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In Vitro Hepatic Metabolism Explains Higher Clearance of Voriconazole in Children *versus* Adults: Role of CYP2C19 and FMO3

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Abbreviation: P450, cytochrome P450; FMO, flavin-containing monooxygenase; K_m, apparent Michaelis constant; V_{max}, maximum reaction velocity; HPLC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; SF, scaling factor; Cl, clearance;

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$Cl_{intrinsic}$, intrinsic clearance; fu, unbound fraction of drug; MPPG, milligram microsomal protein per gram liver; PK, pharmacokinetics; AUC, area under the plasma concentration-time curve.

Abstract

Voriconazole is a broad spectrum antifungal agent for treating life threatening fungal infections. Its clearance is approximately three-fold higher in children compared to adults. Voriconazole is cleared predominantly via hepatic metabolism in adults, mainly by CYP3A4, CYP2C19, and flavin-containing monooxygenase 3 (FMO3). *In vitro* metabolism of voriconazole by liver microsomes prepared from pediatric and adult tissues (n=6/group) mirrored the *in vivo* clearance differences in children *versus* adults, and showed that the oxidative metabolism was significantly faster in children compared to adults as indicated by the *in vitro* half-life (T ½) of 33.8 ± 15.3 *versus* 72.6 ± 23.7 min, respectively. The K_m for voriconazole metabolism to N-oxide, the major metabolite formed in humans, by liver microsomes from children and adults was similar (11.0 ± 5.2 µM *versus* 9.3 ± 3.6 µM, respectively). In contrast, apparent V_{max} was approximately 3-fold higher in children compared to adults (120.5 ± 99.9 *versus* 40.0 ± 13.9 pmol/min/mg). The calculated *in vivo* clearance from *in vitro* data was found to be approximately 80% of the observed plasma clearance values in both populations. Metabolism studies in which CYP3A4, CYP2C19, or FMO was selectively inhibited provided evidence that contribution of CYP2C19 and FMO toward voriconazole N-oxidation was much greater in children than in adults, whereas CYP3A4 played a larger role in adults. While expression of CYP2C19 and FMO3 is not significantly different in children *versus* adults, these enzymes appear to contribute to higher metabolic clearance of voriconazole in children *versus* adults.

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Introduction

Voriconazole (Vfend ™, Pfizer) is a potent triazole antifungal agent with broad activity for treating life-threatening fungal infections in children (Steinback & Benjamin, 2005). In adults, voriconazole is given twice daily: orally 200-300 mg or intravenously 3-4 mg/kg. The absolute bioavailability is estimated to be 96% and its systemic clearance is 2.0 ml/min/kg (Purkins et al., 2002). Voriconazole is cleared predominantly by oxidative metabolism in the liver (EPAR, 2002; Hyland et al., 2003; Roffey et al., 2003; Yanni et al., 2008). CYP2C19, CYP3A4, FMO, and to a lesser extent CYP2C9 contribute to the oxidative metabolism of voriconazole by human liver microsomes. The two major metabolites are the N-oxide and the hydroxymethyl derivative as depicted in Figure 1 (Hyland et al., 2003; Theuretzbacher et al., 2006; Roffey et al., 2003 Murayama et al., 2007). The N-oxide, which accounts for 72% of the circulating metabolites of voriconazole (Roffey et al, 2003), is predominantly formed by CYP3A4 (Hyland et al., 2003) and FMO3 (Yanni et al, 2008); CYP3A5 and CYP3A7 form this metabolite 4- and 15-fold less efficiently than CYP3A4 Murayama et al., 2007). FMO1 also forms voriconazole N-oxide (Yanni et al., 2008); however, it is only expressed in fetal livers (Hines and McCarver, 2002) and therefore only FMO3 was considered in the present study.

In multi dose studies, the AUC and C_{max} of voriconazole in adult subjects increased non-linearly over a narrow dose-range (e.g. 3-5 mg/kg or 200-400 mg) thereby suggesting saturation of clearance (Purkins et al., 2002; Walsh et al., 2004; Leveque et al., 2006). In contrast to adults, the plasma clearance of voriconazole in children age 2-11 years was found to be almost 3-fold higher; furthermore, both the AUC and C_{max} of voriconazole increased linearly in children (Walsh, et al., 2004; Leveque et al., 2006). Recent population pharmacokinetic analyses of voriconazole in children 2-12 years of age have shown that an intravenous dosage of 7 mg/kg in

young children yields a similar exposure to 3-4 mg/kg given to adults. Furthermore, oral bioavailability of voriconazole was found to be much lower (44.6%) in children than in adults (~96%) (Karlsson et al., 2009).

Several factors may contribute to observed differences of drug disposition between children and adults. These include organ maturation, body composition, changes in liver size, liver blood flow, extent of plasma protein binding, changes in the hormonal levels, and alteration in the expression and/or catalytic activity of some of the drug metabolizing enzymes (Hines and McCarver, 2002; Kearns et al., 2003, Chen et al., 2006, Kennedy, 2008). For example, drugs that are metabolized by hepatic CYP2C9/19 such as phenytoin, omeprazole, and sirolimus show higher clearance in children between 2-10 years of age than in adults (Kennedy 2008, Litalien et al, 2005; Filler, et al., 2008).

Voriconazole, because it is cleared predominantly by hepatic metabolism, may have an increased clearance in children due to differences in the expression or catalytic activity of one or more drug metabolizing enzymes. Hence, *in vitro* metabolism of voriconazole by liver microsomes from children between 2-10 years of age was compared to that from adults. Results showed that metabolism of voriconazole by liver microsomes from children *versus* adults mirrored the differences in the clearance of the drug in the two populations. The likely role of CYP2C19, CYP3A4, and FMO3 in altering the metabolic clearance by liver microsomes from adult *versus* children was evaluated.

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Materials and Methods

Chemicals and Reagents: Voriconazole was generously supplied by Pfizer Global Research and Development. Cytochrome P450 (P450) and FMO substrates, metabolites, and inhibitors were obtained from commercial sources. Primary antibodies for immunoblotting analysis and recombinant P450 enzymes and FMO3 were purchased from BD Gentest (Woburn, MA). Voriconazole N-oxide was synthesized as described in Yanni et al. (2008). Frozen liver tissues from adults and children were obtained from Comparative Human Tissue Network (CHTN) (Columbus, OH) under an approved UNC-Chapel Hill Institutional Review Board protocol. The ages of children donors were between 2-8 years, while the adult donors were >18 years of age. The normal liver tissues, free of disease, were snap frozen within 6 hours post mortem.

Preparation and Characterization of Liver Microsomes from Adults and Children: Liver microsomes were prepared from the frozen adult and pediatric tissues by a standard procedure. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL) against bovine serum albumin as a standard. All microsomal preparations were pre-screened for the overall metabolic capacity, and only those preparations that showed robust oxidative metabolism, as indicated by ability to extensively metabolize (>80%, 1 mg protein/ml) 7-ethoxycoumarin (20.0 μM) in 60 min (Shimada et al., 1999), were selected for this study. Six samples of liver microsomes from each group were selected accordingly. The selected microsomal samples were then characterized for P450 activities as previously described (Yanni et al., 2008).

Voriconazole Oxidative Metabolism by Liver Microsomes from Adults and Children: Initial rate of voriconazole (2.0 μM) consumption with respect to time and protein was determined as previously described (Yanni et al., 2008). Liver microsomes (1.0 mg/ml) from

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each subject were mixed with NADPH (2.0 mM), phosphate buffer (100.0 mM, pH 7.4), and magnesium chloride (3.0 mM). The metabolic reaction was initiated by addition of voriocnazole, aliquots were then removed at defined time points from 0.0 – 20.0 min, and mixed with ice cold methanol containing an internal standard (diclofenac) to terminate the reaction. After centrifugation at 10,000xg for 10.0 min, the supernatants were evaporated to dryness, the residues were then dissolved in 10% acetonitrile in water, and analyzed for voriconazole using HPLC-UV (254 nm). Percent of voriconazole remaining was determined by comparing voriconazole/internal standard peak area ratio at each time point to that at time zero (100%). The slope of the log (percent remaining) *versus* incubation time plot was used to determine *in vitro* half-life ($T_{1/2}$) (Obach and Reed-Hagen, 2002). For kinetic analysis, voriconazole over a concentration range of 0.5-500 µM was metabolized as described above, and the voriconazole N-oxide formed was analyzed by HPLC-MS/MS (Yanni, et al. 2008). Non-linear regression analysis (WinNonLin, Pharsight-Cary, NC V.04) was performed using the Michaelis-Menten equation to determine apparent K_m and V_{max} for voriconazole N-oxide formation by liver microsomes from each subject.

Role of P450 and FMO Enzymes in the Formation of Voriconazole N-oxide by Liver Microsomes from Adults and Children: To compare the contribution of CYP2C19, CYP3A4, or FMO in voriconazole metabolism by liver microsomes from adults and children, metabolism of voriconazole by liver microsomes derived from adults (pool of six samples) or from children (pool of six samples) was evaluated in the presence of fluvoxamine (CYP2C19 inhibition, 3.0 µM), ketoconazole (CYP3A4 inhibition, 3.0 µM), or with heat-inactivated microsomes (FMO inhibition, 45 ^{o}C for 5.0 min), respectively. At the concentrations used, the inhibitors were selective for the respective enzymes, and effective under the experimental conditions used (Yanni et al., 2008). Voriconazole N-oxide formed under each condition was measured and the

N-oxidation activity corresponding to CYP2C19, CYP3A4, or FMO3 was estimated by comparing the inhibited activity with the no-inhibition control activity. The percent contribution of these three enzymes to the overall voriconazole N-oxide formation (100%) by adults and children was also estimated.

Expression of CYP2C19, CYP3A4, and FMO3 in liver Microsomes from Children and Adults: A pool of liver microsomes from the six adults or six children was used to determine the differential expression of CYP2C19, CYP3A4, and FMO3 proteins in children *versus* adults. Liver microsomes and recombinant protein standards for CYP3A4, CYP2C19, and FMO3 were subjected to electrophoresis and the proteins transferred to nitrocellulose membranes; subsequently, the nitrocellulose membranes were incubated with anti-CYP3A4, anti-CYP2C19, and anti-FMO3 at a dilution of 1:4000, 1:500, and 1:1000, respectively, for 16 h at 4°C. The bound antibody was detected by BioRad VersaDoc Imaging System. The intensity of the bands corresponding to CYP3A4 (57 KDa), CYP2C19 (52 KDa), or FMO3 (57 KDa) was determined, and expression based on the amount of loaded protein was calculated.

Analytical Methods: Voriconazole in the incubation samples was chromatographically separated using an Agilent 1100 HPLC system (Santa Clara, CA) and C18 Zorbax Eclipse column (150 x 4.6 mm, 5µ - Agilent) as previously reported (Yanni et al., 2008). Briefly, the analytes were eluted from the column with a linear gradient of mobile phase A:B (v/v) starting from 95:5 at 0 min to 30:70 in 10 min at a flow rate 1.1 ml/min; the mobile phase A was 5 mM ammonium acetate with 0.1% formic acid, pH 4.0, and mobile phase B was acetonitrile with 0.1% formic acid. Voriconazole was detected by UV at its λ_{max} of 254 nm; the retention time was 8.2 min. Voriconazole N-oxide in samples from the metabolism studies was analyzed by an HPLC-MS/MS method on an Applied Biosystems, API 4000-triple quadrupole mass

spectrometer (MDS Sciex Instruments - San Francisco, CA) fitted with TurbolonSpray® interface in conjunction with Shimadzu solvent delivery system (Kyoto, Japan) and a CTC PAL auto-sampler (Carborro, NC). The HPLC separation was achieved on an Aquasil C18 analytical column (50 x 2.1, 5µ - Thermo Scientific, Waltham, MA) using gradient elution of mobile phases from 10% B in A (v/v) at 0.5 min to 95% B in A (v/v) at 3 min at a flow rate 0.7 ml/min (injection volume was 5.0 µl); mobile phase A was 0.1% formic acid and mobile phase B was methanol with 0.1% formic acid. Multiple reaction monitoring (MRM) was used to monitor voriconazole (m/z transition 350→127), the N-oxide (m/z transition 366→224), and the internal standard (diclofenac, m/z transition 296→215) in positive ion mode. The retention time for the N-oxide and voriconazole were 2.5 min and 2.8 min, respectively. For quantitative determination of the N-oxide formed, a calibration curve was constructed using the peak area ratio of authentic standard of the N-oxide (Yanni et al., 2008) to the internal standard over a concentration range of 1.0 nM – 5.0 µM (R^2>0.99). The quantitative determination of 4-hydroxymephenytoin (m/z transitions of 235→150) and 6β-hydroxytestosterone (m/z transition 305→269), the metabolites of mephenytoin and testosterone formed by CYP2C19 and CYP3A4, respectively, was achieved by a simultaneous HPLC-MS/MS method as previously described (Yanni et al., 2008). Quantitative determination was based on a calibration curve of corresponding hydroxyl metabolite standards.

Data Analysis: The kinetic parameters K_m and V_{max} determined for six children and six adults were used to estimate the *in vitro* intrinsic clearance (V_{max}/K_m) for voriconazole N-oxidation. The V_{max}/K_m values were scaled to whole body intrinsic clearance (Cl_{int}) according to Equations 1 and 2**.**

$CL_{int} = V_{max} / K_m$ (ml/min/mg) x Scaling Factor (mg/kg) **(Eq. 1)**

Scaling Factor = [MPPG (mg/g) x liver wt (g)] / Body wt (kg) **(Eq. 2)**

where MPPG is milligram microsomal protein per gram liver that was experimentally determined for each subject, liver wt and body wt were obtained from published data of each subject's known age (**Björkman , 2004)**. *In vivo* clearance of voriconazole was calculated based on *in vitro* data from children and adults using the well-stirred model of hepatic disposition (**Björkman, 2006**) as shown in Equation 3:

Predicted Cl = QH * f_u * Cl_{int} / QH + f_u * Cl_{int} **(Eq. 3)**

where QH is hepatic blood flow, f_u is unbound drug fraction, and Cl_{int} is the scaled intrinsic clearance determined from in vitro metabolism study.

Statistical Analysis: All data are expressed as mean ± standard deviation (S.D) of a minimum of three experimental determinations. The statistical significance for the difference between K_m, V_{max}, scaling factor, intrinsic clearance, or total clearance in children (n = 6) *versus* adults (n = 6) was determined by the Wilcoxon rank sum test, while unpaired t-test was used to compare between treated and control in the inhibition studies. In both cases, $p < 0.05$ was accepted for statistic significance.

Results

***In vitro* Oxidative Metabolism of Voriconazole by Liver Microsomes from Adults and Children:** The metabolism of voriconazole by liver microsomes from adults and children as function of time is shown in Figure 2. The half-life of voriconazole metabolism by liver microsomes from children (33.8 ± 15.3 min) was over two-fold shorter than that obtained with liver microsomes from adults (72.6 ± 23.7 min) and the difference in the half-life between the two groups was statistically significant (p <0.05).

The rate of the N-oxide formation by liver microsomes from adults and children as a function of voriconazole concentration followed Michaelis-Menten kinetics (Figure 3). The kinetic parameters for the reaction carried out by liver microsomes from individual subjects in the adult and pediatric group are listed in Table 1. The apparent K_m value for voriconazole metabolism by liver microsomes from children *versus* adults is not significantly different (11.0 ± 5.2 μM and 9.3 ± 3.6 μM, respectively), however the mean apparent V_{max} was three-fold higher with liver microsomes from children *versus* adults (120.5 ± 99.9 pmol/min/mg and 40.0 ± 13.9 pmol/min/mg, respectively) and the difference is statistically significant (p < 0.002).

Role of P450 and FMO in Higher Rate of Voriconazole N-Oxide Formation in Children *versus* Adults: CYP2C19, CYP3A4, and FMO are responsible for most of the voriconazole metabolism by liver microsomes from adults (Yanni et al., 2008). Hence, their role in the quantitative differences in voriconazole N-oxidation by liver microsomes from children *versus* adults was assessed. This was accomplished by measuring the rate of formation of voriconazole N-oxide by liver microsomes from children and adults in the presence of fluvoxamine (CYP2C19 inhibitor), ketoconazole (CYP3A4 inhibitor), or upon heat inactivation (FMO inhibition) (Figure 4A). The CYP3A7 activity (estrone 16α-hydroxylation) was assessed in the

microsomal preparations from pediatric livers used in this study, and was found to be approximately 1000-fold lower than the CYP3A4 activity (testosterone 6β-hydroxylation). Further, Murayama et al. (2007) have showed that the rate of voriconazole N-oxide formation by recombinant CYP3A5 and CYP3A7 is 4-fold and 15-fold lower, respectively, than that by CYP3A4. For these reasons, the involvement of CYP3A5 and CYP3A7 in voriconazole N-oxide formation was not assessed in this study. The estimated N-oxidation by FMO3 and CYP2C19 is five - and three -fold higher, respectively in children compared to adults, but the estimated N-oxidation by CYP3A4 is not different in both groups (Figure 4B). Although the results showed that CYP3A4 contributed predominantly to voriconazole metabolism by microsomes from adult livers, CYP2C19 and FMO3 contributed much more than CYP3A4 to voriconazole metabolism by microsomes from pediatric livers (Figure 4). The expression of CYP2C19 and FMO3 is somewhat higher and that of CYP3A4 is somewhat lower in the liver tissue from children *versus* adult, as determined by the band densities in the Western blot analysis (Figure 4C); the densities of the CYP2C19, FMO, and CYP3A4 bands for the liver tissue from children were 140%, 170%, and 60%, respectively, of that for the liver tissues from adults. When the specific activity of CYP2C19 (S-mepnytoin-4 hydroxylation), CYP3A4 (Testosterone 6-β hydroxylation), and FMO (Benzydamine N-oxidation) in microsomes from adult and pediatric livers was compared (Figure 4C), the results showed that the CYP2C19 and FMO activities were higher in children *versus* adults, whereas the CYP3A4 activity in both groups was comparable.

Calculated *in vivo* Clearance in Children and Adults: The calculated *in vitro* intrinsic clearance (V_{max}/K_m), determined for metabolism of voriconazole by liver microsomes, was found to be three-fold higher in children (11.5 ± 6.2 µl/min/mg) than in adults (4.5 ±1.3 µl/min/mg). The whole body intrinsic clearance was calculated by multiplying the *in vitro* metabolic intrinsic clearance by a scaling factor as shown by equation 1 and 2. The calculated scaling factor, based

on MPPG and liver weight that were normalized to body weight, was somewhat higher for children (878.3 +/-211.3 mg/kg) *versus* adults (650.0 +/- 99.8 mg/kg) but the statistical significance ($p > 0.04$) was marginal (Table 1). The MPPG value was determined experimentally for each subject and ranged from 18 - 40 mg/g with the mean value of 26.0 mg/g in children, while in adults, MPPG ranged from 27- 37 mg/g, with a mean value of 30.3 mg/g. The mean liver weight in children with age from 2-8 years was reported as 575 g (470-740 g) by Bjorkman et al., (2004), while an average value of 1500 g was used for the liver weight for adults (Bjorkman et al., 2004). The body weight of children of age 2-8 years ranged from 12-25.4 kg with a mean value of 17.2 kg, while 70 kg was set as an average body weight for adults. The whole body intrinsic clearance was ~3-fold higher ($p < 0.01$) in children (9.9 ± 5.0 ml/min/kg) compared to adults (2.9 ± 1.0 ml/min/kg) The *in vivo* clearance of voriconazole was calculated to be 5.1 ml/min/kg in children and 1.6 ml/min/kg in adults using the scaled intrinsic clearance values in children and adults, unbound voriconazole fraction (fu) of 0.6 in both adults (Leveque et al., 2006) and children, and the hepatic blood flow value of 37 ml/min/kg in children and of 24 ml/min/kg in adults (Bjorkman et al., 2004) (Equation 3). These values are approximately 80% of the values observed in the pharmacokinetic studies in children (6.7 ml/min/kg) and in adults (2.0 ml/min/kg) that were previously reported by Leveque et al. (2006). Conceivably, inclusion of the kinetic parameters only for the formation of the N-oxide, and not for other routes of voriconazole metabolism, may have contributed to the calculated clearance being 20% lower than the observed clearance.

Discussion

Voriconazole, a frequently prescribed drug for treatment of fungal infection in children, is cleared much more rapidly in children than in adults, and exhibits distinctly different pharmacokinetic profiles in these two populations (Walsh, et al., 2004; Leveque et al., 2006; Karlsson et al., 2009). For example, voriconazole clearance in adults loses linearity over a very small increase in dose from 3 to 5 mg/kg, whereas the clearance remains linear in children over a similar increase in doses. Also, the oral bioavalability of voriconazole is two-fold lower in children (44%) than in adults (96%) (Karlsson et al., 2009), suggesting that voriconazole may be subject to significant first-pass metabolism in children but not in adults.

Interestingly, results on *in vitro* metabolism of voriconazole by microsomes, prepared from livers of children with age 2-8 years and from adults, showed that oxidative enzymes derived from children metabolized voriconazole at three-fold higher rate than those derived from adults. Thus, the microsomal system appears to mimic *in vivo* metabolic clearance of voriconazole. The *in vitro* intrinsic clearance (V_{max}/K_m) of voriconazole N-oxidation by liver microsomes derived from children and adults was used to calculate *in vivo* clearance (Alcorn, McNamara, 2008; Edginton et al., 2006). Box plot analysis of the intra- and inter-group distribution of values of *in vitro* intrinsic clearance, scaling factor, and predicted intrinsic clearance generated by six liver microsomes samples from children and adults are shown in Figure 5A-C, respectively. These results indicated that the distribution of values among children was much broader than that among adults. However the median for the *in vitro* intrinsic clearance and scaled intrinsic clearance in children were significantly greater than in adults. The calculated scaling factor, based on MPPG and liver weights that were normalized to body weight, was marginally different ($p > 0.04$) for children (878.3 +/-211.3 mg/kg) *versus* adults

(650.0 +/- 99.8 mg/kg). The calculated *in vivo* clearance values were approximately 80% of the observed plasma clearance of voriconazole in children and adults. The under prediction by 20% may be due to the fact that the intrinsic clearance was calculated for the formation of the N-oxide, which is a major but not the only metabolite of voriconazole. These results conclude that the higher clearance of voriconazole in children is the result of higher metabolic clearance of voriconazole in children compared to adults.

It has been reported that the oxidative metabolism of voriconazole by liver microsomes from adults generates two major metabolites (see Figure 1), N-oxidation of the fluoropyrimidine ring and hydroxylation of the adjacent methyl group (Roffey et al., 2003; Murayama, et al., 2007). The N-oxide, which is the major circulating metabolite in humans, is formed by CYP3A4, CYP2C19 (Hyland et al., 2003; Murayama, et al., 2007), and FMO (FMO3 > FMO1) (Yanni et al., 2008); while the hydroxymethyl metabolite is formed exclusively by CYP3A4 (Murayama, et al., 2007; Yanni et al., 2008). In the present study, we report that both metabolites are also formed by liver microsomes derived from children. To determine the role of CYP3A4, CYP2C19, and FMO3 in the oxidative metabolism of voriconazole by liver microsomes from adults *versus* children, the N-oxide formation was measured upon inhibition of CYP2C19 (fluoxamine), CYP3A4 (ketoconazole), and FMO3 (heat inactivation) (Figure 4A). The results showed that the relative contribution of these enzymes in the formation of N-oxide is considerably different in children *versus* adults. The estimated rate N-oxidation by FMO3 and CYP2C19 is five - and three -fold higher, respectively in children compared to adults, but the estimated rate of N-oxidation by CYP3A4 is not different in both groups. Consequently, in adults approximately 50% of the N-oxide was formed by CYP3A4, 35% by CYP2C19, and 15% by FMO3, whereas in children 50% of the N-oxide was formed by CYP2C19, 30% by FMO3, and 20% by CYP3A4. To determine if higher voriconazole metabolism in children *versus* adults

is due to higher expression of hepatic CYP2C19 and/or FMO3 in children, the expression of these two proteins and CYP3A4 was determined in adult *versus* pediatric livers. The expression of CYP2C19 and FMO3 in the pediatric liver was somewhat higher (<two-fold) whereas the expression of CYP3A4 was somewhat lower (approximately two-fold) in children compared to adults (Western blot bands at the top of Figure 4C). In comparison, the activity of CYP2C19 and FMO3 toward their respective probe substrates was approximately two-fold higher in liver microsomes from children *versus* adults, with CYP3A4 activity being similar in these two microsomal preparations (Figure 4C).

Clearly, this study shows that higher rate of oxidative metabolism of voriconazole in children *versus* adults contributes to its higher clearance *in vivo*. The microsomal metabolism of voriconazole is able to predict the *in vivo* clearance of voriconazole in adults and in children. Thus, results of the *in vitro* studies to determine the relative contribution of CYP2C19, CYP3A4 and FMO3 should also be indicative of the relative role of these enzymes *in vivo*. It appears that CYP2C19 and FMO3 play a bigger role in the metabolic clearance of voriconazole in children whereas CYP3A4 appears to contribute more in adults. The expression of CYP2C19 and FMO is somewhat higher in children *versus* adults; however, it is not clear whether this difference in expression is sufficient to explain greater metabolic clearance of voriconazole in children *versus* adults. Interestingly other drugs that are predominantly metabolized by hepatic CYP2C9/19 such as phenytoin, omeprazole, and sirolimus show higher clearance in children between 2-10 years of age than in adults (Kennedy 2008, Litalien et al, 2005; Filler, et al., 2008). It would be interesting to identify drugs that are predominantly metabolized by FMO3 and examine their clearance in children *versus* adults. Taken together, such mechanistic studies may lead to significant improvement in our understanding of why certain drugs are cleared more rapidly in children than in adults, and consequently improve our ability to define optimum doses for

children based on pharmacokinetic behavior of these drugs in adults combined with *in vitro* metabolic studies.

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Footnotes:

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Legends

Figure 1. Chemical Structures of Voriconazole and Human Metabolites: Voriconazole N-oxide, the major circulating metabolite, and hydroxymethyl voriconazole as it has been reported by Hyland et al. 2003 and Murayama et al., 2007.

Figure 2. Voriconazole Oxidative Metabolism by Human Liver Microsomes from Adults and Children: Voriconazole oxidative metabolism by liver microsomes prepared from each tissue sample (six adults and six children, 1.0 mg microsomal protein/ml, 20 min) was determined. The oxidative metabolism of Voriconazole was linear with respect to time and protein concentration under the experimental conditions used. Voriconazole remaining as function of time was measured in three separate experiments for each subjects and mean values of six set of data ± S.D. was plotted for adults (●) or children (▲) as function of time.

Figure 3. Voriconazole N-oxide Formation by Liver Microsomes from Adults and Children as a function of Voriconazole Concentration: Voriconazole N-oxide formation by liver microsomes from adults and children, as a function of voriconazole concentration was determined by incubating microsomal protein (1.0 mg/ml) from each subject within the two groups with NADPH and voriconazole (0.5- 500.0 µM). The mean rate of N-oxide formation ± S.D. in adults (n = 6) (●) or children (n = 6) (▲), expressed as pmol of N-oxide formed/min/mg protein was plotted against voriconazole concentration. Non-linear regression analysis (WinNonLin, Pharsight, Cary, NC) was performed using the Michaelis-Menten kinetic equation for the determination of apparent K_m and $V_{max.}$

Figure 4. Role of CYP3A4, CYP2C19, and FMO3 in Voriconazole Metabolism to the N-oxide by Liver Microsomes from Adults and Children: The N-oxide formation by the pooled liver microsomes from adults and children was inhibited by fluoxamine (CYP2C19), ketoconazole (CYP3A4), and heat-treatment (FMO) to determine the contribution of the

respective enzymes (A). The estimated rate of voriconazole N-oxide formation by CYP3A4, CYP2C19, and FMO3 in the liver microsomes from adults and children was calculated from the inhibition experiments in A, and is shown in (B). The approximate relative contribution of the three enzymes in the formation of N-oxide is: 50% by CYP3A4, 35% by CYP2C19, and 15% by FMO3 in adults, and 20% by CYP3A4, 50% by CYP2C19, and 30% by FMO3 in children. The specific activities of CYP2C19 (S-mephenytoin 4-hydroxylation), CYP3A4 (testosterone 6β-hydroxylation), and FMO3 (benzydamine N-oxidation) in the liver microsomes from adults (filled bar) and children (open bar) is shown in C; the data are mean of three determinations ± S.D. The protein expression of CYP2C19, CYP3A4, and FMO3 in pooled liver microsomes from adults and children is shown as Western blot bands (C); the band densities in adults 5060 ± 526 (CYP2C19), 13996 ± 2099 (CYP3A4), and 2310 ± 289 (FMO3), and in children 7084 ± 776 (CYP2C19), 8398 ± 910 (CYP3A4), and 3927 ± 578 (FMO3) were used to estimate relative expression of the respective proteins in adults *versus* children.

Figure 5. Box Plot Analysis of Voriconazole *In Vitro* Intrinsic Clearance, Scaling Factor, and Scaled Intrinsic Clearance in Children Compared to Adults: Graphical presentation of *in vitro* intrinsic clearance, scaling factor, and scaled intrinsic clearance values obtained from analysis of six children and six adults liver microsomes samples is shown by the box plots A, B, C, respectively, where 25% percentile value is indicated by (♦), minimum value is indicated by (■), median value is indicated by (▲), maximum value is indicated by (□), and 75% percentile value is indicated by (●). Non-parametric statistical analysis of children and adults values using Wilcoxon test showed that two-sided exact p-value were 0.0087, 0.041, and 0.0022 for comparison of *in vitro* intrinsic clearance (V_{max}/K_m), scaling factor, and predicted intrinsic clearance, respectively.

Table 1. ***In vitro-in vivo*** **Relationship of voriconazole Clearance in Children and Adults**

Group	Subject	Age (year)	K_m[1] (μM)	V_{max}[1] (pmol/min/mg)	Scaling Factor (mg/kg)	Intrinsic Clearance[2] (ml/min/kg)	*In vivo* Clearance (ml/min/kg)	
Adults	A1	45	8.5 ± 0.9	28.0 ± 0.7	655.7	2.16	**Calculated[3]**	**Observed[4]**
	A2	58	8.8 ± 1.1	44.6 ± 2.3	801.4	3.6		
	A3	24	9.8 ± 2.3	62.5 ± 1.7	600.0	3.8		
	A4	20	8.5 ± 1.0	35.6 ± 0.8	578.6	2.4		
	A5	39	15.7 ± 4.3	45.2 ± 3.0	535.7	1.5		
	A6	46	4.5 ± 0.6	24.2 ± 0.9	728.6	3.9		
	Mean ± S.D.		**9.3 ± 3.6**	**40.0 ± 13.9**	**650.0 ± 99.8**	**2.9 ± 1.0**	**1.6 ± 0.6**	**2.0**
Children	C1	2	3.8 ± 0.4	63.0 ± 2.5	900.8	14.9		
	C2	4	19.7 ± 2.9	315.5 ± 12.7	786.7	16.2		
	C3	8	11.9 ± 1.7	139.1 ± 4.5	844.9	10.7		
	C4	2	12.1 ± 1.5	73.1 ± 1.5	705.0	4.3		
	C5	4	10.0 ± 1.4	68.8 ± 2.3	1311.1	8.3		
	C6	5	8.5 ± 1.6	63.4 ± 2.3	721.1	4.8		
	Mean ± S.D.		**11.0 ± 5.2**	**120.5 ± 99.9**	**878.3 ± 211.3**	**9.9 ± 5.0**	**5.1 ± 2.6**	**6.7**

[1]The K_m and V_{max} values for each subject are reported as mean ± S.D; these values were derived from three determinations of the reaction rate at each substrate concentration.
[2] The (whole body) intrinsic clearance for each subject was calculated from the experimentally determined *in vitro* intrinsic clearance (V_{max}/K_m) values using Equations 1 and 2 (see Materials and Methods Section).
[3]*In vivo* clearance is calculated based on *in vitro* data according to the well-stirred model of hepatic disposition that is described by Equation 3 (see Materials and Methods Section).
[4]Clinically observed clearance values in children and adults were reported by Leveque et al. (2006).

Figure 1

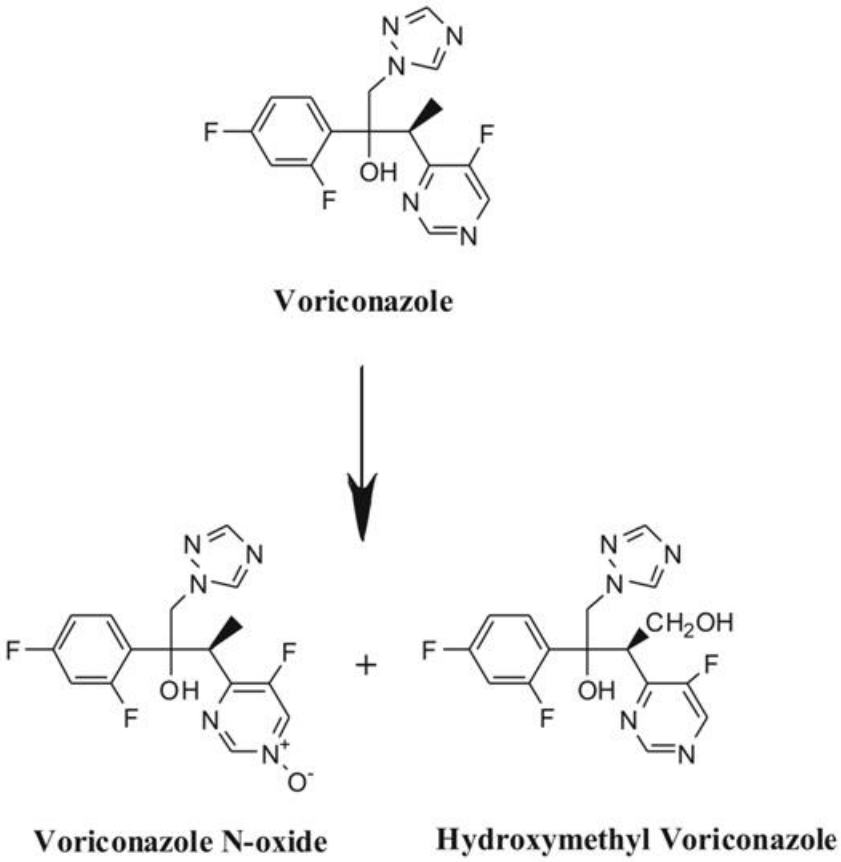


Figure 2

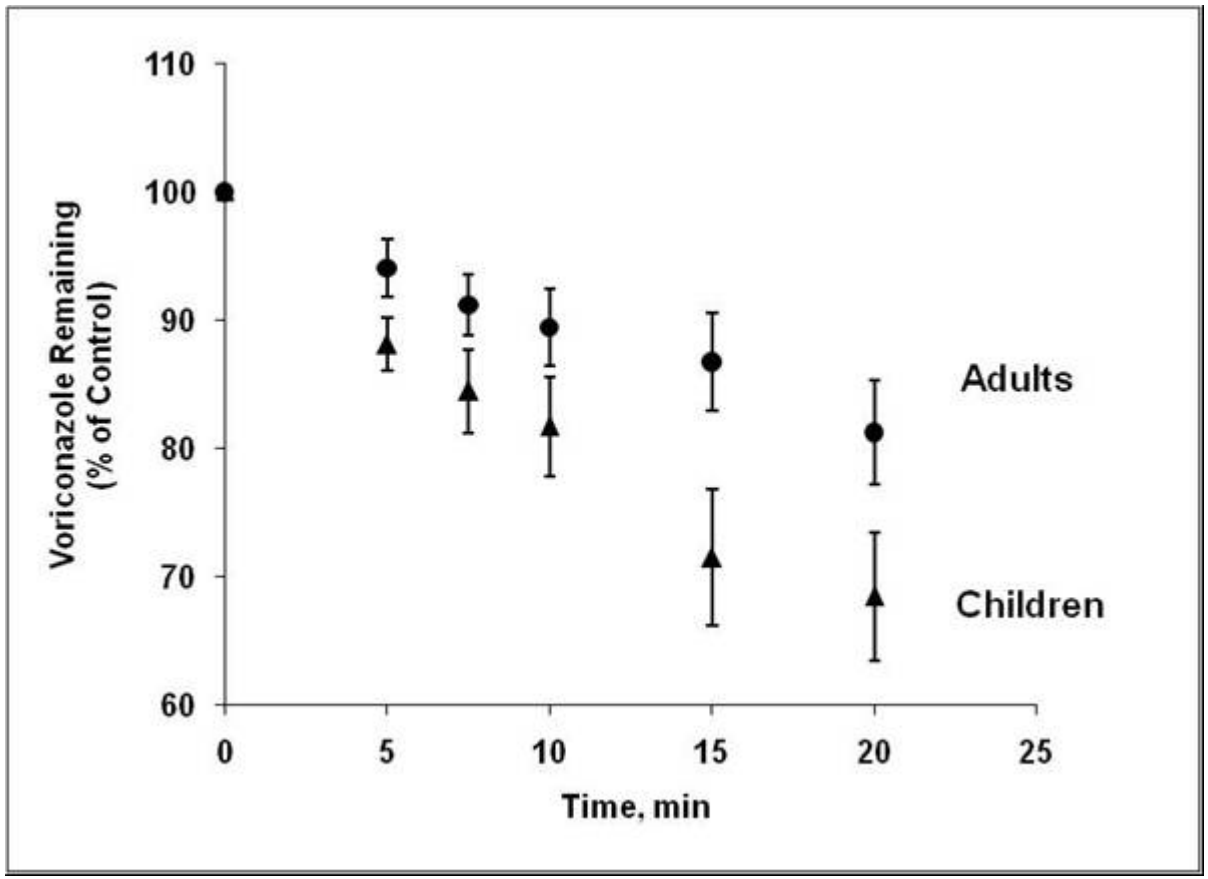
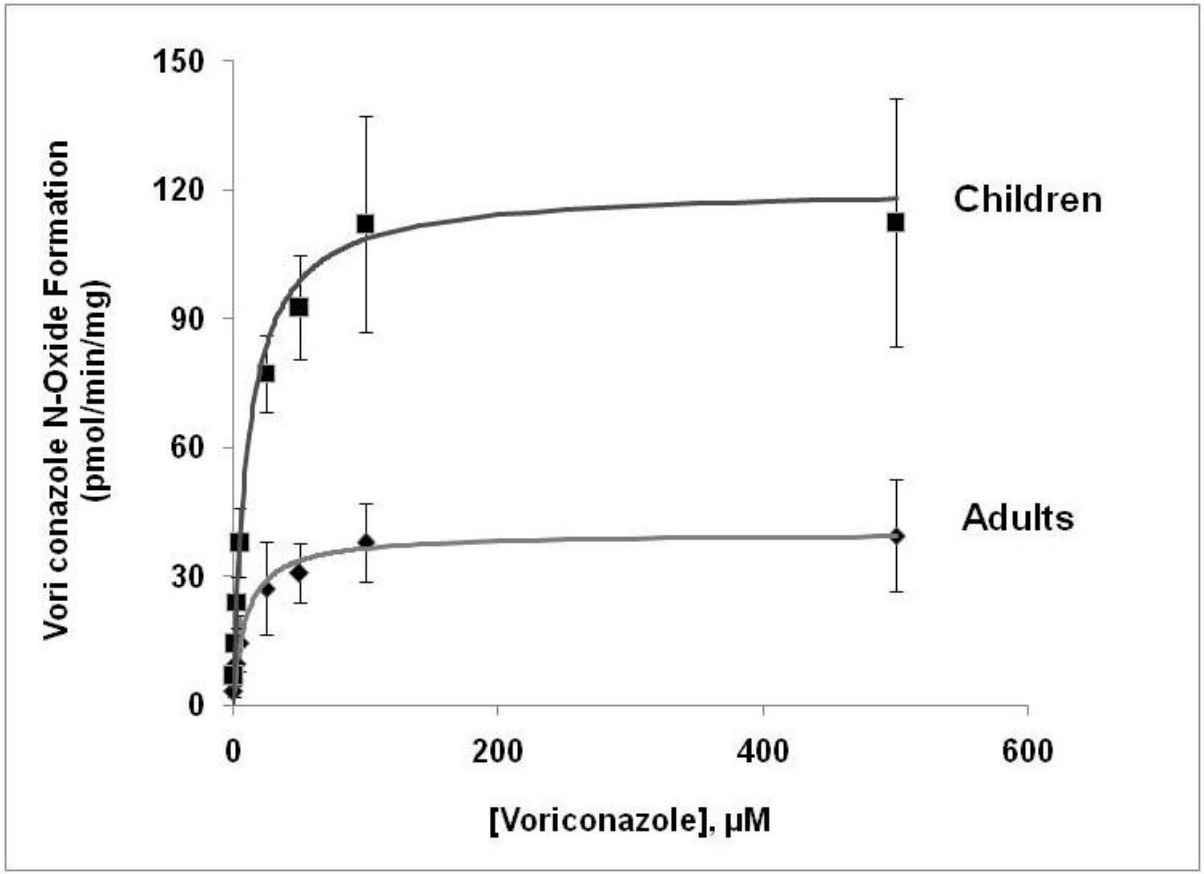


Figure 3



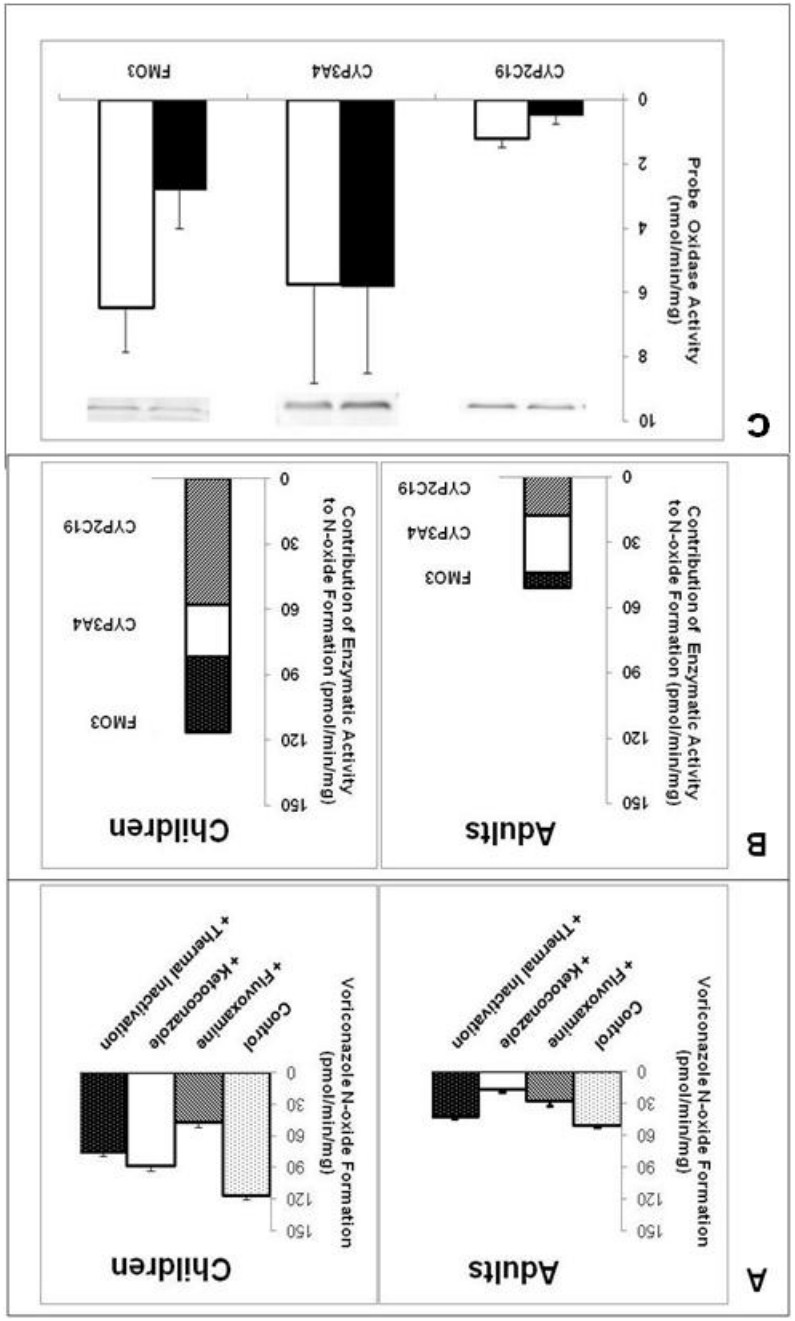


Figure 4

Figure 5