minnesota and was tritiated to a specific activity of 5 Ci/mmol by exchange (Moravek Biochemicals, Inc, Brea California). The radiochemical purity as assessed by C18 reversed phase HPLC was 97.2%. Other compounds were obtained from the following suppliers: chenodeoxycholic acid, sodium

salt, MK571, sodium salt, and taurocholic acid, sodium salt were from Calbiochem; deoxycholic acid, sodium salt was from Difco; cholic acid, sodium salt was from Research Organics; DL-buthionine sulfoximine, bumetanide, cimetidine, 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), estrone 3-sulfate, sodium salt, ethacrynic acid, folic acid, furosemide, genistein, pravastatin, probenecid, progesterone, sulfobromophthalein, tamoxifen, thiamine, hydrochloride, and verapamil were from Sigma. Orange juice (lot: 31001 701820427 NC CT803) and white grapefruit juice (lot: 12-500 1-03:40) were obtained from Winn Dixie (store brand, 100% juice reconstituted from concentrate).

LogP calculations

Molecular calculations including LogP were made with the computer program Marvin Beans, ChemAxon, Budapest, Hungary.

Cell culture and transfection

Caco-2 cells (a transformed human colon epithelial cell line extensively used to model drug permeability in the intestine [20]) were obtained from ATCC (HTB-37). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For expansion, cells were cultured in MEM containing Earle's BSS with sodium bicarbonate, 1 g/L glucose, 1 mM sodium pyruvate, 1X nonessential amino acids, 1X penicillin / streptomycin / glutamine, and 20% fetal bovine serum. For transport experiments, sub-confluent Caco-2 cells were trypsinized and then plated at 8.2×10^4 cells/cm² on either MultiScreen Caco-2 plates (Millipore, treated polycarbonate, 0.4 µm pore, 0.11 cm², 96 well, 8 wells per data point), or ThinCert Cell Culture Inserts (Greiner, treated polyethylene terephthalate, 0.4 µm pore, 0.336 cm², 24 well, 5 wells per data point; 4.25 cm², 6 well, 3 wells per data point) in DMEM with sodium bicarbonate, 4.5 g/L glucose, 1X nonessential amino acids, 1X penicillin / streptomycin / glutamine, 10% Fetal bovine serum, and 10 mM HEPES pH 7.5. Cells were allowed to become confluent and differentiate over the subsequent 21 days. Culture media was replaced every 4 days at initial plating; the frequency increasing to every other day as the cells approached confluence.

MDCKII cells were cultured in MEM containing Earle's BSS with sodium bicarbonate, 1 g/L glucose, 1X penicillin / streptomycin / glutamine and 10% fetal bovine serum. An MDCKII cell line stably expressing OATP2B1 was constructed by calcium phosphate mediated transfection of the OATP2B1 cDNA in pcDNA3.1(+) [21]. Transfectants were selected and expanded in MDCKII medium containing 800 µg/ml G418. For transport experiments, sub-confluent cells resistant to G418 following one month of selection or the parental MDCKII line were trypsinized and then plated at 4.6×10^3 cells/cm² in MDCKII medium with or without G418 as appropriate. Inserts were grown to confluence over the subsequent 3 days. 24 h prior to analysis, transporter expression was induced with 10 mM sodium butyrate [22].

Transport experiments

Transport experiments were conducted in HBSS with CaCl₂, MgCl₂, and 1 g/L glucose, buffered at the appropriate pH by addition of either 10 mM MES (pH 5.0–6.5) or 10 mM HEPES (pH 7.0–8.0). In addition, 4% BSA was added to the buffer of the receiver reservoir [23]. Donor and receiver transport buffers containing the indicated drugs were prepared and equilibrated at the appropriate temperature for 30 minutes. When [³H]-montelukast was present, it was added to 16.7 µCi/mL. Apical and basolateral reservoirs of confluent and differentiated transport plates or tissue culture inserts were washed three times with the appropriate transport buffer without drug additions and were equilibrated in the final wash at the appropriate temperature for 15 minutes. Transport experiments were initiated by aspiration of the apical buffer and relocation of the cell carrier to the pre-equilibrated basolateral buffer reservoir followed by addition of the pre-equilibrated apical buffer. Unless otherwise indicated,

the donor and receiver reservoirs were associated with the apical and basolateral surfaces, respectively. Incubation was continued at the indicated temperature for the indicated time. In total three samples were taken for liquid scintillation counting: donor reservoir load and donor and receiver reservoir post-transport. Cell layer integrity was assessed post-transport by Lucifer yellow permeability exclusion as previously described [24].

Permeability calculations

Apparent permeability (P_{app} , cm/sec) was calculated using the following equation [23], which assumes that the initial amount of soluble montelukast in the donor reservoir can be approximated by the sum of the post-transport donor and receiver reservoir amounts:

$$P_{app} = \left(\frac{V_r}{a \times t} \right) \times \left(\frac{M_{rt}}{m_{dt} + m_{rt} / V_d \times 10^{-3}} \right)$$

where V_d and V_r represent the volume in the donor and receiver reservoir (cm^3), a represents the surface area of the transport membrane (cm^2), t represents time in sec, M_{rt} represents the post-transport receiver reservoir montelukast concentration, and m_{dt} and m_{rt} represent the post-transport moles of montelukast in the donor and receiver reservoirs, respectively.

The apparent permeation rate (PR_{app} , pmole/sec/ cm^2) was calculated using the following equation:

$$PR_{app} = \frac{J_M}{a}$$

where J_M represents the flux of montelukast (pmole/sec) and a represents the surface area of the transport membrane (cm^2).

Subject studies

To evaluate the clinical relevance of carrier mediated transport to the pharmacodynamics of montelukast, we explored associations between *SLCO2B1* SNPs and scores on the Asthma Symptom Utility Index [25] from subjects participating in a large clinical trial comparing the efficacy of low-dose theophylline, montelukast and placebo (LoDo) [26]. The multi-center trial was conducted in accordance with the Declaration of Helsinki and was approved by all relevant IRBs. Briefly, 489 eligible adults diagnosed with asthma were randomized to receive either 10 mg of montelukast, 300 mg of theophylline or placebo at bedtime for 6 months to compare the efficacy of low-dose theophylline in poorly controlled asthma. Additionally, plasma concentrations of montelukast collected on the morning after the evening dose at one and 6 months of treatment were measured to evaluate compliance. Montelukast plasma concentrations were measured by reversed-phase liquid chromatography (detection limit, 5 ng/ml) [27]. The day-to-day coefficient of variation ranged between 3% (3000 ng/mL) and 6% (at 15 ng/mL); the within-day coefficient of variation was 1% and 3% at the high and low concentrations, respectively.

Genotype/Phenotype Associations

To assess the clinical relevance of OATP transport of montelukast, we determined associations between common non-synonymous SNPs in *SLCO2B1* and two phenotypes: plasma concentrations of montelukast, and the asthma symptom utility index (ASUI) after 1 and 6 months of treatment. The ASUI is a validated tool that assesses patient preferences for

combinations of asthma related symptoms and drug effects and correlates with patient perception of asthma control [25].

Subject DNA was genotyped by the LightTyper method using simple probe chemistry [28], (Roche Applied Science, Indianapolis, IN). The primers used were: rs12422149 (*SLCO2B1* {NM_007256.2}:c.935G>A), forward GCCTATCATCCTGACC, reverse TGGAGGGAGCTTACC, probe GTGACTGCTAAGACCTTTCGCCGAACTGAA-P; rs2306168 (*SLCO2B1* {NM_007256.2}:c.1457C>T), forward CTCACATACACGCCAT, reverse GGGTTAAAGCCGTCC, probe CTGGAGCTGTCTCCAAGCTGCATGG-P. Primers were adjusted so that a 10:1 ratio was achieved between probe antisense: probe sense amplicon strands.

Statistics

Significance in the montelukast permeability studies was assessed by analysis of variance (ANOVA) followed by Dunnett's Test [29]. Significance in the MDCKII-*SLCO2B1* permeability experiment was determined by a paired Student's t-test [29]. Chi-Square Goodness of Fit Test was used to determine if *SLCO2B1* alleles were in Hardy-Weinberg equilibrium [29]. ANOVA with the Bonferroni adjustment for multiple comparisons was used to determine significance in the association between *SLCO2B1* genotype and Asthma Symptom Utility Index. Asthma Symptom Utility Index scores were arc-sin transformed to achieve a normal distribution since the data were left skewed and fell between 0–1 [30,31]. Age, gender, and race were analyzed as covariates with genotype, none of which had a statistically significant impact on Asthma Symptom Utility Index. A general linear model with repeated measures was applied to test the association between genotype of *SLCO2B1* and montelukast plasma concentration. Prior to analysis, plasma data were square root transformed to achieve a normal distribution since the data had a right skewed distribution with zero values [30,31]. Multivariate analysis of variance was performed on the data and the Wilks' Lambda statistic was used to determine the significance of the association between *SLCO2B1* genotype and montelukast plasma concentration.

Results and Discussion

Carrier Mediated Uptake of Montelukast

Passive transcellular permeability occurs at activation energies below 4 kcal/mole while carrier mediated transport mechanisms require energies between 7–25 kcal/mole [32]. We measured the temperature dependence for montelukast's transcellular permeability between 17–37°C across a Caco-2 cell monolayer in the apical → basolateral direction. An Arrhenius plot of ln apparent permeability (P_{app}) versus 1/T was constructed from the data (Fig. 1). The plot was found to be linear and the apparent activation energy as estimated from the slope of the least squares fit of the data was 13.7 ± 0.7 kcal/mol suggesting that permeability of montelukast is carrier mediated.

In contrast to passive permeability, carrier mediated permeability displays saturation kinetics. Apparent permeability was assessed at initial montelukast donor reservoir concentrations from ~1–200 μ M. A plot of ln apparent permeability versus donor reservoir concentration was found to deviate from linearity (Fig. 2A). Since the apparent permeability coefficient for passive diffusion is independent of concentration, nonlinearity is indicative of a carrier mediated process. When the corresponding apparent permeation rate (PR_{app}) was plotted against donor reservoir concentration, the relationship could be fit to a rectangular hyperbola suggestive of Michaelis-Menten kinetics ($R^2=0.978$, Fig. 2B). When re-plotted in Eadie-Scatchard form ($PR_{app}/\text{Donor Concentration}$ vs. PR_{app}), the plot was found to be linear ($R^2=0.707$, Fig 2B inset). The apparent kinetic constants for permeation were estimated from the slope and X-

intercept of the least squares fit of the data; apparent Michaelis constant (K_{sapp}) = $120 \pm 40 \mu\text{M}$, maximum permeation rate (PR_{max}) = $0.7 \pm 0.2 \text{ pmole/sec/cm}^2$.

Classification Of Carrier Proteins

Carrier mediated processes are subject to inhibition by compounds that are alternate substrates or inhibitors of the carrier protein. In the Caco-2 model system, the specificity of carrier mediated transport is routinely assessed by competition with micromolar to millimolar levels of alternate substrates or inhibitors [33,34]. We chose to include competitors at $125 \mu\text{M}$ in an attempt to balance maximal inhibition with solubility for several of the compounds. Of the 21 compounds tested (Table I), sulfobromophthalein, estrone 3-sulfate, pravastatin, taurocholic acid, and cholic acid *cis*-inhibit permeability ($p < 0.05$) suggesting that these compounds interact with the putative carrier protein(s) that facilitate transport of montelukast. Significantly, all of these compounds are known substrates or inhibitors of the OATP class of membrane transport proteins expressed in the liver and intestine [33,35–37].

Four compounds tested, cimetidine, MK571, folic acid, and verapamil, enhance permeability ($p < 0.05$) suggesting that these compounds either *cis*-stimulate transport of montelukast through inhibition of metabolism or through inhibition of a membrane transport protein responsible for efflux of montelukast or its metabolites into the donor reservoir. In the present study we cannot discriminate between these two mechanisms. In separate experiments, we have determined that montelukast does not stimulate ATP consumption by membrane preparations containing MDR1 (data not shown), therefore it is likely that MDR1 does not contribute significantly to efflux. However other ABC transporters expressed apically in Caco-2 including members of the ABCC and ABCG gene families remain candidates for a putative efflux transporter.

Components of citrus juice are known inhibitors of two major intestinal OATP transport proteins implicated in transport of xenobiotics, OATP1A2 and OATP2B1 [38,39]. Since the competition data of Table I is consistent with a role for OATP transport proteins in mediating permeability of montelukast, we tested for sensitivity of permeability to citrus juice. Both grapefruit juice and orange juice significantly inhibited permeability of montelukast when included in the donor reservoir at 5% and 10% v/v ($*p < 0.05$). Maximal inhibition by grapefruit juice was obtained at 5% v/v (% of control: 5% v/v = 33 ± 3 ; 10% v/v = 31 ± 2) while orange juice exhibited a dose dependent inhibition achieving the same maximal inhibition as grapefruit juice at 10% v/v (% of control: 5% v/v = 56 ± 5 ; 10% v/v = 30 ± 3). The residual 30% permeability observed at maximal inhibition by citrus juice may be due to passive diffusion, or may be mediated by transport proteins that are refractory to inhibition by the components of citrus juice.

Identification of OATP2B1 as a Carrier Protein for Transport of Montelukast

To determine if montelukast is a substrate for OATP2B1, we tested for activation of permeability by progesterone. Progesterone is known to allosterically activate OATP2B1 by up to 300% for transport of estrone-3-sulfate, however the absolute fold activation appears to be substrate dependent [40]. Permeability of montelukast was assessed in the presence of 5–30 μM progesterone co-incubated in the donor reservoir. Significantly, permeability was activated for all tested concentrations of progesterone (% of control: 5 μM , $150 \pm 10^*$; 15 μM , $140 \pm 10^*$; 30 μM , $160 \pm 10^*$, $*p < 0.05$).

To confirm that OATP2B1 is a transporter of montelukast, we constructed an OATP2B1 expressing MDCKII cell line. Expression of OATP2B1 by this cell line significantly enhanced permeability of montelukast in the basolateral $\rightarrow$ apical direction over the control MDCKII cell line (Fig. 3, % of control: 140 ± 20 , $p < 0.05$). For comparison, permeability of the OATP2B1 model substrate estrone-3-sulfate was enhanced by $280 \pm 40\%$ in the OATP2B1 expressing cell

line relative to control confirming expression of functional OATP2B1. Coincubation of 10% grapefruit juice in the donor reservoir significantly reduced permeability of montelukast in both control* and OATP2B1* expressing cell lines (* $p < 0.05$, % of control: Control = 29 ± 3 ; OATP2B1 = 32 ± 3) suggesting that components in grapefruit juice inhibit both OATP2B1 and endogenously expressed transporters responsible for background permeability. We further confirmed that OATP2B1 is expressed basolaterally in this cell line [41] by measuring a basolateral to apical permeability ratio of 1.5 which we do not observe in control lines suggesting that it isn't mediated by an apically oriented efflux transporter.

In addition to *SLCO2B1*, differentiated Caco-2 monolayers express *SLCO1A2* [42,43]. In similar experiments we find that expression of *SLCO1A2* in MDCKII cell lines leads to an enhancement in permeability that is similar to that observed in *SLCO2B1* expressing cell lines (data not shown). The contribution of OATP1A2 to montelukast permeability in the Caco-2 model system remains to be determined.

SLCO2B1 Genotype/Phenotype Associations

If OATP2B1 contributes significantly to transport of montelukast in the human intestine, then coding region SNPs in *SLCO2B1*, could influence the pharmacokinetics of, and ultimately the response to montelukast. We tested this hypothesis by looking for associations between nonsynonymous SNPs in *SLCO2B1* and response to montelukast therapy in subjects that participated in a clinical trial [26]. *SLCO2B1* contains two documented nonsynonymous SNPs of significant heterozygosity ($\geq 20\%$, rs12422149, *SLCO2B1*{NM_007256.2}:c.935G>A (p.Arg312Gln); rs2306168, *SLCO2B1*{NM_007256.2}:c.1457C>T (p.Ser486Phe)). Subject demographics as well as allele and genotype frequencies are summarized in Table II. In Figure 4, arc-sine transformed mean (ASTM) Asthma Symptoms Utility Index (ASUI) $\pm$ SE scores for each visit are plotted by c.935 genotype. In subjects with the c.[935G]+[935G] genotype, montelukast treatment was associated with an increase in arc-sine transformed mean Asthma Symptom Utility Index score from 0.69 ± 0.02 at randomization to 0.79 ± 0.02 and 0.80 ± 0.02 after one and six months of treatment, respectively ($p < 0.0001$). These data indicate that asthma severity in these subjects improved from moderate to mild-to-moderate during montelukast add-on therapy [25]. In contrast, arc-sine transformed mean Asthma Symptom Utility Index score did not change in subjects with the c.[935G>A]+[935G] genotype suggesting that they did not benefit from montelukast add-on therapy ($p = 0.84$). In addition, there was no association between arc-sine transformed mean Asthma Symptom Utility Index scores for each visit and genotype at c.1457.

Next, we determined if there was an association between genotype of *SLCO2B1* and montelukast plasma concentrations. For c.935G>A, out of the 80 subjects for which we had measured montelukast plasma concentration, 0 were c.[935G>A]+[935G>A], 16 were c.[935G>A]+[935G] and 64 were c.[935G]+[935G] yielding a heterozygosity of 0.20. Multivariate analysis of the data shows that while the square root transformed mean (SQRTM) montelukast plasma concentration was the same for both visits (c.935G>A, $p = 0.32$ and c.1457C>T, $p = 0.56$), there was a statistically significant association between the genotype at c.935 and square root transformed mean montelukast plasma concentration for both visits (Fig. 5; following 1 month of treatment: square root transformed mean plasma concentration $\pm$ SE, c.[935G>A]+[935G] = 2.2 ± 0.8 , c.[935G]+[935G] = 6.0 ± 0.7 , $p = 0.019$; following 6 months of treatment: c.[935G>A]+[935G] = 3 ± 1 , c.[935G]+[935G] = 7.0 ± 0.9 , $p = 0.025$). Correction for race using a general linear model did not affect the association (1 month: $p = 0.03$; 6 months: $p = 0.03$). For subjects with the c.[935G>A]+[935G] genotype this represents a reduction in average plasma concentration to $20 \pm 60\%$ (1 month) or $30 \pm 80\%$ (6 months) of the plasma concentration found in subjects with the c.[935G]+[935G] genotype. In contrast, no association between genotype at c.1457 and plasma concentration was found ($p = 0.54$). These data suggest

that the difference in montelukast plasma concentrations observed between the c.[935G>A]+[935G] and c.[935G]+[935G] genotypes of *SLCO2B1* are directly associated with clinical outcome.

Finally, we plotted response (arc-sine transformed mean Asthma Symptom Utility Index score) as a function of montelukast plasma concentration at one month and 6 months of treatment (data not shown), and tested for a direct relationship by linear regression. A positive trend between arc-sine transformed mean Asthma Symptom Utility Index and plasma concentration is observed at each visit, however the relationship is only significant at the 6 month interval (data not shown, 1 month of treatment: $p=0.12$; 6 months of treatment: $p=0.042$).

Conclusions

Montelukast is a safe and effective alternative to inhaled corticosteroid (ICS) monotherapy of mild persistent asthma or as add-on to ICS in moderate to severe persistent asthma poorly controlled by ICS [44]. Once daily montelukast is also an acceptable step-down strategy for patients with mild persistent asthma who are being treated with twice daily ICS and who have concerns about the safety of ICS and/or long acting beta agonists [45]. However the poor response rate to montelukast, which can approach 60 to 70% [7,8], continues to be a concern. This large variability in response to montelukast and to leukotriene modifiers in general, may be attributable in part to genetic variation [9], and has been the focus of several pharmacogenetic studies. Associations have been reported between several polymorphisms in genes that encode leukotriene pathway enzymes and response to LTRAs [10–14] thus establishing a genetic basis for response heterogeneity in patients.

The long-range goal of the present study was to determine if sequence variants in genes that code for transport proteins contribute to the interpatient variability in response to montelukast. The rationale for this study was based on the physical and pharmacokinetic properties of montelukast, which suggest that it will be a substrate for carrier mediated transport [15], and on the variability in plasma concentrations observed in clinical trials or in pharmacokinetic studies [7,14,26].

Our in vitro Caco-2 studies demonstrate that permeability of montelukast requires 13.7 kcal/mol (Fig. 1), is subject to saturation at high concentrations (Fig. 2), and can be inhibited by coincubation of specific drugs in the donor chamber (Table 1), all characteristics of carrier mediated transport. Permeability is enhanced by low concentrations of progesterone suggesting a role for OATP2B1 since progesterone is a known activator of this transporter [40]. The role of OATP2B1 in absorption of montelukast was confirmed by assessing permeability in OATP2B1 expressing MDCKII cell lines (Fig 3.). In similar experiments we also found that expression of OATP1A2 enhances permeability of montelukast to a level equivalent to that of *SLCO2B1*. Thus our studies have identified two transporters of montelukast: OATP2B1 and OATP1A2. While both of these transporters are known to be expressed in Caco-2 [46,47], their relative expression levels may vary depending on culture conditions or cell passage [48]. Therefore, characterization of the relative contribution of these two transporters, as well as that of other expressed members of the SLCO transporter family, to the permeability of montelukast in Caco-2s, awaits a more systematic study.

OATP2B1, a member of the SLCO family of organic anion membrane transport proteins, is coded for by *SLCO2B1* located on chromosome 11q13. OATP2B1 is expressed in the liver, spleen, placenta, lung, kidney, heart, ovary, small intestine, and brain [19]. A small number of drugs that have physical and pharmacokinetic properties similar to montelukast are substrates for the SLCO transporters, including the statin-like drugs pravastatin and atorvastatin [36,49, 50] and fexofenadine [38]. Importantly, we report an association between the non-synonymous

SNP *SLCO2B1*{NM_007256.2}:c.935G>A (p.Arg312Gln) and plasma concentrations of montelukast after one and 6 months of montelukast treatment. Plasma concentrations in p.[Arg312]+[Arg312] homozygotes on both occasions were significantly higher compared to p.[Arg312Gln]+[Arg312] heterozygotes (Fig. 5). In keeping with our finding of higher plasma concentrations in p.[Arg312]+[Arg312] homozygotes, ASUI scores improved after one and 6 months of montelukast treatment compared to baseline just prior to starting treatment (Fig. 4). In contrast, ASUI scores in p.[Arg312Gln]+[Arg312] heterozygotes did not improve with montelukast treatment compared to baseline. These data suggest that SNP *SLCO2B1*{NM_007256.2}:c.935G>A can influence the pharmacokinetics of, and the response to montelukast. Further studies are required to replicate these findings.

We used the Asthma Symptom Utility Index as the phenotype in this study rather than other more common phenotypes like measures of lung function because montelukast is used in the treatment of asthma as controller medication. The Asthma Symptom Utility Index is a validated tool that assesses patient preferences for combinations of asthma related symptoms and drug effects on a scale from worst to best possible state [25], and has been used as an outcome measure for clinical trials in asthma [26,51,52]. The use of the Asthma Symptom Utility Index as an outcome is consistent with recently published guidelines in the Expert Panel Report 3, which support a new approach to assessing a patient's level of asthma control through multiple measures [44]. We therefore believe the asthma symptom utility index is superior to lung function tests as an outcome measure in a pharmacogenetic study of montelukast.

The present study has several limitations. Expression levels and localization of OATP2B1 were not confirmed by immunohistochemistry in either our Caco-2 cultures or in our cell lines. Since OATP2B1 is expressed in both the intestine and the liver, c.935G>A may influence clearance as well as absorption. In addition other hepatically expressed transporters including OATP1B1 and OATP1B3 could potentially play a role in clearance.

Gene variants that contribute to variable drug response in complex phenotypes, like asthma, may have modest effects thus requiring large sample sizes to detect associations [53]. Our sample size was small, and it is possible that the associations we observed between c.935G>A and responsiveness to montelukast could represent false positives. We did not stratify our subject population by ethnicity, which could contribute to false positive associations [54]. Medication adherence may confound our results. Subjects not taking montelukast would have negligible plasma concentrations and negligible response. However, it is unlikely that non-adherent subjects would be of a single genotype. Lastly, we did not have a replicate cohort and we acknowledge that our findings require replication before we can conclude that c.935G>A influences response to montelukast. Nevertheless, our findings that montelukast undergoes carrier mediated transport by OATP2B1, and that c.935G>A influences Asthma Symptom Utility Index and plasma concentrations in subjects with asthma are novel and require further study.

Our calculations suggest that at the lowest area under the concentration time curve values observed in our studies [7], the plasma concentration of montelukast will be below the K_i for Cys-LTD4 binding to the human CYS-LT1 receptor [1] for a significant fraction of the inter-dose 24 hour period, suggesting that significant activation of the receptor may occur in these individuals. It is reasonable to hypothesize that these individuals may be at risk for poor response to the standard dose of montelukast, and could potentially benefit from individualization of montelukast therapy. The results of this study suggest a way to identify these patients.

Acknowledgements

This work was supported by NIH grants R01HL071394 and R01HL074755. The American Lung Association sponsored the LODDO clinical trial conducted by the Asthma Clinical Research Centers. The OATP2B1 cDNA construct was kindly provided by Dr. Dietrich Keppler, German Cancer Research Center, Heidelberg, Germany. We would like to acknowledge Astride Altomare, and Karl Mann for their HPLC expertise, and Beth Maguire for expert technical assistance.

References

1. Jones TR, Labelle M, Belley M, Champion E, Charette L, Evans J, et al. Pharmacology of montelukast sodium (Singulair), a potent and selective leukotriene D4 receptor antagonist. *Can J Physiol Pharmacol* 1995;73:191–201. [PubMed: 7621356]
2. Drazen JM. Asthma therapy with agents preventing leukotriene synthesis or action. *Proc Assoc Am Physicians* 1999;111:547–559. [PubMed: 10591083]
3. Kanaoka Y, Boyce JA. Cysteinyl leukotrienes and their receptors: cellular distribution and function in immune and inflammatory responses. *J Immunol* 2004;173:1503–1510. [PubMed: 15265876]
4. Blake KV. Montelukast: data from clinical trials in the management of asthma. *Ann Pharmacother* 1999;33:1299–1314. [PubMed: 10630831]
5. Jarvis B, Markham A. Montelukast: a review of its therapeutic potential in persistent asthma. *Drugs* 2000;59:891–928. [PubMed: 10804041]
6. Muijsers RB, Noble S. Montelukast: a review of its therapeutic potential in asthma in children 2 to 14 years of age. *Paediatr Drugs* 2002;4:123–139. [PubMed: 11888359]
7. Lima JJ. Treatment heterogeneity in asthma: genetics of response to leukotriene modifiers. *Mol Diagn Ther* 2007;11:97–104. [PubMed: 17397245]
8. Weiss ST, Litonjua AA, Lange C, Lazarus R, Liggett SB, Bleecker ER, et al. Overview of the pharmacogenetics of asthma treatment. *Pharmacogenomics J* 2006;6:311–326. [PubMed: 16568148]
9. Drazen JM, Silverman EK, Lee TH. Heterogeneity of therapeutic responses in asthma. *Br Med Bull* 2000;56:1054–1070. [PubMed: 11359637]
10. Asano K, Shiomi T, Hasegawa N, Nakamura H, Kudo H, Matsuzaki T, et al. Leukotriene C4 synthase gene A(-444)C polymorphism and clinical response to a CYS-LT(1) antagonist, pranlukast, in Japanese patients with moderate asthma. *Pharmacogenetics* 2002;12:565–570. [PubMed: 12360108]
11. Klotsman M, York TP, Pillai SG, Vargas-Irwin C, Sharma SS, van den Oord EJ, et al. Pharmacogenetics of the 5-lipoxygenase biosynthetic pathway and variable clinical response to montelukast. *Pharmacogenet Genomics* 2007;17:189–196. [PubMed: 17460547]
12. Sampson AP, Siddiqui S, Buchanan D, Howarth PH, Holgate ST, Holloway JW, et al. Variant LTC (4) synthase allele modifies cysteinyl leukotriene synthesis in eosinophils and predicts clinical response to zafirlukast. *Thorax* 2000;55:S28–S31. [PubMed: 10992553]
13. Whelan GJ, Blake K, Kissoon N, Duckworth LJ, Wang J, Sylvester JE, et al. Effect of montelukast on time-course of exhaled nitric oxide in asthma: influence of LTC4 synthase A(-444)C polymorphism. *Pediatr Pulmonol* 2003;36:413–420. [PubMed: 14520724]
14. Lima JJ, Zhang S, Grant A, Shao L, Tantisira KG, Allayee H, et al. Influence of leukotriene pathway polymorphisms on response to montelukast in asthma. *Am J Respir Crit Care Med* 2006;173:379–385. [PubMed: 16293801]
15. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* 2001;46:3–26. [PubMed: 11259830]
16. Thibert R, Mach H, Meisner DR, Vadas EB. Characterization of the self-association properties of a leukotriene D4 receptor antagonist, MK-0476. *Int J Pharm* 1996;134:59–70.
17. Artursson P, Palm K, Luthman K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv Drug Deliv Rev* 2001;46:27–43. [PubMed: 11259831]
18. Zhao JJ, Rogers JD, Holland SD, Larson P, Amin RD, Haesen R, et al. Pharmacokinetics and bioavailability of montelukast sodium (MK-0476) in healthy young and elderly volunteers. *Biopharm Drug Dispos* 1997;18:769–777. [PubMed: 9429741]

19. Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/ functional properties. *Pflugers Arch* 2004;447:653–665. [PubMed: 14579113]
20. Artursson P. Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *J Pharm Sci* 1990;79:476–482. [PubMed: 1975619]
21. Kopplow K, Letschert K, Konig J, Walter B, Keppler D. Human hepatobiliary transport of organic anions analyzed by quadruple-transfected cells. *Mol Pharmacol* 2005;68:1031–1038. [PubMed: 16046661]
22. Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein MRP2, permanently expressed in human and canine cells. *Mol Pharmacol* 1999;55:929–937. [PubMed: 10220572]
23. Youdim KA, Avdeef A, Abbott NJ. In vitro trans-monolayer permeability calculations: often forgotten assumptions. *Drug Discov Today* 2003;8:997–1003. [PubMed: 14643163]
24. Hidalgo JJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 1989;96:736–749. [PubMed: 2914637]
25. Revicki DA, Leidy NK, Brennan-Diemer F, Sorensen S, Togias A. Integrating patient preferences into health outcomes assessment: the multiattribute Asthma Symptom Utility Index. *Chest* 1998;114:998–1007. [PubMed: 9792568]
26. ALA-ACRC. Clinical trial of low-dose theophylline and montelukast in patients with poorly controlled asthma. *Am J Respir Crit Care Med* 2007;175:235–242. [PubMed: 16998094]
27. Amin RD, Cheng H, Rogers JD. Determination of MK-0476 in human plasma by liquid chromatography. *J Pharm Biomed Anal* 1995;13:155–158. [PubMed: 7766722]
28. Bennett CD, Campbell MN, Cook CJ, Eyre DJ, Nay LM, Nielsen DR, et al. The LightTyper: high-throughput genotyping using fluorescent melting curve analysis. *Biotechniques* 2003;34:1288–5
29. Glantz, SA. *Primer of Biostatistics*. Vol. 6th edition. New York, NY: McGraw-Hill Medical Pub.; 2005.
30. Oehlert, GW. *A first course in design and analysis of experiments*. Vol. 1 edition. New York, NY: W.H. Freeman; 2000.
31. Rao, PV. *Statistical Research Methods in the Life Sciences*. Vol. 1 edition. Pacific Grove, CA: Duxbury Press; 1998.
32. Hofer, M. *Transport Across Biological Membranes*. Marshfield, Mass: Pitman Publishing, INC; 1981.
33. Nishimura T, Kubo Y, Kato Y, Sai Y, Ogihara T, Tsuji A. Characterization of the uptake mechanism for a novel loop diuretic, M17055, in Caco-2 cells: involvement of organic anion transporting polypeptide (OATP)-B. *Pharm Res* 2007;24:90–98. [PubMed: 17103337]
34. Sai Y, Kaneko Y, Ito S, Mitsuoka K, Kato Y, Tamai I, et al. Predominant contribution of organic anion transporting polypeptide OATP-B (OATP2B1) to apical uptake of estrone-3-sulfate by human intestinal Caco-2 cells. *Drug Metab Dispos* 2006;34:1423–1431. [PubMed: 16714376]
35. Kullak-Ublick GA, Ismail MG, Stieger B, Landmann L, Huber R, Pizzagalli F, et al. Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* 2001;120:525–533. [PubMed: 11159893]
36. Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J Pharmacol Exp Ther* 2004;308:438–445. [PubMed: 14610227]
37. Yamaguchi H, Okada M, Akitaya S, Ohara H, Mikkaichi T, Ishikawa H, et al. Transport of fluorescent chenodeoxycholic acid via the human organic anion transporters OATP1B1 and OATP1B3. *J Lipid Res* 2006;47:1196–1202. [PubMed: 16534140]
38. Bailey DG, Dresser GK, Leake BF, Kim RB. Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice. *Clin Pharmacol Ther* 2007;81:495–502. [PubMed: 17301733]
39. Satoh H, Yamashita F, Tsujimoto M, Murakami H, Koyabu N, Ohtani H, et al. Citrus juices inhibit the function of human organic anion-transporting polypeptide OATP-B. *Drug Metab Dispos* 2005;33:518–523. [PubMed: 15640378]

40. Grube M, Kock K, Karner S, Reuther S, Ritter CA, Jedlitschky G, et al. Modification of OATP2B1-mediated transport by steroid hormones. *Mol Pharmacol* 2006;70:1735–1741. [PubMed: 16908597]
41. Letschert K, Faulstich H, Keller D, Keppler D. Molecular Characterization and Inhibition of Amanitin Uptake into Human Hepatocytes. *Toxicol Sci.* 2006
42. Landowski CP, Anderle P, Sun D, Sadee W, Amidon GL. Transporter and ion channel gene expression after Caco-2 cell differentiation using 2 different microarray technologies. *AAPS J* 2004;6:e21. [PubMed: 15760106]
43. Nielsen M, Bressendorff S, Møller J, Olsen J, Troelsen JT. Mapping of HNF4a binding sites, acetylation of histone H3 and expression in Caco2 cells. [GSE7745]. National Center for Biotechnology Information. Geo Datasets. 2008
44. Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. *J Allergy Clin Immunol* 2007;120:S94–S138. [PubMed: 17983880]
45. Peters SP, Jones CA, Haselkorn T, Mink DR, Valacer DJ, Weiss ST. Real-world Evaluation of Asthma Control and Treatment (REACT): findings from a national Web-based survey. *J Allergy Clin Immunol* 2007;119:1454–1461. [PubMed: 17481716]
46. Sai Y, Kaneko Y, Ito S, Mitsuoka K, Kato Y, Tamai I, et al. Predominant contribution of organic anion transporting polypeptide OATP-B (OATP2B1) to apical uptake of estrone-3-sulfate by human intestinal Caco-2 cells. *Drug Metab Dispos* 2006;34:1423–1431. [PubMed: 16714376]
47. Maubon N, Le Vee M, Fossati L, Audry M, Le Ferrec E, Bolze S, et al. Analysis of drug transporter expression in human intestinal Caco-2 cells by real-time PCR. *Fundam Clin Pharmacol* 2007;21:659–663. [PubMed: 18034668]
48. Sambuy Y, de A I, Ranaldi G, Scarino ML, Stamatii A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* 2005;21:1–26. [PubMed: 15868485]
49. Grube M, Kock K, Oswald S, Draber K, Meissner K, Eckel L, et al. Organic anion transporting polypeptide 2B1 is a high-affinity transporter for atorvastatin and is expressed in the human heart. *Clin Pharmacol Ther* 2006;80:607–620. [PubMed: 17178262]
50. Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J Pharmacol Exp Ther* 2003;306:703–708. [PubMed: 12724351]
51. The safety of inactivated influenza vaccine in adults and children with asthma. *N Engl J Med* 2001;345:1529–1536. [PubMed: 11794219]
52. Peters SP, Anthonisen N, Castro M, Holbrook JT, Irvin CG, Smith LJ, et al. Randomized comparison of strategies for reducing treatment in mild persistent asthma. *N Engl J Med* 2007;356:2027–2039. [PubMed: 17507702]
53. Long AD, Langley CH. The power of association studies to detect the contribution of candidate genetic loci to variation in complex traits. *Genome Res* 1999;9:720–731. [PubMed: 10447507]
54. Freedman ML, Reich D, Penney KL, McDonald GJ, Mignault AA, Patterson N, et al. Assessing the impact of population stratification on genetic association studies. *Nat Genet* 2004;36:388–393. [PubMed: 15052270]

Figure 1. Apparent activation energy for transport of montelukast

Caco-2 cells were plated on ThinCert transwell inserts with a surface area of 0.34 cm². Permeability was assessed following a one-hour incubation at temperatures from 17–37°C. The 125 µL donor reservoir (apical) was fixed at pH 5.0 and contained ~2 µM [H³]-montelukast while the 700 µL receiver reservoir was fixed at pH 7.5. Permeability was assessed at three different time points for each temperature (20, 40, and 60 min) and the apparent permeability reported was the average of these three readings. The following equation was

used to calculate apparent activation energy (E_{app}): $\ln(P_{app}) = -\frac{E_{app}}{RT} + C$ where P_{app} represents the measured apparent permeability at a given temperature (T , Kelvin), R is the gas constant, and C is the pre-exponential factor.

Figure 2. Concentration dependence of permeability

Caco-2 cells were plated on MultiScreen transport assay plates. For permeability measurements, the donor reservoir was fixed at pH 5.0 and contained [H^3]-montelukast at a final concentration of from ~1–200 μM . The receiver reservoir was fixed at pH 7.5. Permeability was assessed following a 1-hour incubation at 37°C. A) Plot of $\ln$ apparent permeability versus donor reservoir concentration. The best fit curve was modeled using the function: $y = a - b \ln(x + c)$. B) Plot of apparent permeation rate versus donor reservoir concentration. The best-fit curve was modeled using a rectangular hyperbola function. The inset is an Eadie-Satchard replot of the data.

Figure 3. Transport of montelukast by an OATP2B1 expressing MDCKII cell line
MDCKII or MDCKII-OATP2B1 cells were plated on ThinCert transwell inserts with a surface area of 4.25 cm². Permeability was assessed following a one-hour incubation at 37°C. The 1500 μ L donor reservoir (basolateral) was fixed at pH 7.5 and contained either [³H]-montelukast or [³H]-estrone-3-sulfate while the 1000 μ L receiver reservoir (apical) was fixed at pH 7.5. Apparent permeability is reported as a percentage of control (parental MDCKII cell line). Significant results are indicated by an * ($p < 0.05$).

Figure 4. Association between Asthma Symptom Utility score and genotype at *SLCO2B1* {NM_007256.2}:c.935 in LoDo subjects

Asthma Symptom Utility score was measured by standard questionnaire and the arc-sine transformed mean Asthma Symptom Utility score $\pm$ SE is plotted as a function of genotype at *SLCO2B1*{NM_007256.2}:c.935 for each sampling interval. Arc-sine transformed mean Asthma Symptom Utility score significantly improves (*) for subjects with the c.[935G] + c.[935G] genotype at one month of montelukast treatment, and at 6 months of montelukast treatment, relative to randomization (randomization vs. one month of treatment: $p < 0.0001$; randomization vs. 6 months of treatment: $p < 0.0001$). Patients with the c.[935G>A] + c.[935G] genotype did not show a statistically significant improvement in arc-sine transformed mean Asthma Symptom Utility score at either sampling interval.

Figure 5. Association between montelukast plasma concentration and genotype at *SLCO2B1* {NM_007256.2}:c.935 in LoDo subjects

Montelukast plasma concentration was measured by HPLC on the morning following an evening dose (10 mg) and the square root transformed mean plasma concentration (SQRTM $\pm$ SE ng/ml) is plotted as a function of genotype at *SLCO2B1* {NM_007256.2}:c.935 for both sampling intervals. Subjects with the c.[935G>A] + c.[935G] genotype had significantly reduced plasma concentrations of montelukast (*) at both sampling intervals relative to the consensus c.[935G] + c.[935G] genotype (one month of treatment $p=0.019$, 6 months of treatment $p=0.025$).

Table I**Competition of transport**

Transport of montelukast was assessed in the presence of 125 μ M of the individual competitors. Apparent permeability is reported as a percentage of control in which no additional drug was present. Values are sorted in ascending order;

Competitor	$P_{app} \pm SD$ (% of control)
Sulfobromophthalein	72 $\pm$ 7 *
Estrone 3-sulfate	79 $\pm$ 6 *
Pravastatin	83 $\pm$ 8 *
Taurocholic acid	84 $\pm$ 9 *
Cholic acid	86 $\pm$ 7 *
Probenecid	90 $\pm$ 10
Chenodeoxycholic acid	90 $\pm$ 10
Furosemide	95 $\pm$ 8
Ethacrynic acid	100 $\pm$ 10
DL-Buthionine sulfoximine	100 $\pm$ 20
DIDS	100 $\pm$ 20
Bumetanide	100 $\pm$ 10
Deoxycholic acid	110 $\pm$ 10
Tamoxifen	110 $\pm$ 20
Thiamine, hydrochloride	110 $\pm$ 10
Genistein	110 $\pm$ 10
Cimetidine	120 $\pm$ 10 *
MK571	130 $\pm$ 10 *
Folic acid	130 $\pm$ 20 *
Verapamil	160 $\pm$ 20 *

* designates a result that is significantly different from the control ($p \leq 0.05$).

Table II
Frequency of *SLCO2B1*{NM_007256.2}:c.935G>A in LoDo Subject Population
Demographics and allele / genotype frequencies for *SLCO2B1*{NM_007256.2}:c.935G>A in the LoDo subject population. SNP c.935G>A is in Hardy-Weinberg equilibrium for all populations ($p < 0.05$ is the cutoff for SNPs not in Hardy-Weinberg equilibrium).

Race(N)	Polymorphism	Allele	Allele frequency (%)	Genotype	Genotype Frequency N (%)	pvalue for Hardy-Weinberg
African American (20)	rs12422149	A	5 (13.16)	AA	0 (0)	0.51
		G	33 (86.84)	AG	5 (26.32)	
				GG	14 (73.68)	
Caucasia n (55)	rs12422149	A	9 (8.18)	AA	0 (0)	0.51
		G	101 (91.82)	AG	9 (16.36)	
				GG	46 (83.64)	
Hispanic (5)	rs12422149	A	2 (20)	AA	0 (0)	0.58
		G	8 (80)	AG	2 (40)	
				GG	3 (60)	