Characterization of Atomoxetine Biotransformation and Implications for Development of PBPK Models for Dose Individualization in Children

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NONSTANDARD ABBREVIATIONS

Atomoxetine = ATX
4-hydroxyatomoxetine = 4-OH-ATX
N-desmethylatomoxetine = NDM-ATX
2-hydroxymethylatomoxetine = 2-CH₂OH-ATX
ABSTRACT

Atomoxetine (ATX) is a second-line non-stimulant medication used to control symptoms of attention deficit hyperactivity disorder (ADHD). Inconsistent therapeutic efficacy has been reported with ATX, which may be related to variable CYP2D6-mediated drug clearance. We characterized ATX metabolism in a panel of human liver samples as a basis for a bottom-up PBPK model to aid in ATX exposure prediction and control. $K_m$, $V_{max}$, and $Cl_{int}$ values in pooled human liver microsomes (HLMs) were 2.4 µM, 479 pmol/min/mg protein, and 202 µL/min/mg protein respectively. Mean population values of kinetic parameters are not adequate to describe variability in population, given that $K_m$, $V_{max}$, and $Cl_{int}$ values from single-donor HLMs ranged from 0.93 – 79.2 µM, 20.0 – 1600 pmol/min/mg protein, and 0.3 – 936 µL/min/mg protein. All kinetic parameters were calculated from 4-hydroxyatomoxetine (4-OH-ATX) formation. CYP2E1 and CYP3A contributed to 4-OH-ATX formation in livers with CYP2D6 intermediate and poor metabolizer status. In HLMs with lower CYP2D6 activity levels, 2-hydroxymethylatomoxetine (2-CH$_2$OH-ATX) formation became a more predominant pathway of metabolism, which appeared to be catalyzed by CYP2B6. ATX biotransformation at clinically relevant plasma concentrations was characterized in a panel of pediatric HLM (n = 116) samples by evaluating primary metabolites. Competing pathways of ATX metabolism ($N$-desmethylatomoxetine (NDM-ATX) and 2-CH$_2$OH-ATX formation) had increasing importance in livers with lesser CYP2D6 activity, but, overall ATX clearance was still compromised. Modeling ATX exposure to individualize therapy would require comprehensive knowledge of factors that affect CYP2D6 activity as well as an understanding of competing pathways, particularly for individuals with lower CYP2D6 activity.
INTRODUCTION

Attention deficit/hyperactivity disorder (ADHD) is the most prevalent neurodevelopmental disorder among children. In the United States, the prevalence of ADHD among school-aged children is approximately 9.5%, and worldwide prevalence is approximately 5.3% (Feldman and Reiff, 2014). Pharmacological intervention is a key component of ADHD symptomatic relief (Feldman and Reiff, 2014). Children who receive sub-optimal care for their symptoms may fail to thrive academically and socially, the effects of which can persist into adulthood (Asherson et al., 2012). Currently, first-line therapies are stimulants, which include methylphenidate and amphetamine derivatives. Non-stimulants, such as atomoxetine (ATX) and centrally acting α2-adrenergic agonists, are considered second-line therapies. ATX was originally considered to be a viable non-stimulant alternative to stimulants, particularly where first-line therapy was contraindicated. However, its use has declined since being introduced into the market in 2003 (Chai et al., 2012), which may be due to variable efficacy. A retrospective analysis of pooled randomized control trials involving ATX (IDEA study) found that response during the acute phase of treatment (6-9 weeks) to be bimodal, with 40% of patients being non-responders (Newcorn et al., 2009). An understanding the mechanistic reasons for variability in ATX response may permit more precise drug selection and dosing strategies.

ATX exposure, as measured by maximum concentration or calculating area under the plasma concentration-time curve (AUC), has been associated with response (Michelson et al., 2007). CYP2D6-mediated oxidative metabolism is the major route of ATX clearance, and polymorphisms of the CYP2D6 gene likely contribute to the variability in drug exposure. Data from clinical studies indicated that individuals who are genotyped as poor metabolizers (PMs) have a longer half-life, and higher $C_{\text{max}}$, $C_{\text{min}}$, and drug exposure compared to extensive
metabolizers (EMs) (Sauer et al., 2005). The current labeling for ATX does not require any change in starting dose based on CYP2D6 genotype or phenotype because the results of initial studies were interpreted as indicating that physicians could adequately dose ATX without prior knowledge of projected CYP2D6 activity (Trzepacz et al., 2008). Alternatively, dose adjustment should be based on weight of the child to accommodate the effect of growth and development on the dose-exposure relationship (Witcher et al., 2003). A recent review concluded that considerable time is needed to achieve a therapeutic response, which can be a frustrating period for patients and their families (Savill et al., 2015).

A CYP2D6 genotype-stratified pharmacokinetic study of ATX conducted by our group (Brown et al., 2015) revealed an 11.4-fold difference in mean dose-corrected AUCs between CYP2D6 PMs and EMs. More importantly, there was a 30-fold range in dose-corrected AUC values amongst all participants given nominally the same dose. Of particular note was the 4-5 fold variability in dose-corrected AUC within each of the EM groups (one and two functional CYP2D6 allele groups), and the considerable absolute inter-individual variability in AUC within the PM group. Furthermore, urinary recovery of parent drug and metabolites revealed that 4-hydroxyatomoxetine (4-OH-ATX), the primary CYP2D6-mediated metabolite, was the single most abundant metabolite formed, regardless of CYP2D6 genotype, including the PM group. In addition, carboxyl-hydroxyl ATX metabolites resulting from sequential metabolism of initial 2-methylhydroxylation, which have not been quantified in earlier human studies, represented a significant percentage of recovered metabolites in CYP2D6 intermediate metabolizers (IMs, defined as genotypes consisting of a partial function allele and a null allele) and PMs. Formation of N-desmethylatomoxetine (NDM-ATX) metabolite was a relatively minor compared to that of other metabolites. The relative contribution of competing ATX metabolic pathways (N-
demethylation and 2-methylhydroxylation) was a function of CYP2D6 activity, and the results of this study further emphasized the importance of characterizing the CYPs contributing to 4-OH-ATX and 2-methyl-hydroxyl-ATX (2-CH₂OH-ATX) formation, especially under conditions where CYP2D6 activity is compromised (Figure 1).

Evaluating the interplay of pathways responsible for ATX metabolism in a comprehensive pediatric system is also critical for developing tools to better dose children. In preparation for building a bottom-up physiologically-based pharmacokinetic (PBPK) model as a computational engine to estimate and predict ATX exposure in children, the purpose of this study was to 1) investigate the sources of variability in ATX biotransformation within the same CYP2D6 genotype/activity score class; 2) identify other CYP isoforms contributing to ATX metabolism, particularly in the scenario of lower CYP2D6 activity; and 3) characterize the relative contribution of all pathways of ATX metabolism in a pediatric context.
MATERIALS AND METHODS

Materials and reagents. ATX, 4-OH-ATX, 4-OH-ATX-d3, NDM-ATX, NDM-ATX-d7, montelukast sodium salt, and (S)-(+)N-3-benzyl nirvanol were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Ketoconazole was obtained from Research Biochemicals International (Natick, MA, USA). β-Nicotinamide adenine dinucleotide 2’-phosphate reduced tetrasodium salt hydrate (NADPH), α-naphthoflavone, tranylcypromine, thiotepa, montelukast, sulfaphenazole, and quinidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were purchased from Fisher Scientific (Waltham, MA, USA). Heterologously expressed cytochrome P450 enzymes (Cypex Bactosomes), adult and pediatric single-donor HLMs, and pooled human HLMs (n = 200 donors) were purchased from XenoTech LLC (Lenexa, KS, USA). HLMs prepared from liver samples from pediatric donors were obtained through the Liver Tissue Cell Distribution System (Minneapolis, MN, USA; Pittsburgh, PA, USA), NIH Contracts #N01-DK-7-0004/HHSN267200700004C, #HHSN276201200017C, the University of Maryland Brain and Tissue Bank for Developmental Disorders, and XenoTech LLC, and have been described elsewhere (Pearce et al., 2015).

Michaelis-Menten Kinetic Parameter Experiments. Kinetic experiments were initially conducted in pooled HLMs to obtain a mean population reference. In addition, kinetic experiments were also performed in 21 single-donor HLMs stratified by genotype using activity score (AS) as a qualitative measure of predicted phenotype (Gaedigk et al., 2008). Briefly, a CYP2D6 AS is calculated from the CYP2D6 genotype. Fully functional alleles having similar activity to wild-type are given a score of one. Allelic variants that have been reported to result in a decreased activity are given a score of 0.5, and non-functional allelic variants are assigned a value of 0. The CYP2D6 AS of an individual is the sum of the scores for each copy of the
CYP2D6 gene present. These experiments were conducted to evaluate variability in the kinetic parameters of associated with ATX metabolism. CYP2D6 AS and number of HLMs are as follows: AS = 0, n = 2; AS = 0.5, n = 1; AS = 1, n = 8; AS = 2, n = 8; AS = 3, n = 2. Metabolite formation was linear with respect to protein (up to 0.25 mg/mL) and time (up to 10 minutes) in pooled HLMs. For single-donor adult HLMs with a CYP2D6 AS ≥ 1, incubations were performed in the following manner: 0.03 mg/mL protein (final concentration) was pre-incubated at 37 °C with ATX (0.2 – 20 μM, final concentration) for 5 minutes in 100 mM potassium phosphate buffer. The reaction was initiated with 1 mM NADPH (final concentration). The reaction volume was 100 μL. The reaction was quenched after 5 minutes with ice cold acetonitrile (50 μL) containing internal standards and vortexed. Because substrate turnover is reduced in single-donor adult HLMs with a CYP2D6 AS of 0 and 0.5, incubations were modified to contain a final increased protein concentration of 0.1 mg/mL and an increased range of ATX concentrations (0.2 – 1500 μM) to approach enzyme saturating conditions. Kinetic parameters in pooled HLMs were determined using both conditions mentioned previously. Negative control reactions were prepared in pooled HLMs. These included reactions that did not contain one of the following: substrate, enzyme, or NADPH. Reaction vials were subjected to centrifugation at 16,300 g for 10 minutes to precipitate protein using a Spectrafuge 24D (Labnet Internation Inc., Edison, NJ, USA). Supernatant was collected and analyzed via UPLC-MS/MS. All reactions were performed in duplicate.

**Heterologously Expressed Cytochrome P450 Screen.** A screen of heterologously expressed CYP enzymes was conducted at clinically relevant concentrations of ATX to determine CYP isoforms involved in formation of the three primary ATX metabolites. Two picomoles of heterologously expressed enzyme (Cypex Bactosomes) were pre-incubated with ATX (1, 3, or
10 μM) at 37 °C for 5 minutes in 100 mM potassium phosphate buffer and 100 μL volumes. The reaction was initiated with 1 mM NADPH (final concentration) and was quenched after 5 minutes with ice cold acetonitrile (50 μL) containing internal standards and vortexed. Each reaction vial was subject to centrifugation at 16,000 g for 10 minutes to precipitate protein using an AccuSpin 1R centrifuge (Fisher Scientific, Lenexa, KS, USA). Supernatant was collected and analyzed via UPLC-MS/MS. All reactions were performed in duplicate. The following CYP enzymes + reductase (+/− cytochrome b₅) were screened: CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9 + cytochrome b₅, CYP2C18 , CYP2D6 , CYP2E1 + cytochrome b₅, CYP2J2, CYP3A4, CYP3A4 + cytochrome b₅, CYP3A5 + cytochrome b₅, CYP3A7 + cytochrome b₅, CYP4A11, and vector (negative enzyme control).

**Chemical Inhibition Experiments:** Inhibition experiments were conducted in pooled HLMs, an adult single-donor HLM with a CYP2D6 AS = 0.5 (CYP2D6 *4/*41), and an adult single-donor HLM with a CYP2D6 AS = 0 (CYP2D6 *4/*5) to understand CYP contribution to ATX metabolism with decreasing CYP2D6 activity. For pooled HLM incubations, 0.03 mg/mL protein was pre-incubated with 2 μM ATX + inhibitor at 37 °C for 5 minutes in 100 mM potassium phosphate buffer. The reaction was initiated with 1 mM NAPDH (final concentration). The reaction volume was 100 μL. The reaction was quenched after 5 minutes with 50 μL ice cold acetonitrile containing internal standards and vortexed. For the adult single-donor HLM with a CYP2D6 AS = 0.5, reaction conditions were modified to increase substrate turnover, as described above. The final protein and substrate concentration achieved was 0.2 mg/mL and 5 μM, respectively. The total reaction time was 10 minutes. For the adult single-donor HLM with a CYP2D6 AS = 0, reaction conditions were modified to contain a final protein concentration of 0.5 mg/mL, final ATX concentration of 10 μM, and the total reaction time was increased to 30 minutes.
minutes. Inhibition experiments with single-donor adult HLMs did not include a pre-incubation period. All reactions were performed in triplicate. The following CYP isoforms were assayed (specific chemical inhibitor; final concentrations in incubations): CYP1A (α-napthoflavone; 0.15, 0.5, 1.5, and 5 μM); CYP2A6 (tranylcypromine; 0.18, 0.6, 1.8, and 6 μM); CYP2B6 (thiotepa; 1.5, 5, 15, 50 μM); CYP2C8 (montelukast; 0.15, 0.5, 1.5, 5 μM); CYP2C9 (sulfaphenazole; 0.3, 1, 3, 10 μM); CYP2C19 ((S)-(+)N-benzylnirvanol; 0.3, 1, 3, and 10 μM); CYP2D6 (quinidine; 0.15, 0.5, 1.5, 5 μM); CYP2E1 (4-methylpyrazole; 1.8, 6, 18, 60 μM); and CYP3A (ketoconazole; 0.03, 0.1, 0.3, 1 μM). The lowest concentration of inhibitor was chosen to approximate $K_i$, which would result in 50% inhibition of the selected CYP isoform at a substrate concentration that was less than or equal to $K_m$ and therapeutically relevant (whichever was lower). The highest concentration of inhibitor was selected so that most enzymatic activity is inhibited (Khojasteh et al., 2011). An incubation containing only solvent (i.e., without inhibitor) was performed for each specific inhibitor experiment as a positive control. Negative controls reactions were prepared in pooled HLMs. These included reactions that did not contain one of the following: substrate, enzyme, or NADPH. Reaction vials were subject to centrifugation at 16,300 $g$ for 10 minutes to precipitate protein using a Spectrafuge 24D (Labnet International Inc., Edison, NJ, USA). Supernatant was collected and analyzed via UPLC-MS/MS.

**Genotyping of Pediatric HLMs:** All pediatric livers were genotyped for common allelic variants of CYP2D6, CYP2B6 and CYP2C19. CYP2D6 genotyping was carried out as previously described (Gaedigk et al., 2008; Gaedigk et al., 2007; Gaedigk et al., 2010b; Gaedigk et al., 2010a; Gaedigk et al., 2006; Gaedigk et al., 2005; Gaedigk et al., 2012) . Briefly, CYP2D6 genotyping was conducted by amplifying a 6.6 kb long-range (XL) fragment with CYP2D6-specific primers and subsequently utilized as a template for TaqMan (*2-4, *6, *7, *9, *10, *17,
*29, *36 and *40-*42; Thermo-Fisher Scientific, formerly Life Technologies, Foster City, CA) genotyping assays. Six µl reactions were conducted in 96-well plates using the TaqMan Genotyping Master Mix (Life Technologies) or the KAPA Probe qPCR Master Mix (KAPA Biosystems, Wilmington, MA). Cycling was performed on an Applied Biosystems 7900 Real Time PCR System, according to manufacturer’s specifications and data analyzed with the SDS2.4 software. The exon 9 conversion defining CYP2D6*36 was genotyped from genomic DNA using a duplex PCR assay (Gaedigk et al., 2006) and the presence of a *45 or *46 allele was determined by PCR-RFLP (Gaedigk et al., 2005) . The CYP2D6*5 gene deletion, the presence of gene duplications/multiplications and CYP2D7/2D6 hybrid genes were assayed by XL-PCR and/or our quantitative gene copy number assay (Gaedigk et al., 2012). To genotype gene duplications, an 8.6 kb long XL-PCR product encompassing the duplicated gene copy was generated (Gaedigk et al., 2012) and the amplified fragment genotyped for the presence of sequence variations as described above. Genotypes were translated into activity scores as described in the Michaelis-Menten Kinetic Parameter Experiments section according to Gaedigk et al. 2008 (Gaedigk et al., 2008). CYP2B6 genotyping was carried out as described (PMID 23746185 and 26608082). Briefly, rs34223104 (-82T>C), rs3745274 (516G>T, Q172H), rs28399499 (983T>C, I328T) and rs3211371 (1459C>T R487C) were genotyped using TaqMan assays and rs2279343 (785A>G, K262R) was genotyped by high resolution melt (HRM) analysis. PHASE (version 2.1) software was used to construct haplotypes from genotype data as described (Stephens and Donnelly, 2003; Stephens et al., 2001). CYP2C19*2-*4 and *17 were genotyped using TaqMan genotyping assays (Thermo Fisher Scientific) assays. Alleles were defined according to the Human Cytochrome P450 Allele Nomenclature Database at www.cypalleles.ki.se/.
Characterization of ATX Biotransformation by Pediatric HLMs. ATX biotransformation by all three primary routes of oxidative biotransformation was characterized in a set of pediatric HLMs ranging in age from 0.01-18 years with known CYP2D6, CYP2B6, and CYP2C19 genotype to determine the relative contribution of age and genotype to the observed variability in metabolite formation. Pediatric HLMs (0.03 mg/mL final concentration; n = 116), regardless of CYP2D6 AS, were pre-incubated with ATX (1, 3, or 10 μM, final concentration) at 37 °C for 5 minutes in 100 mM potassium phosphate buffer. The reaction was initiated with 1 mM NADPH (final concentration). The reaction volume was 100 μL. The reaction was quenched after 5 minutes with 50 μL ice cold acetonitrile containing internal standards and vortexed. Negative controls reactions were prepared in pooled HLMs. These included reactions that did not contain one of the following: substrate, enzyme, or NADPH. Each reaction vial was subject to centrifugation at 16,000 g for 10 minutes to precipitate protein using an AccuSpin 1R centrifuge (Thermo Fisher Scientific, Lenexa, KS, USA). Supernatant was collected and analyzed via UPLC-MS/MS. All reactions were performed in duplicate.

UPLC-MS/MS Sample Processing. All samples were analyzed using an UPLC-MS/MS method previously developed and validated in our laboratory (Van Haandel et. al, manuscript in progress). Briefly, 10 μL of deproteinated samples were applied to a Waters Acquity UPLC C18 reversed phase column (BEH 1.7 μm, 2.1 x 100 mm) in tandem with a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters, Manchester, UK). Aqueous mobile phase consisted of 90% 10 mM ammonium formate pH = 3.5, 10% methanol (solvent A), and organic mobile phase consisted of 10% 10 mM ammonium formate pH = 3.5, 90% methanol (solvent B). The UPLC gradient used is as follows: initial conditions at the beginning of the gradient were 30% solvent B for 0.10 min; the percentage of B was increased linearly to 70% at 1.30 minutes and this
percentage was maintained until 2.30 minutes; the percentage of B decreased linearly to initial conditions at 2.35 minutes and the column was allowed to re-equilibrate until 3.00 minutes. The following mass and corresponding fragments were monitored: 4-OH-ATX (m/z 272.2 > 44), 4-OH-ATX-d3 (m/z 275.2 > 47), NDM-ATX (m/z 242.04 > 134), NDM-ATX-d7 (m/z 249.1 > 133.98), 2-CH₂OH-ATX (m/z 272.2 > 150).

**Data Analysis.** Kinetic parameter determination in pooled and adult single donor HLMs were performed using GraphPad Prism version 5.02 (GraphPad Software Inc., San Diego, CA, USA). 4-OH-ATX formation rate, calculated as picomole 4-OH-ATX formed, corrected for time and protein content, was plotted against ATX concentration and analyzed using a single-enzyme + one substrate Michaelis-Menten model. Values of $K_m$ and $V_{max}$ were obtained from the fitted model. $Cl_{int}$ was then calculated as $V_{max}/K_m$. Linear regression analysis was subsequently used to describe the association between CYP2D6 activity as measured by dextromethorphan O-demethylation activity (data provided by XenoTech LLC, Lenexa, KS, USA) and calculated $Cl_{int}$. Formation rate of 4-OH-ATX and NDM-ATX metabolites in heterologously expressed enzymes was calculated as picomole of metabolite formed, corrected for both time in minutes and picomole of CYP enzyme in the reaction. Because an authentic standard for 2-CH₂OH-ATX was commercially unavailable, the formation rate was determined as NDM-ATX equivalents. These values were compared to the mean formation rate of metabolite of the vector negative control to determine formation rate of metabolite. Only formation rates and area ratios that were higher than 20% of negative controls are reported. For specific chemical inhibition experiments, mean percentage inhibition was calculated by taking the ratio of mean picomole metabolite formed in experiments that included inhibitor and mean picomole metabolite formed in no inhibitor positive control. This value was subtracted from 1, and multiplied by 100 (equation A).
Mean and standard deviation are reported in results. Inhibition that was greater than 20% was considered experimentally credible.

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(A) \% \text{Inhibition} = \left(1 - \frac{\text{metabolite formed with inhibitor}}{\text{mean metabolite formed with no inhibitor control}}\right) \times 100
\]

Formation rates of 4-OH-ATX and NDM-ATX metabolites in pediatric HLMs were calculated as picomole of metabolite formed, corrected for both time in minutes and milligram of protein in the reaction. As no standard for 2-CH₂OH-ATX was commercially available, 2-CH₂OH-ATX formation was reported as NDM-ATX equivalents. Median and range of formation rates are reported for each concentration of ATX screened (Table 2, Supplemental Table 2, Supplemental Table 3). One-way analysis of variance (one-way ANOVA) analyses were performed to determine genetic factors that could influence formation of ATX metabolites (Norman, 2010). A p-value below 0.05 was considered statistically significant. These analyses were done using data generated with an ATX concentration of 10 µM because this concentration approximates \( V_{\text{max}} \) and differences in metabolism rates due to varying CYP2D6 activity can be detected. Additional one-way ANOVA analyses were conducted on log transformed data (excluding CYP2D6 AS = 0) as the data from formation rates is not normally distributed. The conclusions from the statistical tests from the raw and log-transformed data were in agreement and this data is not shown.
RESULTS

Michaelis-Menten Experiments. $K_m$ and $V_{max}$ values using 4-OH-ATX formation from pooled HLMs were 2.3 $\mu$M and 479 pmol/min/mg protein using conditions for HLMs with a CYP2D6 AS > 1, resulting in a $Cl_{int} = 202$ $\mu$L/min/mg protein. $K_m$ and $V_{max}$ determined from this same pool using experimental conditions used for HLMs with a CYP2D6 AS = 0 or 0.5, values of 2.1 $\mu$M and 357 pmol/min/mg protein were obtained ($Cl_{int} = 168$ $\mu$L/min/mg protein). The decrease in $V_{max}$ in the latter experiment may be due to either substrate or metabolite inhibition at higher ATX concentrations (data not shown). Overall, $K_m$ values varied ~100 fold (range = 0.7-79.2 $\mu$M), and $V_{max}$ values varied ~80 fold (range = 20 – 1600 pmol/min/mg protein) among all single-donor HLM samples analyzed (Table 1, Figure 2). Values of $Cl_{int}$ varied ~3000 fold in the single-donor HLMs (range = 0.3 – 936 $\mu$L/min/mg protein). Linear regression analysis showed a statistically significant association between CYP2D6 activity (dextrorphan formation) and ATX $Cl_{int}$ ($r^2 = 0.74$, $p < 0.0001$) (Figure 3). Interestingly, less variation was noted within HLM preparations where CYP2D6 AS $\geq$ 1 for $K_m$, which varied only ~6-fold (0.7 – 4.5 $\mu$M). However, for these same livers, $V_{max}$ exhibited considerably more variation, ~ 60-fold (range = 28 – 1600 pmol/min/mg protein) (Table 1).

Heterologously Expressed Cytochrome P450 Screen. CYP2D6 generated 4-OH-ATX to the greatest extent regardless of the substrate concentration evaluated (1, 3, or 10 $\mu$M), followed by CYP2J2. Formation rate of 4-OH-ATX for CYP2J2 were approximately 100-fold lower than that of CYP2D6. No other CYP isoforms studied formed 4-OH-ATX. NDM-ATX was formed by CYP1A1, CYP2B6, CYP2C18, CYP2C19, CYP2D6, CYP2J2, and CYP3A4. Of these isoforms, CYP2C18 generated NDM-ATX to the greatest extent, in contrast to previous studies citing that formation of this metabolite is mainly mediated by CYP2C19 (Ring et al., 2002). It should be...
noted that previous studies did not evaluate CYP2C18, most likely due to the fact that CYP2C18 has been shown to have low levels of hepatic expression (Lapple et al., 2003). Allelic variation in this gene is not likely to impact NDM-ATX formation. CYP2C18 and CYP2C19 generated this metabolite to a comparable extent, with CYP2C19 formation rate being 85% of CYP2C18 formation rate at the highest concentration of ATX used. All other isoforms had formation rates that were 40-75% of CYP2C18 formation rate at the highest concentration of ATX studied. CYP2B6 and CYP2D6 had the highest rates of 2-CH₂OH-ATX formation, CYP1A1, CYP2J2, CYP3A4 (with and without cytochrome b₅) also formed 2-CH₂OH-ATX, however to a lesser extent than CYP2B6 and CYP2D6 (Figure 4, Supplemental Table 1).

**Chemical Inhibition Experiments.** In pooled HLMs, only quinidine and 4-methylpyrazole inhibited the formation of 4-OH-ATX in a concentration dependent manner. At the highest concentration of quinidine, the percent inhibition of 4-OH-ATX formed was 98.2 ± 0.6%. Percent inhibition of 4-OH-ATX formed was 74.0 ± 1.8% at the highest concentration of 4-methylpyrazole used. The same two CYP isoforms also contributed to 4-OH-ATX formation in the adult HLM with a CYP2D6 AS = 0.5. Percent inhibition at the highest concentration of quinidine and 4-methylpyrazole was 93.3 ± 0.9% and 58.5 ± 3.4%, respectively. In the adult HLM with a CYP2D6 AS = 0, tranylcypromine, 4-methylpyrazole, and ketoconazole inhibited 4-OH-ATX formation in a concentration dependent manner. At the highest concentration of each of the three inhibitors, 4-OH-ATX formation inhibition was 40.6 ± 3.3% with tranylcypromine, 47.8 ± 1.6% with 4-methylpyrazole, and 80.4 ± 0.6% with ketoconazole (Figure 5). 2-CH₂OH-ATX formation inhibition was only analyzed in single-donor adult livers. In the HLM preparation with a CYP2D6 AS = 0.5, only incubations with tranylcypromine exhibited some concentration dependent inhibition of 2-CH₂OH-ATX formation, implying a role for CYP2A6 in
the formation of this metabolite. However, the CYP2A6 contribution to metabolite formation of metabolite appears to be low, with a mean percent inhibition at the highest concentration of tranylcypromine being 23.6 ± 4.9%. In the HLM preparation with a CYP2D6 AS = 0, CYP2A6 and CYP2B6 appear to contribute to formation of 2-CH$_2$OH-ATX. At the highest concentration of tranylcypromine and thiotepa, percent inhibition was 53.6 ± 4.5% and 51.0% ± 5.0% (Figure 5).

**Characterization of ATX Biotransformation by Pediatric HLMS.** ATX biotransformation by pediatric HLMS was conducted at three concentrations of ATX that reflected systemic plasma drug levels, bracketing an inferred target C$_{\text{max}}$ of 3 µM (Kratochvil et al., 2007; Michelson et al., 2007). Concentration dependent metabolite formation was observed for all metabolites analyzed and the results are shown in Table 2, Supplemental Tables 2, and 3. In general, median formation rates of 4-OH-ATX increased in parallel with CYP2D6 AS, although the pediatric HLMS with AS = 0.5 (n = 5) had higher median formation rates than livers with AS = 1 (n = 34). The median value for HLMS with AS = 0.5 may not be accurate, due to the small sample size. The 4-OH-ATX formation rates exhibited a wider range of values with increasing CYP2D6 AS at each concentration of ATX assayed. Within each CYP2D6 AS group and ATX concentration screened, formation rates of NDM-ATX and 2-CH$_2$OH-ATX did not vary appreciably. However, the relative contribution of these competing pathways to overall ATX biotransformation increased with decreasing CYP2D6 AS (Table 2, Supplemental Tables 2 and 3). Formation rates of metabolites were stratified by AS (CYPD6) or genotype ($CYP2C19$ and $CYP2B6$) (Figure 6). One-way ANOVA analyses were conducted using data from the 10 µM screen, i.e. $V_{\text{max}}$ concentrations, to better understand how genotype affects CYP activity. Mean formation rates of 4-OH-ATX differed significantly between livers of differing CYP2D6 AS scores ($p = 0.03$).
Subsequent correction for multiple testing using the Bonferonni method evaluating livers containing CYP2D6 activity (i.e. AS = 0 livers not included in this analysis) found that significant differences between livers with an AS score = 1 and an AS = 2 persisted. However, there were no other significant differences between other genotype-predicted phenotype groups. There was not a significant association between age and 4-OH-ATX formation rates (Figure 7). Although data from the current investigation indicates that CYP2C18 also plays a substantial role in NDM-ATX formation, much of the current literature suggests that CYP2C19 is the major isoform that mediates formation of this metabolite. For this reason, only CYP2C19 genotype effects were evaluated. For ease of data interpretation, CYP2C19 genetic variants *2-4 were classified as no function alleles (*0) as these are associated with no activity according to the CPIC term standardization project (https://www.pharmgkb.org/page/cpicTermProject).

CYP2C19 genotype groups were stratified in the following manner: *0/*0 (class 1), *0/*1 (class 2), *0/*17 (class 3), *1/*1 (class 4), *1/*17 (class 5), and *17/*17 (class 6). There was no statistically significant effect of CYP2C19 genotype on mean formation rate of NDM-ATX was observed ($p = 0.10$). However, formation of NDM-ATX in livers genotyped as CYP2C19 poor metabolizers (*0/*0) did appear to be lower, while CYP2C19 *17/*17 formation rates appeared higher, consistent with genotype-predicted phenotype. It is important to note that NDM-ATX formation was still present in livers genotyped as class 1, most likely because other CYP isoforms can form this metabolite (Figure 6). Similar to 4-OH-ATX formation, no statistically significant effect of age on NDM-ATX formation was observed (Figure 7). Formation of 2-CH$_2$OH-ATX was stratified by CYP2B6 genotype. Because of the numerous CYP2B6 allelic variants detected and because CYP2B6 genotype effects may result in substrate dependent phenotypes, all allelic variants of this gene were collated and categorized as, “*X.”
genotype groups were stratified in the following manner: \(*X/*X, *X/*I, and *I/*I. One-way ANOVA analysis provided evidence that CYP2B6 genotype does not appear to influence mean 2-CH₂OH-ATX formation \((p = 0.30)\) (Figure 6). As with the other metabolites analyzed, age did not influence formation of this metabolite (Figure 7). A complete list of patient demographics and metabolite formation rates are included in supplemental information (Supplemental Table 4).
DISCUSSION

Although a predominant role for CYP2D6 in the disposition of ATX has been documented by several studies, both in vitro (Ring et al., 2002) and in vivo (Witcher et al., 2003; Cui et al., 2007; Matsui et al., 2012; Brown et al., 2015) other factors also contribute to variability in the dose-exposure relationship as 2- to 5-fold variability in apparent oral clearance can be observed within a CYP2D6 genotype group in vivo (Matsui et al., 2012; Brown et al., 2015). The objectives of this investigation were three-fold: 1. investigate the sources of variability in ATX biotransformation within a CYP2D6 genotype/activity score group; 2. identify other CYP isoforms contributing to ATX biotransformation, particularly in the scenario of lower CYP2D6 activity; and 3. characterize the relative contribution of all pathways of ATX metabolism in a pediatric context, where the influences of CYP genotype, maturation, and development may be evaluated. Further understanding of these three aims will help individualize ATX dosing in children. The first major observation from these studies is that CYP2D6 activity is associated with 74% of the variability observed with ATX Clint when calculated using the kinetic parameters of 4-OH-ATX formation in vitro (Figure 3). In livers genotyped with at least one functional CYP2D6 allele (i.e. AS ≥ 1), variability in Clint appeared to be due to variability in V_max, which correlates to CYP2D6 activity. In vitro (Stevens et al., 2008) and longitudinal phenotyping (Blake et al., 2007) studies have demonstrated that beyond the first week or two of life, age is not a significant determinant of variability in CYP2D6 activity. However, investigations of the regulation of CYP2D6 gene expression have revealed that a long-range single nucleotide polymorphism (SNPs) can influence the expression of CYP2D6 through enhancement of gene transcription (Wang et al., 2014; Wang et al., 2015). Expanding genotyping panels to include this SNP could potentially aid to improve prediction of ATX exposure. Data on
additional factors impacting CYP2D6 transcription are sparse, although farnesoid X receptor (FXR) transactivation of small heterodimer partner (SHP) has been demonstrated to play a role in CYP2D6 regulation (Pan et al., 2015). FXR acts as a bile acid sensor and high levels of ligand promote transactivation of SHP, which reduces expression of proteins involved in bile acid synthesis. CYP2D6 gene expression also appears to be reduced with bile acid binding of FXR (Pan et al., 2015; Pan and Jeong, 2015). Finally, there has been recent interest in the role that variability in cytochrome P450 oxido-reductase (POR) may have on inter-individual variability in CYP-mediated oxidative biotransformation reactions. POR variant proteins affected the turnover of three different CYP2D6 substrates, with essentially no effect on $K_m$, but effects on $V_{max}$ ranging from an inability to support catalytic activity (R457H) to a 4- to 6-fold range in $V_{max}$ values, depending on the individual substrate (Sandee et al., 2010). Additional work is required to determine the relative contribution of these factors to observed variability in ATX clearance in vitro and in vivo.

The second contribution provided by this investigation is the identification of the CYP isoforms responsible for 4-OH-ATX formation in livers with lower CYP2D6 activity. $K_m$ and $V_{max}$ determined from 4-OH-ATX formation in livers with an AS scores of 0 and 0.5 had increased $K_m$ and decreased $V_{max}$ values. Different CYP isoforms are likely responsible for the Michaelis-Menten profile for 4-OH-ATX formation, as previously published (Sauer et al., 2003). Heterologously expressed enzyme CYP2J2 was the only CYP, other than CYP2D6, to form 4-OH-ATX, albeit with rates 100-fold lower than CYP2D6. Hepatic abundance of CYP2J2 is low, but its importance may be relevant in individuals who have low CYP2D6 activity, as with the anti-viral drug ritonavir (Kaspera et al., 2014). Studies using specific chemical inhibitors of CYPs in the HLMs samples genotyped as intermediate metabolizers (AS = 0.5) found that
CYP2D6 and CYP2E1 were the major isoforms involved in 4-OH-ATX formation. CYP2E1 metabolism of ATX is unusual, given that the drug is bigger than a prototypical CYP2E1 substrate (< 100 gram/mol) (Sridhar et al., 2012). The sum percent inhibition of 4-OH-ATX between CYP2D6 and CYP2E1 is greater than 100%, suggesting loss of specificity with quinidine or 4-methylpyrazole. Specific chemical inhibition is not well studied for CYP2E1 (Khojasteh et al., 2011). Linear regression analysis evaluating the association between CYP2E1 activity (chlorzoxazone 6-hydroxylation) and Clint was analyzed to further elucidate this relationship. A statistically significant association was noted ($r^2 = 0.46$, $p = 0.042$). Visual examination of this analysis found what appears to be a biased outlier. Removal of this observation resulted in a non-significant association ($r^2 = 0.36$, $p = 0.12$). Additionally, recombinant CYP2E1 did not form 4-OH-ATX. In the CYP2D6 PM (AS = 0) HLM, inhibition studies suggested a role for CYP3A in the 4-hydroxylation of ATX, which was apparent with higher concentrations of ATX and increased incubation time.

This is the first investigation to examine 2-CH$_2$OH-ATX formation. Heterologously expressed CYP2B6 formed this metabolite to the greatest extent, followed by CYP2D6. Inhibition studies in HLMs with low CYP2D6 AS confirmed the role for CYP2B6 and also implicated CYP2A6 in formation of 2-CH$_2$OH-ATX. Cumulatively, in the absence of CYP2D6, it appears that CYPs 2B6, 2C18, 2C19, 2E1, 2J2, and 3A4 are the primary pathways contributing to hepatic ATX biotransformation. Thus, factors contributing to variability in the activity of these pathways in children - ontogeny and genetic variation - could cause inter-individual variability in ATX disposition in children with reduced CYP2D6 activity. While the exact percent contribution of these pathways cannot be calculated at this time, the data from the pediatric HLM screen suggests that the competing pathways can contribute to ~98% of ATX
metabolism on average in PM livers. In non-PM livers the contribution of competing pathways decreases to 33%. On average, we found that 4-OH-ATX was the most abundant metabolite, followed by 2-CH$_2$OH-ATX, and NDM-ATX. However, these values are tentative considering that the study design was optimized for CYP2D6-mediated 4-OH-ATX formation, i.e. we did not have a standard to measure 2-CH$_2$OH-ATX metabolite formation.

The third issue addressed in our study was characterization of the relative contributions of age and genotype in each of the three primary pathways of ATX biotransformation. As expected, $CYP2D6$ genotype had a major impact on 4-OH-ATX formation rate. Age had no effect on 4-OH-ATX formation. Neither age nor genetic variants of $CYP2C19$ and $CYP2B6$ appeared to affect formation rates of NDM-ATX and 2-CH$_2$OH-ATX metabolites. Changes in expression and activity of enzymes, due to ontogeny or genetic variation, involved in competing pathway would not be captured if an individual liver also has high CYP2D6 activity. However, multiple CYP isoforms can form NDM-ATX and 2-CH$_2$OH-ATX and there was not a high differential in the rates of formation for the metabolites among CYP isoforms studied. This may cause genotype or ontogeny effects to be dampened. The reason why CYP2D6 differs is because the formation rate of 4-OH-ATX was at least 100-fold higher compared to other isoforms. It is likely that differences in ontogeny and genetic variation in competing pathways will be more apparent in individuals who have low CYP2D6 activity.

This investigation guides future directions of research needed to build tools to individualize ATX use in a pediatric population. Although we confirmed that ATX metabolism and $C_l_{\text{int}}$ is primarily associated with $CYP2D6$ genotype and phenotype, substantial variability is still unexplained, which is tied to a partial understanding of factors that influence CYP2D6 activity. Therefore, understanding the mechanisms of $CYP2D6$ gene regulation and CYP2D6
activity modulation are important for understanding ATX pharmacokinetics in this population. Incorporating genetic and ontogeny information of these factors may enhance the ability to predict CYP2D6 \textit{in vivo} activity, and hence also result in improved ability to predict drug-exposure relationships for an individual child. Accurately predicting CYP2D6 activity would also allow for proper weighting of other CYPs involved in ATX metabolism. Differences in CYP2E1, CYP2J2 and CYP3A enzymatic activities will particularly influence clearance through the 4-OH-ATX pathway for individuals with low CYP2D6 activity. The importance to ATX clearance is only relative with decreasing CYP2D6 activity. In order to build a comprehensive PBPK model of ATX, the nuances of ATX metabolism in genetic variation and ontogeny must be weighted as function of CYP2D6 activity that takes into account data from genotype, factors that affect transcription and translation, and variability of accessory proteins. The increased percent contribution of competing pathways with decreasing CYP2D6 activity (i.e. AS = 0-0.5) must also be considered. Variability in NDM-ATX and 2-CH$_2$OH-ATX formation becomes influential when formation of these metabolites are rate limiting steps to overall ATX clearance. Both ontogeny and genetic polymorphisms have the potential to affect these minor routes of metabolism. As such, for certain CYPs, further investigation of ontogenic trajectory and/or genetic variability is needed for an accurate bottom-up model. In summary, a comprehensive PBPK model that can be used to predict individual ATX clearances must include \textit{a priori} knowledge of factors that influence CYP2D6 activity which can then be weighted against the contribution and variability of competing CYP isoforms.
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AUTHORSHIP CONTRIBUTION

Participated in research design: Dinh, Gaedigk, Leeder, Pearce, Van Haandel

Conducted Experiments: Dinh, Gaedigk, Pearce, Van Haandel

Contributed new reagents or analytical tools: not applicable

Wrote or contributed to the writing of the manuscript: Dinh, Gaedigk, Leeder, Pearce, Van Haandel
REFERENCES


FOOTNOTES

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FIGURES LEGENDS

**Figure 1.** Scheme of ATX metabolism *in vivo*. Figure reproduced and modified from Mattiuz et al. with permission from *Drug Metabolism and Disposition* (Mattiuz et al., 2003).

**Figure 2.** Michaelis-Menten plots of 4-OH-ATX formation in 21 single-donor HLMs of varying CYP2D6 phenotype. Extensive and ultrarapid metabolizers are plotted in panel (A), while intermediate and poor metabolizers are plotted in panel (B). Extensive metabolizers were individuals with a CYP2D6 AS = 1-2. Extensive metabolizers were subcategorized as EM1 (green plots) or EM2 (blue plots), depending on CYP2D6 AS. Ultra-rapid metabolizers (UMs) were individuals with an AS > 2 (purple plots). Intermediate metabolizers (IMs) are individuals with a CYP2D6 AS = 0.5, which indicates one copy of a *CYP2D6* allele with compromised activity (yellow plot). Poor metabolizers (PMs) have two no function *CYP2D6* alleles (red plots). (PM, n = 2; IM, n = 1; EM1, n = 8, EM2 = 8, UM, n = 2).

**Figure 3.** Linear regression analysis evaluating ATX intrinsic clearance (Clint) and CYP2D6 activity measured by dextromethorphan (DM) *O*-demethylation activity. Clint was calculated by taking the ratio of \( V_{\text{max}}/K_m \) from data obtained from 4-OH-ATX formation. Data from CYP2D6 phenotypic activity was provided by Xenotech, LLC. Data from 20 HLMs was included in this analysis, because one PM HLM preparation did not produce a reliable estimate of \( K_m \) (\( r^2 = 0.74, ***p < 0.0001 \)). UM = purple dots, EM2 = blue dots, EM1 = green dots, IM = yellow dot, PM = red dot.

**Figure 4.** Formation rate of 4-OH-ATX (A), NDM-ATX (B), and 2-CH\(_2\)OH-ATX (expressed as NDM-ATX equivalents) (C) in heterologously expressed CYP enzymes incubated with ATX (1, 3, and 10 \( \mu \)M). Only those CYPs isoforms that displayed a concentration dependent increase of
metabolite formation that was greater than the limit of quantitation or the rate of formation by the negative control (vector) are shown. All experiments were conducted in duplicate.

**Figure 5.** Relative percentage of 4-OH-ATX and 2-CH₂OH-ATX formed when ATX co-incubated with chemical inhibitors of specific CYP isoforms. Relative formation of 4-OH-ATX are reported with pooled HLMs (A), as well as adult single-donor HLMs with CYP2D6 AS = 0.5 (B) and 0 (C). Relative formation of 2-CH₂OH-ATX are reported with adult single donor HLMs with CYP2D6 AS = 0.5 (D) and 0 (E). All HLM preparations were obtained from Xenotech, LLC. All experiments were conducted in triplicate.

**Figure 6.** ATX metabolite formation stratified by CYP2D6, CYP2C19 and CYP2B6 genotype. These three CYPs are the most important contributors to 4-OHATX formation. Data from the 10 µM ATX pediatric HLM screen were used for these analyses. 4-OH-ATX formation rate is stratified by CYP2D6 activity score (A), NDM-ATX formation rate by CYP2C19 genotype (B), and 2-CH₂OH-ATX formation rate by CYP2B6 genotype (C). Livers genotyped as CYP2C19 *2, *3, or *4 were collated as non-functional alleles (i.e. *0) as all have been reported to result in loss of activity. Due to the numerous number of allelic variants detected for CYP2B6, genotype groups were categorized as homozygous wild-type (*1/*1), heterozygous (*1/*X), and homozygous variant (*X/*X). All experiments were conducted in duplicate.

**Figure 7.** Formation of 4-OH-ATX (A), NDM-ATX (B), and 2-CH2OH-ATX (C) as a function of age. This figure was generated using data from the pediatric HLM screen with 10 µM screen. A total of 116 individual livers were evaluated in duplicate.
Table 1. Demographic information and kinetic parameters derived from 4-OH-ATX formation in 21 individual human liver microsomal preparations of varying CYP2D6 genotype and activity score.

<table>
<thead>
<tr>
<th>HLM #</th>
<th>CYP2D6 Genotype</th>
<th>Activity Score</th>
<th>M/F</th>
<th>Age</th>
<th>Race</th>
<th>Km (µM)</th>
<th>Vmax (pmol/min/mg protein)</th>
<th>Clint (µL/min/mg protein)</th>
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<td>44</td>
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<td>49</td>
<td>C</td>
<td>59.1*</td>
<td>4.6*</td>
<td>NA</td>
</tr>
<tr>
<td>H0446</td>
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<td>0.9</td>
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<tr>
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<td>H0028</td>
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<td>Age</td>
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<td>GMN</td>
<td>Focal Cortex</td>
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<td>2nd Layer</td>
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Table 2. Formation rate of 4-OH-ATX, NDM-ATX, and 2-CH$_2$OH-ATX in NDM-ATX equivalents, stratified by CYP2D6 genotype. Data is from incubations conducted at ATX 3 μM. All experiments conducted in duplicate.

<table>
<thead>
<tr>
<th>CYP2D6 AS (n)</th>
<th>4'-hydroxy-ATX Formation Rate (pmol/min/mg)</th>
<th>N-desmethyl-ATX Formation Rate (pmol/min/mg)</th>
<th>2-methyl-OH-ATX Formation Rate (pmol/min/mg)</th>
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<tr>
<td>0 (10)</td>
<td>0 (0 - 0)</td>
<td>25.1 (18.9 - 55.0)</td>
<td>15.4 (5.7 - 185.0)</td>
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<tr>
<td>0.5 (5)</td>
<td>89.6 (10.5 - 328.2)</td>
<td>38.5 (29.6 - 49.2)</td>
<td>48.7 (29.5 - 98.7)</td>
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<td>1 (34)</td>
<td>93.3 (6.1 - 445.4)</td>
<td>37.6 (18.0 - 62.8)</td>
<td>42.1 (5.8 - 251.5)</td>
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<td>1.5(20)</td>
<td>212.6 (28.9 - 489.0)</td>
<td>38.4 (18.1 - 54.6)</td>
<td>54.0 (8.7 - 481.4)</td>
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<td>2+(47)</td>
<td>185.9 (0 - 1540.9)</td>
<td>29.5 (15.7 - 74.2)</td>
<td>29.8 (5.4 - 382.5)</td>
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</table>
Figure 1
Figure 2
Figure 3

The graph shows a positive correlation between ATX Clint (µL/min/mg protein) and CYP2D6 Activity (DM O-demethylation pmol/min/mg). The correlation coefficient $r^2 = 0.74$ and the significance level is $***P < 0.0001$. The data points are represented with different colors to indicate different groups or conditions.
Figure 4
Figure 5

Chemical Inhibitor Key

- α-NF = α-naphthoflavone
- BN = benzynirvanol
- TCP = tranylcypromine
- Quin = quinidine
- TTP = thiotepa
- 4-MP = 4-methylpyrazole
- MLK = montelukast
- Keto = ketoconazole
- SPZ = sulfaphenazole
Figure 6
Figure 7