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Differential Genotype Dependent Inhibition of CYP2C9 in Humans

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Non-Standard Abbreviations:

CL2C9 – CYP2C9 metabolic clearance

CLnon2C9 – non-CYP2C9 clearance

$CL_{f,m}$ – formation clearance of 4'-hydroxyflurbiprofen by CYP2C9

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Abstract

The effect of genetic polymorphisms in drug metabolizing enzymes (e.g., CYP2C9*3) on drug clearance have been well characterized but much less is known about whether these polymorphisms alter susceptibility to drug-drug interactions. Previous *in-vitro* work has demonstrated genotype-dependent inhibition of CYP2C9 mediated flurbiprofen metabolism, suggesting the possibility of genotype-dependent inhibition interactions *in-vivo*. In the current study, flurbiprofen was used as a probe substrate and fluconazole as a prototypical inhibitor to investigate whether genotype dependent inhibition of CYP2C9 occurs *in-vivo*. From 189 healthy volunteers who were genotyped for CYP2C9 polymorphisms, eleven control subjects (*CYP2C9*1/*1*), nine heterozygous and two homozygous for the CYP2C9*3 allele participated in the pharmacokinetic drug interaction study. Subjects received a single 50 mg oral dose of flurbiprofen alone or after administration of either 200 or 400 mg fluconazole for seven days using an open, randomized, cross-over design. Flurbiprofen and fluconazole plasma concentrations along with flurbiprofen and 4'-hydroxyflurbiprofen urinary excretion were monitored. Flurbiprofen apparent oral clearance differed significantly among the three genotype groups ($p<0.05$) at baseline but not after pretreatment with 400mg fluconazole for seven days. Changes in flurbiprofen apparent oral clearance after fluconazole co-administration were gene-dose dependent, with virtually no change occurring in **3/*3* subjects. Analysis of fractional clearances suggested that fraction metabolized by CYP2C9, as influenced by genotype, determined the degree of drug interaction observed. In summary, the presence of *CYP2C9*3* alleles (either one or two alleles) can alter the degree of drug interaction observed upon co-administration of inhibitors.

The cytochrome P450 (CYP) superfamily of enzymes plays an important role in the oxidation of numerous xenobiotics, with CYP2C9 accounting for 10-20% of the CYP protein content in human liver. CYP2C9 has been reported to catalyze approximately 20% of the CYP mediated drug oxidation reactions (Miners and Birkett, 1998;Rendic and Dicarlo, 1997;Shimada, et al., 1994), including agents such as the non-steroidal anti-inflammatory drugs (NSAIDs), tolbutamide, losartan and the narrow therapeutic index drugs warfarin and phenytoin (Bajpai, et al., 1996;Hamman, et al., 1997;Miners and Birkett, 1996;Niemi, et al., 2002;Rettie, et al., 1992;Stearns, et al., 1995;Tracy, et al., 1995;Yan, et al., 2005). To date, 30 CYP2C9 allelic variants located within the coding region have been reported (http://www.imm.ki.SE/CYPalleles/). In particular, the **3* allele (Ile359Leu) that is expressed at an allele frequency of 15% (Lee, et al., 2002) results in significantly reduced oral clearance for several CYP2C9 substrates (Guo, et al., 2005;Lee, et al., 2003b;Perini, et al., 2005;Vianna-Jorge, et al., 2004;Aithal, et al., 1999) and has been associated with an increased frequency of adverse events following warfarin or phenytoin administration (Higashi, et al., 2002;Kidd, et al., 2001).

Certain diseases or the presence of co-morbid conditions may necessitate co-administration of multiple medications, increasing the chances of drug-drug interactions. However, genetic polymorphisms in drug metabolizing enzymes are not routinely evaluated for their impact on drug interactions during clinical studies. Genotype dependent inhibition has been demonstrated with CYP2D6 and CYP2C19 genetic polymorphisms (Hamelin, et al., 2000;Lessard, et al., 2001;Lindh, et al., 2003;Llerena, et al., 2001;Uno, et al., 2006;Yasui-Furukori, et al., 2004a;Yasui-Furukori, et al., 2004b), but these polymorphisms resulted in expression of inactive proteins that should not be subject to inhibition. No clinical studies have explored the effect of single nucleotide polymorphisms (SNPs) resulting in reduced activity enzymes (e.g., *CYP2C9*3*)

and their effect on the degree of inhibition interactions observed. Recently, *in vitro* studies from our laboratory using five commonly used probe substrates and 28 inhibitors demonstrated genotype- as well as substrate- dependent inhibition of CYP2C9 (Kumar, et al., 2006b). To evaluate the potential *in vivo* significance of these findings, a clinical study utilizing an open, randomized, cross-over design, was conducted with the CYP2C9 probe substrate flurbiprofen (Greenblatt, et al., 2006;Hutzler, et al., 2001;Zgheib, et al., 2007) and the prototypical CYP2C9 inhibitor fluconazole (Venkatakrishnan, et al., 2000) in individuals of the *CYP2C9*1/*1*, **1/*3*, and **3/*3* genotypes to characterize the magnitude of the drug interaction in subjects with different *CYP2C9* polymorphisms.

Materials and Methods

Subject Selection. The study protocol was reviewed and approved by the Institutional Review Board, University of Minnesota, Minneapolis, MN. 189 healthy subjects were enrolled in the genotype screening phase of the study. After obtaining written informed consent, 30 ml of blood was drawn and stored at room temperature for DNA isolation. All subjects were non-smokers and were not taking any medicine at the time of enrollment, except oral contraceptives.

DNA isolation. The whole blood sample was mixed thoroughly with red blood cell lysis buffer (preheated to 37^{o}C). The mixture was allowed to incubate in the water bath at 37^{o}C for 15 minutes. The samples were then centrifuged for 15 minutes at 3000 rpm. The pellets were washed repeatedly with RBC lysis buffer until the pellet became white in color. The pellet was then dissolved in 3 ml of white blood cell lysis buffer and incubated at 37^{o}C overnight. To each tube, 10 µl proteinase K was added, vortexed and incubated for 24 hours at 37^{o}C. Ammonium acetate (1.5 ml of 7.5M) was added to precipitate the DNA. Proteins were then removed by centrifuging at 3000 rpm for 20 minutes and 10 ml of absolute ethanol was added to the supernatant. The mixture was then gently inverted to allow the DNA to precipitate. The precipitated DNA was then suspended in 3 ml of TE buffer (Sigma, St louis, MO). Quantitation of DNA was carried out by measuring the sample absorbance at 260 nm.

Determination of CYP2C9 Genotype. DNA samples were diluted to 20 ng/µl and single nucleotide polymorphisms were determined by the Taqman® based allele discrimation assay kits (Applied Biosystems, Foster City, CA). The genotyping reaction was conducted in 96 well plates and the reaction components were 1 µl diluted DNA, 1.25 µl of primer, 12.5 µl of Taqman universal master mix (Applied Biosystems) and water to a total volume of 25 µl. The thermocycler protocol was as follows: 50^{o}C for 2 min, 95 ^{o}C for 10 min and 40 cycles of 92^{o}C

for 15 sec and 60^{o}C for 1 min. Reactions were analyzed on a Prism model 7500 sequence detection system (Applied Biosystems). The db SNP reference numbers were *CYP2C9*2* = rs1799853 and *CYP2C9*3* = rs 1057910.

Drug Interaction Study design. From the 189 genotyped subjects, eleven *CYP2C9*1/*1*, nine *CYP2C9*1/*3* and two *CYP2C9*3/*3* subjects further consented to participate in the drug interaction study. All subjects were healthy as determined by physical examination, medical history, vital signs, and routine biochemical and urinalysis tests. Subjects were instructed to abstain from alcohol for 3 days and caffeine containing food, chocolate and beverages, for 2 days prior to the pharmacokinetic study. Female subjects of child bearing potential underwent a urine pregnancy test.

An open, randomized, crossover design with a one week wash out was employed. Subjects received either 50mg flurbiprofen as a tablet (Mylan Pharmaceuticals Inc., Morgantown, WV, US) alone or 200mg or 400mg fluconazole as a tablet (Ivax Pharmaceuticals Inc., Miami, Fl, US) for seven days followed by 50mg flurbiprofen on the seventh day. Compliance with fluconazole administration was assessed by pill count as well as examination of subject diaries. Subjects were required to fast overnight prior to the study day. On the morning of each period, subjects were admitted to the Clinical Research Center and remained there for twelve hours, were dismissed, and then returned the next morning for collection of the 24 hour blood sample and return of the urine collection. Fluconazole was administered in the morning as scheduled and flurbiprofen administered orally two hours later. A light snack was allowed two hours after flurbiprofen dosing.

Plasma and Urine Collection. Blood samples (seven mL) were collected into heparinized tubes at 0 min (prior to flurbiprofen administration), 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 hours after

the administration of flurbiprofen, immediately centrifuged for 10 mins at 3200 rpm, and the resulting plasma aliquoted and stored at -20^{o}C. Total voided urine was collected before flurbiprofen administration and at intervals of 0-12 h and 12-24 h after flurbiprofen administration and kept refrigerated throughout the collection period. At the end of each collection interval, total urine volume was recorded and two 25 ml aliquots were stored at -20^{o}C for later analysis.

Analysis of flurbiprofen concentrations in plasma and flurbiprofen and 4'OH flurbiprofen concentrations in urine. Flurbiprofen and 4'OH flurbiprofen concentrations were quantified by high-performance liquid chromatographic (HPLC) according to methods previously developed in our laboratory (Hutzler, et al., 2000). Acid hydrolysis, as previously described, was used to cleave glucuronides of flurbiprofen and 4'-hydroxyflurbiprofen, prior to analysis.

Analysis of fluconazole in plasma. Fluconazole plasma concentrations were quantitated by a previously described method (Cociglio, et al., 1996).

In vitro assessment of genotype-dependent inhibition. The ability of fluconazole to inhibit expressed, reconstituted CYP2C9.1, an equimolar mixture of CYP2C9.1/CYP2C9.3 and CYP2C9.3 was carried out according to methods previously established in our laboratory (Kumar, et al., 2006a). Briefly, fluconazole (0, 10, 50 and 100 μM) was incubated with three concentrations of flurbiprofen (5, 25 and 50 μM) with each of the enzyme preps listed above and the formation of 4'-hydroxyflurbiprofen monitored.

Noncompartmental pharmacokinetic analysis. Flurbiprofen pharmacokinetic parameters were estimated from plasma concentration time data by standard non-compartmental methods (Win Nonlin v5.2, Pharsight, Palo Alto, CA). The area under the concentration-time curve ($AUC_{0\text{-}inf}$) of flurbiprofen was calculated using the linear trapezoidal rule with extrapolation to

infinity. Apparent oral clearance (CL/F) of flurbiprofen was calculated as dose / $AUC_{0\text{-}inf}$. The 4'OH flurbiprofen formation clearance ($CL_{f,m}$) was calculated using equation 1.

$$CL_{f,m} = \frac{Amt.\ 4'OHFexcreted_{0-24hr}}{FlurbiprofenAUC_{0-24hr}} \quad \text{equation 1}$$

where *Amt. 4'OHFexcreted 0-24hr* is the amount of 4'-hydroxyflurbiprofen metabolite (both as 4'-hydroxyflurbiprofen and its glucuronide conjugate) excreted in the urine during the 24 hour collection interval.

Statistical Analysis. Sample size calculations were based on the study conducted by Zgheib et al. in which 78% inhibition of 4' OH flurbiprofen formation clearance was observed after pretreatment with 7 doses of 400mg fluconazole (Zgheib, et al., 2007). To detect a 30% difference in inhibition between *CYP2C9*1/*1* and *CYP2C9*1/*3* with power of 90% and 5% chance of type I error, it was estimated that a sample size of at least N = 8 per group would be necessary. One-way ANOVA was applied to compare demographics such as age, body weight, height and creatinine concentration between the three CYP2C9 genotype groups. Prior to statistical analysis, flurbiprofen pharmacokinetic parameters (e.g., CL/F, $AUC_{0\text{-}inf}$ and half-life) were log transformed and two-way repeated-measure ANOVA then applied. Pair wise multiple comparisons were conducted using the Holm-Sidak method. A Friedman repeated measure ANOVA on ranks was performed on $CL_{f,m}$, C_{max}, T_{max} and V/F because of non-normalized distributions. Pair wise comparisons were made using the Tukey-Kramer test. Fluconazole pharmacokinetic parameters ($AUC_{0\text{-}24}$, C_{max} and half-life) across groups and treatment periods were compared using one way analysis of variance with a p-value of 0.05 or less regarded as statistically significant. All statistically analyses were conducted with SigmaStat 3.1 (Systat Software, Point Richmond, CA).

Model-based Pharmacokinetic Analysis. A nonlinear mixed-effects modeling approach was used for both pharmacokinetic parameter estimation and to develop a global model for the flurbiprofen data that included a pharmacokinetic submodel and a drug interaction submodel. All flurbiprofen concentrations from all drug interaction arms in subjects with all three genotypes were simultaneously modeled using NONMEM VI (ICON U.S., Ellicott City, MD) (Beal, et al., 2006) implemented with Compaq Visual Fortran v6.6 and PDxPop 2.2a, using a first-order conditional estimation algorithm that allowed for an interaction between the ETA and EPSILON levels of random effects. For the pharmacokinetic submodel, a one-compartment model with first-order absorption and first-order elimination adequately described the flurbiprofen concentration-time profiles. The link between the drug interaction model and the pharmacokinetic model was adapted from the Rowland-Matin equation (Rowland and Matin, 1973). Flurbiprofen clearance was modeled as a function of the genotypic CL2C9, CLnon2C9, the fluconazole concentration (I), and the Ki for the fluconazole/CYP2C9 interaction.

$$CL = \frac{CL2C9}{1+\frac{I}{Ki}} + CLnon2C9 \qquad \text{equation 2}$$

It is important to note that CL2C9 and Ki can both take on different values across different genotypes.

In the population model, the typical values of the uninhibited CL2C9 for each of the three genotypes were estimated as regression parameters. The typical value of CLnon2C9 was also estimated as a regression parameter, assuming that it is not dependent upon genotype and is shared across all genotypes and inhibitor concentrations. Flurbiprofen volume of distribution, first-order absorption rate constant, and an absorption lag time were also estimated and assumed to be log-normally distributed in the population. The between-subject variability for each was

expressed as a coefficient of variation (CV%). Residual unexplained variability was modeled with a proportional error model and expressed as a CV%.

Parameters of the drug interaction submodel were based on previous literature (Kunze and Trager, 1996), wherein the average steady-state concentration was reported to be 60µM following fluconazole 400mg/day for six days. This value was used in the regression equation as I for the 400mg fluconazole interaction treatment arm. Assuming linear pharmacokinetics of fluconazole, the value for I in the 200mg fluconazole treatment arm was taken to be 30µM. This same study reported the average *in vivo* Ki for the fluconazole/CYP2C9 interaction to be 22µM. We assumed all these subjects were of the predominant **1/*1* genotype. Our laboratory determined the *in vitro* Kis to have a **1/*3* to **1/*1* ratio of 1.55; similarly, the **3/*3* to **1/*1 in vitro* ratio was 2.00 (unpublished data). Assuming the *in vitro* ratio approximates the *in vivo* ratio, these ratios were used to estimate the value of Ki in **1/*3* and **3/*3* genotypes from the literature *in vivo* value.

Since there were only two **3/*3* subjects, a likelihood ratio test was applied to determine if the information from these two subjects was sufficiently strong to reject the hypothesis that CL2C9 in **3/*3* subjects was no different than the CL2C9 in **1/*3* subjects. Using the maximum likelihood objective function in NONMEM, the difference in objective function values between a full and nested model is approximately chi-square distributed with degrees of freedom equal to the difference in the number of parameters between the two competing models. A decrease in objective function value of 3.8 or greater indicates the more complicated model is superior to the nested model ($p<0.05$, χ^2, df=1). It was also determined whether the model that independently estimated the clearances of each genotypic group in each treatment arm (9 clearance parameters) was significantly different from the model that included all the

assumptions made in the drug interaction submodel (4 clearance parameters). With 5 degrees of freedom, a decrease in objective function value of 11.1 results in alpha=0.05.

Results

After obtaining informed consent, 189 healthy volunteers were genotyped for CYP2C9 alleles. Two subjects were not further evaluated as one subject presented with hypertension and in the other case, phlebotomy was unsuccessful. Of these subjects, 40.2% were male, 72.3% were white, 12.7% were African-American, 8.5% were Asian, two individuals were Pacific-Islander and three did not self-identify. The median age was 25 (range 18-61). The allele frequencies for all individuals screened are reported in Table 1 . Twenty two subjects (eleven *CYP2C9*1/*1*, nine *CYP2C9*1/*3* and two *CYP2C9*3/*3*) were then enrolled into the drug interaction study and their demographics are presented in Table 2. Prior to flurbiprofen administration, one *CYP2C9*1/*3* genotype subject withdrew from the study due to personal reasons and hence, was excluded from the analysis. No significant adverse events were reported by any subjects.

Mean flurbiprofen plasma concentration-time profiles before and after pre-treatment with either 200 or 400mg fluconazole in *CYP2C9*1/*1*, *CYP2C9*1/*3* and *CYP2C9*3/*3* genotype individuals are depicted in Figures 1A, 1B and 1C, respectively. Pharmacokinetic parameters derived from non-compartmental analysis of individual data are presented in Table 3. With respect to genotype, comparing the control period vs. seven day pre-treatment with 200mg fluconazole, statistically significant differences were noted in apparent oral clearance, AUC and half-life between treatment periods in the *CYP2C9*1/*1* and *CYP2C9*1/*3* genotype groups ($p<0.01$). However, no statistically significant differences in oral clearance or AUC were noted in the *CYP2C9*3/*3* group comparing control vs 200mg fluconazole.

Flurbiprofen oral clearance, AUC and half-life differed significantly between baseline and 400mg fluconazole pretreatment in subjects with either the *CYP2C9*1/*1* or *CYP2C9*1/*3*

genotypes ($p<0.01$). These differences remained, when the parameters for the 200mg fluconazole and 400mg doses of fluconazole were compared. However, no statistically significant differences in the above parameters between any of the periods (baseline vs. 200mg vs. 400mg fluconazole) were observed in the *CYP2C9*3/*3* genotype group. Finally, no statistically significant differences were noted in C_{max}, T_{max} and V/F across periods in any of the three genotype groups.

Figure 2 is an interaction plot of median oral clearance (25^{th} and 75^{th} percentile) for each of the three genotype groups during the three periods (baseline, 200mg or 400mg fluconazole). At baseline, median oral clearance in *CYP2C9*1/*1* individuals differed significantly ($p <0.05$) from the *CYP2C9*1/*3* and *CYP2C9*3/*3* individuals. After 200mg fluconazole, individual oral clearances in all three genotype groups decreased, but the median oral clearance in *CYP2C9*1/*1* individuals was not statistically different ($p = 0.084$) from that of *CYP2C9*1/*3* individuals. However, median oral clearance in the *CYP2C9*1/*1* group was statistically different ($p = 0.015$) from *CYP2C9*3/*3* individuals after 200mg fluconazole. Finally, no differences were noted ($p >0.05$) in flurbiprofen median oral clearance across all genotypes following 400mg fluconazole pretreatment. In the *CYP2C9*3/*3* individuals, no significant decrease in apparent oral clearance was observed at either of the fluconazole doses.

The 4'-hydroxyflurbiprofen formation clearances ($CL_{f,\ m}$) for the three periods are presented in Table 3. At both baseline and 200mg fluconazole co-administration, the formation clearance in individuals with either one or two **3* alleles varied significantly from wild type individuals. However, after 400mg fluconazole co-administration, only individuals with two **3* alleles differed statistically from those carrying only the **1* allele.

Mean plasma concentration-time profiles of fluconazole after seven doses of 200mg and 400mg in the three genotype groups are shown in Figure 4 and the pharmacokinetics parameters are summarized in Table 4. No statistically significant differences were noted in AUC_{0-24}, half-life or C_{max} for a given dose of fluconazole among the three genotype groups.

In vitro studies of fluconazole inhibition of flurbiprofen metabolism were conducted to provide the framework for the *in vitro-in vivo* correlations and model-based analysis (see below). In expressed enzyme preparations of CYP2C9.1 (simulate wild-type **1/*1* individuals), the Ki value was 11 μM for fluconazole inhibition of flurbiprofen 4'-hydroxylation. In an equimolar mixture of CYP2C9.1/CYP2C9.3 (simulate heterozygote **1/*3* individuals) the fluconazole Ki was 17 μM. Finally, with the CYP2C9.3 protein (simulate homozygote **3/*3* individuals) the fluconazole Ki was 23 μM. Thus, the Ki of fluconazole inhibition was altered in a gene-dose dependent fashion.

Results from the model-based analysis (equation 2) of the drug interaction are presented in Table 5. Only the values of the uninhibited clearance, CL2C9, for each genotype were estimated. The clearances as the result of the drug interaction (i.e., the reduced clearances) were modeled in the drug interaction submodel as 1/(1+I/Ki). These values of flurbiprofen clearance and the magnitude of the drug interaction are all consistent with the noncompartmental results. Diagnostic plots of weighted residuals vs. time or predicted concentration were randomly distributed around zero and suggested no reason to reject the model. The plot of observed vs. predicted concentration indicated a good fit of the observed data under the model. The attempt to combine CL2C9 in the **3/*3* subjects with the CL2C9 in **1/*3* subjects as a single parameter resulted in a significantly inferior model ($p<0.05$, χ^2, df=1) as indicated by an increase in the objective function value of 5.2 units. When nine CL2C9 typical values were estimated (one for

each genotype and level of drug interaction), the objective function value decreased by 4.8 units compared to the model incorporating the *in vivo* and *in vitro* drug interaction model (not statistically significant ($p>0.05$, χ^2, df=5).

Discussion

The effects of genetic polymorphisms on rates of drug metabolism are well known but how these polymorphisms impact susceptibility to drug-drug interactions is less clear. In particular, whether reduced function proteins (e.g., CYP2C9.3) are inhibited to the same extent *in vivo* as wild type proteins (e.g., CYP2C9.1). This knowledge is potentially clinically important since differential dosage adjustments may be needed in individuals with the *CYP2C9*3* genotype as compared to the more prevalent wild type expressing individuals, when a known interacting drug is co-administered. Results of the present *in vivo* interaction study demonstrated that genotype dependent inhibition of flurbiprofen oral clearance occurs when co-administered with fluconazole in subjects with none, one or two *CYP2C9*3* alleles.

CYP2C9 exclusively catalyzes the oxidative metabolism of flurbiprofen to its 4'-hydroxy (primary) oxidative metabolite (Tracy, et al., 1996;Tracy, et al., 1995) and this reaction has been demonstrated to be a reliable probe of CYP2C9 activity *in vivo* (Lee, et al., 2003b;Zgheib, et al., 2007). Differences in flurbiprofen pharmacokinetics have previously been evaluated in individuals genotyped for *CYP2C9*1/*2* and *CYP2C9*1/*3* and reduced oral clearance was noted in both genotypes as compared to individuals with the *CYP2C9*1/*1* genotype (Lee, et al., 2003a). Fluconazole is a prototypical CYP2C9 inhibitor, with nearly complete bioavailability and less than 10% of a dose being metabolized (Shiba, et al., 1990). Greenblatt et al., reported a reduction of flurbiprofen oral clearance, after two doses of fluconazole 200mg, to approximately 55% of the baseline value (Greenblatt, et al., 2006), similar to the current results in wild-type individuals. In another study, the formation clearance of 4'-hydroxyflurbiprofen (as an indicator of CYP2C9 activity) decreased by 69 and 78% from baseline after single and seven doses of

200mg fluconazole, respectively (Zgheib, et al., 2007), again similar to the current results. However, in neither study were the subject's genotypes known.

In vitro studies from our laboratory (Kumar, et al., 2006b) reported genotype-dependent inhibition of CYP2C9 and in the current work we report a 2-fold difference in fluconazole Ki values (with flurbiprofen as a substrate) between the CYP2C9.1 and CYP2C9.3 proteins, suggesting that genotype-dependent inhibition *in vivo* was possible. Pretreatment with seven doses of either 200mg or 400mg fluconazole significantly decreased flurbiprofen oral clearance in *CYP2C9*1/*1* and *CYP2C9*1/*3* individuals (Figure 1A and B) in a gene-dose dependent manner, as compared to the control phase. In contrast, flurbiprofen plasma concentrations were unchanged among the three periods in the two individuals homozygous for *CYP2C9*3* (Figure 1C). Though fluconazole is largely excreted unchanged, it was necessary to determine if CYP2C9 genotype in any way altered fluconazole clearance and thus, accounting for the genotype dependent inhibition. However, neither fluconazole plasma concentrations nor clearance were impacted by genotype demonstrating that the inhibition observed was not due to altered fluconazole disposition.

Clearance values estimated from the population model-based drug interaction analysis were consistent with the noncompartmental analysis. It is particularly noteworthy that the clearances of flurbiprofen at two different levels of fluconazole interaction were closely predicted from a drug interaction model. For example, in the *CYP2C9*1/*3* group, the uninhibited clearance was estimated to be 0.9L/hr. In the model-based approach, a value of 0.81L/hr was estimated. In the 200mg fluconazole drug interaction arm, the noncompartmental approach provided a clearance of 0.5L/hr; this compares to 0.428L/hr under the drug interaction model. Values that were comparable between the two analysis approaches were also noted at the 400mg fluconazole

interaction level. These findings are encouraging and provide support to the notion that *in vivo* drug interactions may be reasonably predicted from *in vitro* data under an appropriate model.

With respect to 4'-hydroxyflurbiprofen formation clearances ($CL_{f,m}$), significant differences were noted at baseline (no fluconazole) among the three genotype groups (Table 4). These differences disappeared at the highest dose of fluconazole (400mg) between the **1/*1* and **1/*3* groups, but the $CL_{f,m}$ value for the *CYP2C9*3/*3* individuals remained statistically different. Interestingly, a substantial reduction in $CL_{f,m}$ was noted with administration of either 200mg or 400mg fluconazole, regardless of genotype, as compared to the control situation (no fluconazole). For example, in *CYP2C9*1/*1* individuals, the $CL_{f,m}$ was reduced 75% at the 400mg fluconazole dose, and $CL_{f,m}$ was reduced 77% and 60% in the *CYP2C9*1/*3* and *CYP2C9*3/*3* groups, respectively. Given that fluconazole at this dose inhibits several CYPs (Venkatakrishnan, et al., 2000), it cannot be discounted that this reduction in formation clearance may be due to inhibition of residual flurbiprofen hydroxylase activity carried out by other CYPs, such as CYP3A4 or CYP2C19. Thus, fluconazole does inhibit the CYP2C9.3 protein by a similar percentage as with the CYP2C9.1 protein, yet no change in oral clearance of flurbiprofen is noted in *CYP2C9*3/*3* individuals. This apparent paradox can be explained by the relatively low percentage contribution of this $CL_{f,m}$ of 4'-hydroxyflurbiprofen to the total clearance in *CYP2C9*3/*3* individuals. For example, formation of 4'-hydroxyflurbiprofen accounted for 42% of total clearance in the absence of fluconazole and 34% of total clearance with 400mg fluconazole in **1/*1* subjects. In contrast, in *CYP2C9*3/*3* individuals, though the $CL_{f,m}$ was reduced by 60% following co-administration of 400mg fluconazole, this pathway of clearance was only 10% of total clearance in the control period and reduced to 5% with 400mg fluconazole. Since this pathway represented such a small proportion of the total clearance, the

effect of CYP2C9 inhibition was minimal in these homozygotic variant individuals. This has important implications for prediction of drug-drug interactions since one must consider both genotype and fraction metabolized by a given pathway when predicting these interactions. Further studies are needed to test this hypothesis with drugs that have greater and lesser fractions metabolized by a given pathway in individuals with variant genotypes.

Some studies have reported the impact of genetic polymorphisms in CYP2C19 and CYP2D6 on drug-drug interaction in humans. Yasui-Furukori et al., studied the degree of inhibition of omeprazole and lansoprazole metabolism following co-administration of the CYP2C19 inhibitor fluvoxamine, in subjects genotype for CYP2C19 polymorphisms. This study, in concurrence with similar studies by others (Uno, et al., 2006;Yasui-Furukori, et al., 2004b;Yasui-Furukori, et al., 2004a), concluded that extensive metabolizers experience a greater extent of CYP2C19 inhibition upon co-administration of inhibitors as compared to CYP2C19 poor metabolizers. Similarly, studies have been conducted in extensive versus poor metabolizers of CYP2D6 and the extent of drug-drug interactions compared. These studies have consistently reported a greater degree of inhibition of wild type CYP2D6 enzyme compared with variant forms of CYP2D6 (Lessard, et al., 2001;Lindh, et al., 2003;Llerena, et al., 2001;Hamelin, et al., 2000). The variant alleles of CYP2C19, as well as CYP2D6, in the above studies cause either a splicing defect or a frame shift resulting in either premature termination of translation or a truncated protein. Thus, these genotype-dependent inhibition results for CYP2C19 and CYP2D6 polymorphisms identified in these studies are not surprising given that the polymorphisms result in the expression of inactive proteins, such that no residual activity is present to be inhibited. The present findings suggest that despite CYP2C9.3 protein maintaining ~20-30% residual activity as compared to the CYP2C9.1 enzyme, with respect to fluconazole inhibition, the CYP2C9.3

enzyme, *in vivo*, behaves as if it is inactive with respect to inhibition in that the contribution to overall clearance of the drug is so minor that inhibition has little effect on overall clearance.

These findings of genotype dependent inhibition in an enzyme with residual activity have potentially important clinical implications. If one were to reduce the dose of a target drug based on interaction potential of the inhibitor without considering genotype, the net result could be under-dosing of the patient and potentially sub-optimal therapy. Thus, if these results are more generally applicable, one may also need to consider both genotype status and fraction metabolized for drugs metabolized by polymorphic enzymes in situations where known interacting combinations must be used. Additional studies are underway to determine whether this phenomenon in *CYP2C9*3* subjects occurs with other substrates or is limited to the studied combination of flurbiprofen and fluconazole.

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Footnotes

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Figure Legends

Figure 1. Mean ± SD plasma flurbiprofen concentration-time profiles after oral administration of flurbiprofen tablet (50 mg) alone (closed circles) and after seven doses of fluconazole: 200mg (open circles) and 400mg (closed triangles) in (A) eleven *CYP2C9*1/*1* subjects, (B) eight *CYP2C9*1/*3* subjects and (C) two *CYP2C9*3/*3* subjects.

Figure 2. Interaction plot of flurbiprofen apparent oral clearance (median ± 25th percentile) of the three genotype groups at each of the three treatment periods. Values are taken from Table 3.

Figure 3. Ratio of individual $AUC_{0\text{-}inf}$ (Flurbiprofen with fluconaozole (200 or 400mg)/ flurbiprofen alone with means (line) of the three genotype groups. A = Baseline, B = 200mg fluconazole, C = 400mg fluconazole.

Figure 4. Mean ± SD plasma fluconazole concentration versus time profiles after oral administration of seven doses of 200mg or 400mg fluconazole tablets in eleven *CYP2C9*1/*1*, eight *CYP2C9*1/*3* and two *CYP2C9*3/*3* subjects.

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Table 1. Prevalence of CYP2C9*2 and CYP2C9*3 alleles in the study population (individuals screened for genotype, prior to inclusion in the pharmacokinetic study).

SNPs	Number of Subjects (n = 187)†	
	Homozygous	**Heterozygous**
CYP2C9*2	2	23
CYP2C9*3	2	18

†CYP2C9*1*1 - n = 142

Table 2. Demographics of subjects participating in drug interaction study (n = 21).

Demographics	***CYP2C9*1/*1***	***CYP2C9*1/*3***	***CYP2C9*3/*3***
Number of subjects	11	8	2
Age (yrs)	25 (19-36)	23 (19-28)	(25, 29)
Gender (Male/Female)	4/7	3/5	2/0
Body Weight (Kg)	73.7 (51-108)	66.9 (49-84)	(77, 85)
Height (cm)	166 (154, 193)	167 (160-189)	(177, 179)
Creatinine Concentration	0.87 (0.7-1.27)	0.86 (0.76-1.03)	(1, 1.1)

Data are presented as median (range), except for *CYP2C9*3/*3* genotype group in which individual data are presented.

No significant differences among the CYP2C9 genotype groups, *CYP2C9*1/*1*, *CYP2C9*1/*3* and *CYP2C9*3/*3*, were noted with respect to age ($F(2, 18) = 0.95$, $p = 0.41$), body weight ($F(2, 18) = 1.38$, $p = 0.28$), height ($F(2, 18) = 0.48$, $p = 0.63$) or creatinine concentrations ($F(2, 18) = 1.2$, $p = 0.32$).

Table 3. Noncompartmental pharmacokinetic parameters of flurbiprofen before (baseline) and after pre-treatment with 200mg or 400mg fluconazole for seven days.

Pharmacokinetic Parameters	**Units**	***CYP2C9*1/*1*** **(n =11)**	***CYP2C9*1/*3*** **(n = 8)**	***CYP2C9*3/*3*** **(n = 2)**
Baseline				
CL/F	$L.hr^{-1}$	1.6 (1.2 – 2.2)	0.9 (0.5 – 2)*	(0.4, 0.6)*,#
$AUC_{0\text{-}inf}$	$\mu g.ml^{-1}.hr$	30.8 (23.2 – 42.7)	53.7 (24.6 – 112)*	(85.8, 119) *,#
$T_{1/2}$	hr	4.8 (4.2 – 6.1)	7.2 (5.5 – 7.5)*	(9.7, 13.8) *,#
C_{max}	$\mu g.ml^{-1}$	7.6 (4.1 – 9.5)	8.9 (4.2 – 10.7)	(8, 9.4)
T_{max}	hr	1.5 (0.5 – 2.0)	2.0 (1.5 – 6.1)	(2.0, 2.0)
V/F	L	10.8 (7.8 – 13.8)	9.2 (3.9 – 22)	(8.2, 8.4)
$CL_{f,\,m}$	$L.hr^{-1}$	0.67 (0.43 – 0.95)	0.35 (0.15 – 0.86) *	(0.05, 0.05)*,#
200mg Fluconazole co-administration				
CL/F	$L.hr^{-1}$	0.8 (0.3 – 1.1)	0.5 (0.3 – 1.1)	(0.3, 0.4) *
$AUC_{0\text{-}inf}$	$\mu g.ml^{-1}.hr$	62.3 (46.1 – 150)	96.1 (44.1 – 197)	(136, 152) *
$T_{1/2}$	hr	7.8 (6.1 – 16.3)	9.2 (8.0 – 13.2)	(18.9, 22.6) *,#
C_{max}	$\mu g.ml^{-1}$	5.9 (4 – 10.2)	7.7 (5.2 – 15.5)	(8.6, 8.6)
T_{max}	hr	2.0 (1.0 – 4.0)	2.0 (1.8 – 4.0)	(1, 1.5)
V/F	L	8.3 (6.1 – 13.7)	7.5 (3.2 – 14.6)	(10, 10.8)
$CL_{f,\,m}$	$L.hr^{-1}$	0.28 (0.13 – 0.37)	0.14 (0.06 -0.34) *	(0.02, 0.03) *
400mg Fluconazole co-administration				
CL/F	$L.hr^{-1}$	0.5 (0.24 – 0.82)	0.4 (0.2 – 0.8)	(0.4, 0.4)
$AUC_{0\text{-}inf}$	$\mu g.ml^{-1}.hr$	93.2 (60.7 – 206)	133 (65.4 – 257)	(119, 133)
$T_{1/2}$	hr	9.9 (7.4 – 12.5)	13.7 (12.2 – 17.1)*	(16.1, 16.1)*

C_{max}	$\mu g.ml^{-1}$	7.5 (4.7 – 14.4)	8.4 (3.7 -12.2)	(4.3, 8.5)
T_{max}	hr	2.0 (1.0 – 4.0)	1.5 (1.0 – 4.0)	(1.5, 8)
V/F	L	7.5 (4.2 – 11.0)	7.1 (4.5 – 16.4)	(8.8, 9.8)
$CL_{f, m}$	$L.hr^{-1}$	0.17 (0.07 - 0.34)	0.08 (0.04 – 0.23)	(0.02, 0.02)*

Data are shown as median (range), except for *CYP2C9*3/*3* genotype group in which individual data are presented.

*P < 0.05 between *CYP2C9*1/*1* and *CYP2C9*1/*3* or *CYP2C9*1/*3*.

# P < 0.05 between *CYP2C9*1/*3* and *CYP2C9*3/*3*.

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Table 4. Noncompartmental pharmacokinetic parameters of fluconazole after 200mg or 400mg co-administration for seven days.

Pharmacokinetic Parameters	**Units**	***CYP2C9*1/*1*** **(n =11)**	***CYP2C9*1/*3*** **(n = 8)**	***CYP2C9*3/*3*** **(n = 2)**
200mg Fluconazole				
AUC_{0-24}	$\mu g.ml^{-1}.hr$	265 (187-395)	248 (142-293)	158, 262
$T_{1/2}$	hr	31.6 (13.3–46.4)	27.3 (14.7-46.7)	34.6, 51.6
C_{max}	$\mu g.ml^{-1}$	14.5 (11.3-19.6)	14.4 (9.2-16.0)	9.6, 20.3
400mg Fluconazole				
AUC_{0-24}	$\mu g.ml^{-1}.hr$	543 (362-742)	515 (474-722)	330, 490
$T_{1/2}$	hr	35.4 (17.9-54.9)	42.0 (15.5, 55.2)	(49.5, 51.1)
C_{max}	$\mu g.ml^{-1}$	27.8 (19.6-42.1)	26.4 (23.3-47.0)	18.8, 24.6

Data are shown as median (range), except for *CYP2C9*3/*3* genotype group in which individual data are presented.

Table 5. Model-based population pharmacokinetic parameters of flurbiprofen.

Parameter	Estimate	95% Confidence Interval
CL2C9, uninhibited (L/hr)		
*CYP2C9*1/*1*	1.38	(1.15, 1.61)
*CYP2C9*1/*3*	0.805	(0.580, 1.03)
*CYP2C9*3/*3*	0.384	(0.215, 0.553)
CLnon2C9 (L/hr)	0.198	(0.0792, 0.317)
V (L)	8.40	(7.34, 9.46)
Ka (hr^{-1})	2.13	(1.18, 3.08)
LAG (hr)	0.200	(0.181, 0.219)
Variability (CV%)		
CL	25.6%	(5.9, 35.8%)
V	26.1%	(12.4, 34.6%)
Ka	94.2%	(63.1, 117%)
LAG	14.0%	(1.4, 19.7%)
RUV	47.7%	(43.5, 51.7%)

V- flurbiprofen volume of distribution, Ka – flurbiprofen first-order absorption rate constant, LAG – flurbiprofen absorption lag time, RUV – residual unexplained variability, CV – coefficient of variation

Figure 1

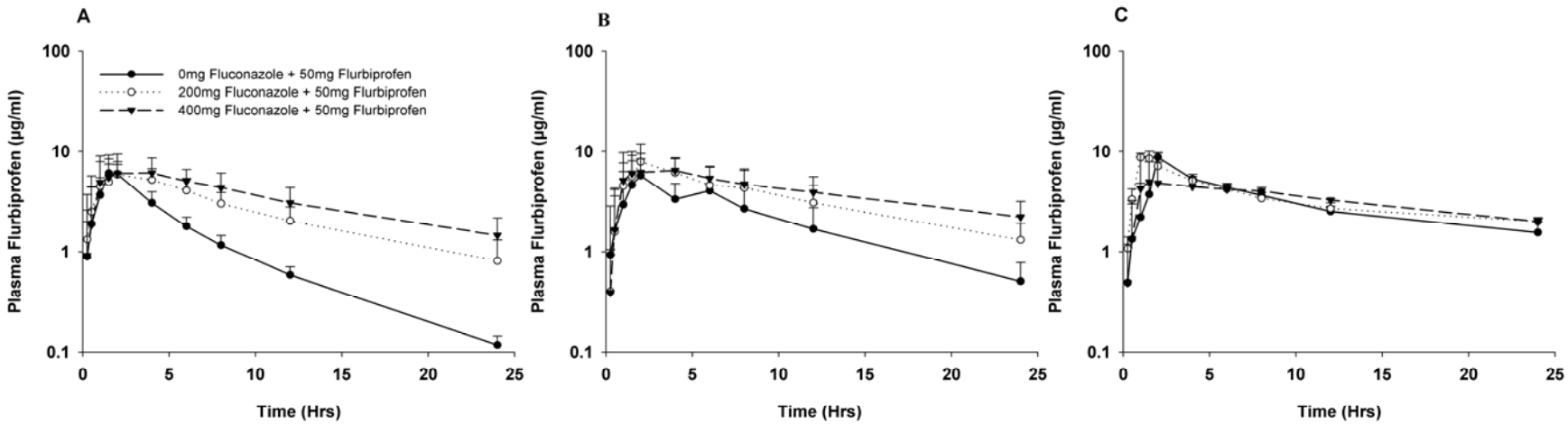

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Figure 2

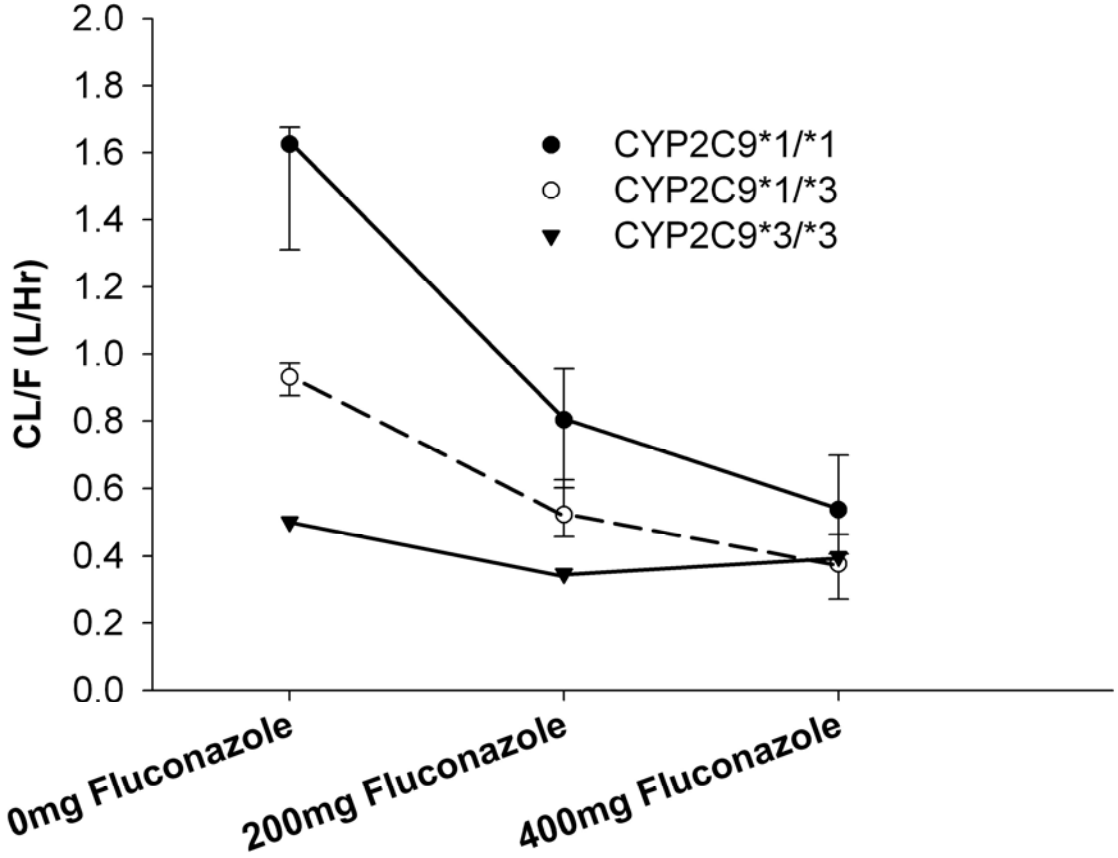

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Figure 3

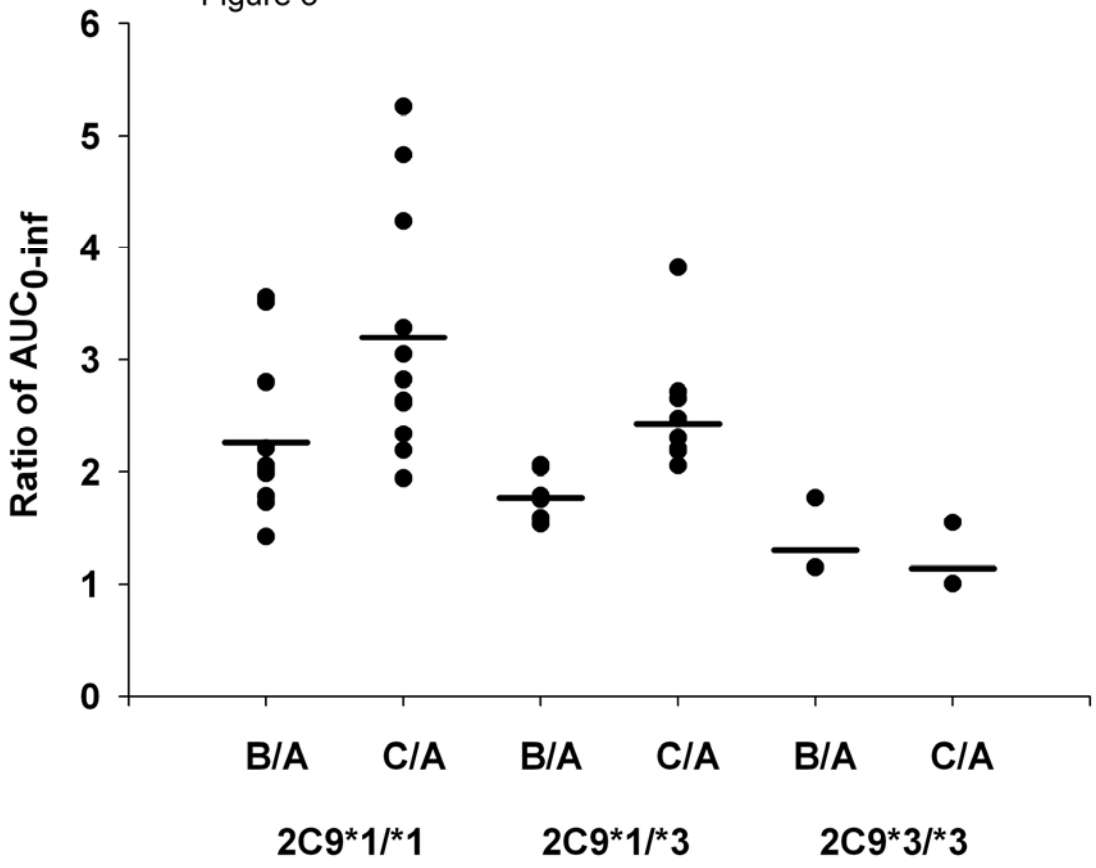

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Figure 4