IDENTIFICATION OF THE CYTOCHROME P450 ENZYMES INVOLVED IN THE N-OXIDATION OF VORICONAZOLE

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ABSTRACT:

Voriconazole is a triazole antifungal agent with potent activity against a broad spectrum of clinically significant pathogens. In vivo and in vitro studies have demonstrated that voriconazole is extensively metabolized, with the major circulating metabolite resulting from N-oxidation. In the present study, we report on the human cytochrome P450 enzymes responsible for the generation of this metabolite. In human liver microsomes voriconazole N-oxidation exhibited biphasic kinetics with $K_m$ of 8.1 $\mu$M, and $K_{m2}$ of 835 $\mu$M. Studies at 2500 $\mu$M voriconazole identified CYP3A4 as the low-affinity component, with activity correlating strongly with CYP3A4 activity in a bank of human liver microsomes ($r = 0.90$) and inhibited by ketoconazole. At 25 $\mu$M, voriconazole N-oxidation showed strong correlation with CYP2C19 and CYP3A4 activity ($r = 0.77$ and 0.74, respectively) and was inhibited by both sulfaphenazole and ketoconazole. Incubations with recombinant enzymes suggested both CYP2C9 and CYP2C19 as high-affinity enzymes ($K_m$ values of 20 and 3.5 $\mu$M, respectively). Further studies used chemical inhibitors in human liver microsomes prepared from individual donors, including two CYP2C19 poor metabolizers. No inhibition was observed with sulfaphenazole, indicating a minor role for CYP2C9 in human liver, but inhibition by ketoconazole was most potent in the CYP2C19 poor metabolizer livers, suggesting an increased role for CYP3A4 in individuals lacking CYP2C19. These data indicate that voriconazole is a substrate for CYP2C9, CYP2C19, and CYP3A4, with CYP2C19 involvement being minimal in human liver microsomes. Genotype status for CYP2C19 and/or coadministration of drugs that modulate CYP2C19 or CYP3A4 activities could effect voriconazole plasma levels.

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Materials and Methods

Drugs and Chemicals. Voriconazole (UK-109,496), authentic metabolite (UK-121,265), and structurally similar internal standard for HPLC analysis were synthesized at Pfizer Central Research (Sandwich, UK). [14C]Voriconazole was prepared by Amersham Biosciences (Cardiff, UK) with a radiochemical purity >98% (by thin layer chromatography and HPLC) and a specific activity of 16.2 $\mu$Ci $\mu$mol$^{-1}$. Furafylline, (S)-mephenytoin, 4-hydroxy mephentoin, butafuralol, 1’-hydroxybufuralol, 6-hydroxychloroxazone, and sulfaphenazole were obtained from Salford Ultrafine Chemicals and Research Ltd (Manchester, UK). Specific P450 cDNA-transfected human β-lymphoblastoid-derived microsomes were obtained from BD Gentest (Woburn, MA). All other reagents were obtained from Sigma Chemical (Poole, Dorset, UK).

Preparation of Liver Microsomes. Transplant-quality human liver tissue was obtained from the International Institute for the Advancement of Medicine (Exton, PA). All tissues were procured with informed consent in accordance with the Uniform Anatomical Gift Act U.S.A. The donors ranged in age from 16 to 70 years old and included 13 males and 10 females. No donor had a known drug history with the exception of one donor who had been treated with phenobarbitone, a known CYP3A4 inducer. None of the donors had a history of alcohol abuse. Hepatic microsomes were prepared from individual human livers or a combination of four human livers by the process of differential centrifugation (Hyland et al., 2001). Cytochrome P450 content was determined using the method of Ormro and Sato (1964), and the protein concentration was determined using the method of Lowry et al. (1951) with bovine serum albumin as the protein standard.

CYP2C19 poor metabolizer liver microsomes were kindly provided by Dr. David J. Greenblatt (Tufts University School of Medicine, Boston, MA).

In Vitro Metabolism of [14C]Voriconazole. Incubation (5 ml) was performed in human liver microsomes at 1 $\mu$M [14C]voriconazole and 0.5 $\mu$M

Abbreviations used are: HPLC, high performance liquid chromatography; P450, cytochrome P450.
cytochrome P450, for 60 min. The assay was terminated using 10 ml of methanol to precipitate the microsomal protein. After centrifugation, the supernatant was evaporated to dryness under nitrogen and resuspended in methanol. The methanolic sample was injected onto a gradient HPLC system comprising a 25 x 0.46-cm Spherisorb 5-µm ODS2 column (Hichrom, Reading, UK) eluted by a gradient of 0 to 100% methanol in 0.1 M ammonium acetate over 40 min at 1 ml/min. The eluate was monitored for UV absorbance (254 nm) and by an on-line radioactivity detector (β-Ram; Lablogic Ltd, Sheffield, UK). Metabolic characterization was by comparison of retention times on the gradient HPLC system with authentic standards.

In Vitro Metabolism of Voriconazole by “Poor Metabolizer” Supermix.

Three specially commissioned preparations of BD Gentest Supermix (control, lacking CYP2D6 and lacking CYP2C19) were incubated at 0.5 µM P450 for 1 h in the presence of 1 µM voriconazole. Nine samples (100 µl) were taken over the 1-h incubation and analyzed by HPLC-tandem mass spectrometry for voriconazole. Disappearance rate constants were determined in the CYP2D6 and CYP2C19 poor metabolizer preparations and compared with those obtained in the control.

Microsomal Incubations. The conversion of voriconazole to its fluoropyrimidine N-oxide metabolite (UK-121,265) by human liver microsomes or expressed recombinant cytochromes P450 was determined according to the following method. Each incubation (final volume, 1 ml) was comprised of Tris-HCl (pH 7.4), 5 mM MgCl₂, and 5 µM MnCl₂. Reducing equivalents required for P450 metabolism were provided by NADPH, which was regenerated in situ by an isocitric acid/isocitric acid dehydrogenase system. The incubation mixture was preincubated at 37°C for 5 min in the presence of substrate before addition of NADPH. At the end of the incubation, the reaction was terminated by the addition of 7 ml of dichloromethane followed by structurally related internal standard (0.1 mg/ml, 10 µl). Samples were rotary mixed for 15 min, followed by centrifugation to separate the aqueous and organic layers. The aqueous layer was discarded and the organic layer was transferred to tapered tubes and evaporated to dryness under nitrogen at 40°C. Samples were reconstituted in mobile phase (100 µl of HPLC mobile phase and 80 µl of HPLC mobile phase) for HPLC analysis.

Analytical Method. Samples were chromatographed on a 25-cm Inertsil ODS2 column eluted at 1 ml/min with an isotropic mixture of acetic acid (0.5%/acetonitrile/methanol, 85:5:10 (v/v/v)). Detection was by UV absorbance (SPD-10A, Shimadzu, Kyoto, Japan) at 274 nm. Under these conditions paraxanthine had a retention time of approximately 9 min, β-hydroxyethyltheophylline approximately 12 min, and caffeine approximately 17 min. The remaining probe substrate activities used were as described previously in the literature. Caffeine N-demethylase activity was determined at 1000 µM caffeine and 1 mg/ml microsomal protein for 60 min. After 60 min, the incubations were terminated by the addition of 7 ml of dichloromethane/propan-2-ol (85:15, v/v) followed by internal standard (100 µg/ml β-hydroxyethyltheophylline, 10 µl). After extraction, the organic layer was removed and evaporated to dryness under nitrogen. The residue was resuspended in 100 µl of HPLC mobile phase and 80 µl was injected onto the HPLC. Samples were chromatographed on a 25-cm Inertsil ODS2 column eluted at 1 ml/min with an isotropic mixture of acetic acid (0.5%/acetonitrile/methanol, 85:5:10 (v/v/v)). Detection was by UV absorbance (SPD-10A, Shimadzu, Kyoto, Japan) at 274 nm. Under these conditions paraxanthine had a retention time of approximately 9 min, β-hydroxyethyltheophylline approximately 12 min, and caffeine approximately 17 min. The remaining probe substrate activities used were as described previously in the literature. Cumarin 7-hydroxylase activity was determined at a substrate concentration of 100 µM (Maurice et al., 1991), (S)-warfarin 7-hydroxylase activity was determined at a substrate concentration of 500 µM (Doekes et al., 1991), (S)-mephenytoin 4-hydroxylase activity was determined at a substrate concentration of 1000 µM (Maurice et al., 1991), (S)-warfarin 7-hydroxylase activity was determined at a substrate concentration of 500 µM (Meier et al., 1985), bufuralol 1’-hydroxylase was determined at a substrate concentration of 10 µM (Kronbach et al., 1987), 4-nitrophenol 3-hydroxylase activity was determined at a substrate concentration of 1000 µM (Tassaneeyakul et al., 1993), and testosterone 6β-hydroxylase activity was determined at a substrate concentration of 250 µM (Funae and Imaoka, 1987).

Chemical Inhibition Studies. The effect of specific cytochrome P450 inhibitors furafylline (CYP1A2), sulfaphenazole (CYP2C9), quinidine (CYP3D6), and ketoconazole (CYP3A4) on voriconazole N-oxidation was investigated in human liver microsomes. The inhibitors were chosen on the basis of selective inhibition of a particular cytochrome P450 enzyme and the concentrations used had been previously established to cause significant inhibition of that cytochrome P450. For the mechanism-based inhibitor furafylline a 15-min preincubiation period was used. These inhibitors were coincubated

Voriconazole and Its Fluoropyrimidine N-Oxide Metabolite (UK-121,265).

Voriconazole

UK-121,265

**FIG. 1.** Structure of voriconazole and its fluoropyrimidine N-oxide metabolite (UK-121,265).
with voriconazole at 25 and 2500 μM and their influence on the rate of UK-121,265 formation was determined.

**Metabolism by Recombinant Cytochromes P450.** The formation of UK-121,265 from voriconazole (25 and 2500 μM) was assessed in microsomes derived from specific P450 cDNA-transfected human β-lymphoblastoid cells. In addition, the kinetics of UK-121,265 formation was determined in lymphoblastoid-derived microsomes expressing CYP2C9, CYP2C19, and CYP3A4.

**Metabolism by CYP2C19 Poor Metabolizer Liver Microsomes.** To elucidate the metabolism of voriconazole in a CYP2C19 poor metabolizer population, the effect of a CYP2C9 and CYP3A4 inhibitor on the formation of UK-121,265 in human liver microsomes prepared from five “normal” and two CYP2C19 poor metabolizer livers was investigated. Voriconazole (25 μM) was incubated with the specific inhibitors sulfaphenazole (30 μM) and ketoconazole (3 μM) (Baldwin et al., 1995; Newton et al., 1995), at 1 mg/ml microsomal protein for 60 min. UK-121,265 formation was expressed as the percentage of activity remaining, compared with uninhibited value.

**Analysis of Results.** All results are presented as mean ± standard deviation of three determinations. Determination of apparent K_m and V_max values were obtained by the use of Grafit (version 3.01). Statistical analysis to investigate the effects of inhibitors was carried out in Microsoft Excel (version 5) by using a two-sided t test for independent samples. Correlations and multi linear regression analysis was also carried out in Microsoft Excel (version 5).

**Results**

**In Vitro Metabolism of [14C]Voriconazole.** The profile of radioactive components was investigated in extracts from human liver microsomal incubations with [14C]voriconazole (Fig. 2). Two major components were identified as parent compound (region 2) and active components was investigated in extracts from human liver microsomal incubations. Peak 1 has been identified as UK-121,265 and peak 2 as voriconazole.

**In Vitro Metabolism of Voriconazole by Poor Metabolizer Supermix.** Voriconazole disappearance rate constants were determined in three Supermix preparations: control, CYP2D6 poor metabolizer, and CYP2C19 poor metabolizer. Disappearance rate constants of 0.0365 and 0.0408 min⁻¹ were measured in the control and CYP2D6 poor metabolizer preparations. This was extended to 0.0138 min⁻¹ in the preparation lacking CYP2C19. From these data, the involvement of CYP2C19 in the metabolism of 1 μM voriconazole was estimated at 62%.

**Kinetics of Voriconazole N-Oxidation in Human Liver Microsomes.** The rate of formation of UK-121,265 in human liver microsomes was found to be linear with time up to 60 min and protein up to 2 mg/ml. Enzyme kinetics (n = 2, for each individual donor) was therefore determined at a protein concentration of 1 mg/ml over 60 min. The apparent kinetic constants were estimated using voriconazole concentrations up to 5000 μM. The conversion followed Michaelis-Menten kinetics, and examination of the Eadie-Hofstee transformation revealed a biphasic plot, suggesting at least two enzymes are involved in this reaction (Fig. 3, for the liver designated HM22).

The apparent Michaelis-Menten kinetic parameters were estimated with the assumption that two enzymes were involved, by fitting the data to the following equation:

\[ v = \frac{V_{\text{max1}} \cdot [S]}{K_m1 + [S]} + \frac{V_{\text{max2}} \cdot [S]}{K_m2 + [S]} \]  

(1)

where v is the velocity of formation of UK-121,265, S is the concentration of voriconazole in the incubation mixture, K_m1 and K_m2 are the Michaelis constants for the two enzymatic components, and V_max1 and V_max2 are the respective maximum velocities. The kinetic parameters obtained for the N-oxidation in the three human livers investigated are detailed in Table 1. The mean apparent K_m values for the two components were 8 and 835 μM, with V_max values of 1.4 to 22 and 3.6 to 60 pmol/mg/min, respectively.

Further studies were performed at 25 and 2500 μM voriconazole to identify the cytochrome P450 enzymes involved. By substitution of mean K_m and V_max values into eq. 1, it can be calculated that at 2500 μM voriconazole N-oxidation will primarily be mediated through a low-affinity, high K_m enzyme (approx.73%), whereas at 25 μM there...
will be a greater role for a high-affinity, low $K_m$ enzyme (approx. 93%).

**Characterization of Voriconazole N-Oxidation in Human Liver Bank.** A bank of 23 human livers was used to assess the rate of UK-121,265 formation from voriconazole. The correlation data for voriconazole N-oxidation and the probe reactions for specific P450 enzymes are shown in Table 2. To avoid liver samples with very low or very high activities giving undue weighting to the correlation, all activities were correlated as their logarithm. At both concentrations of voriconazole there was a strong correlation with CYP3A4 ($r = 0.79$ at 25 $\mu$M voriconazole, $r = 0.89$ at 2500 $\mu$M voriconazole). At 25 $\mu$M voriconazole there was also a strong correlation with CYP2C19 ($r = 0.77$) activity.

From multivariate analysis, the rate of UK-121,265 formation at 25 $\mu$M voriconazole was shown to correlate strongly with both the rate of ($S$)-mephenytoin 4-hydroxylation (CYP2C19) and testosterone 6β-hydroxylation (CYP3A4), according to the following equation:

$$\log \text{UK-121,265 formation} = -1.2 + 0.53 \log \text{CYP2C19} + 0.39 \log \text{CYP3A4}$$

$R = 0.95, \text{S.E.} = 1.4, F = 161$

This correlation is illustrated in Fig. 4A as observed values against predicted values, calculated from the equations. The correlation was of greater statistical significance ($P < 0.001$) than the correlations with individual parameters at 25 $\mu$M voriconazole. These data further support an involvement of both CYP3A4 and CYP2C19.

**Inhibition of Voriconazole Metabolism in Human Liver Microsomes.** The effect of specific cytochrome P450 inhibitors on voriconazole metabolism at 25 and 2500 $\mu$M was investigated using furafylline (CYP1A2), sulfaphenazole (CYP2C9), quinidine (CYP2D6), and ketoconazole (CYP3A4). These studies were carried out in microsomes produced from a combination of four human livers. The results of this investigation are illustrated in Fig. 5.

Voriconazole metabolism is clearly inhibited by ketoconazole at both substrate concentrations. From statistical analysis using a two-sided $t$ test,
inhibition is significant at the 1% significance level. At the low-substrate concentration (25 \( \mu \)M), this is also true for the CYP2C9 inhibitor sulfaphenazole (25 \( \mu \)M). At this voriconazole concentration, inhibition was also observed with the lower concentration of quinidine; however, this did not translate to more potent inhibition as the quinidine concentration increased. Overall, the inhibition data suggest an involvement of CYP2C9 and CYP3A4 in the \( N \)-oxidation of voriconazole.

Metabolism in Expressed Recombinant Cytochromes P450. The \( N \)-oxidation of voriconazole (25 and 2500 \( \mu \)M) was investigated in microsomes derived from human B-lymphoblastoid cells expressing recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 (Fig. 6).

At both voriconazole concentrations, CYP2C9, CYP2C19, and CYP3A4 formed UK-121,265. At 25 \( \mu \)M voriconazole, the rate of CYP2C19-mediated UK-121,265 formation was approximately 16- and 25-fold greater than that of CYP2C9 and CYP3A4, respectively, determined on a picomoles of UK-121,265 per picomoles of P450 per minute basis. However, when expressed enzyme activity was normalized using relative activity factors (determined in house; data not shown), the rates were 3.1-fold that of CYP2C9 and 1.4-fold that of CYP3A4. This apparent CYP3A4 involvement arises from the contribution of the low-affinity enzyme at 25 \( \mu \)M voriconazole. The rate of UK-121,265 formation (normalized values) mediated by CYP3A4 increased 7.3-fold in going from 25 to 2500 \( \mu \)M voriconazole, whereas the increase was only 2.4-fold for CYP2C9 and 1.3-fold for CYP2C19. These data are indicative of CYP2C9 and CYP2C19 being...
voriconazole was characterized by a $K_m$ value of 20 $\mu$M and a $V_{max}$ value of 0.056 pmol of UK-121,265/min/pmol of P450. CYP2C19 mediated this transformation with a $K_m$ value of 3.5 $\mu$M and a $V_{max}$ value of 0.39 pmol of UK-121,265/min/pmol of P450, and CYP3A4 mediated $N$-oxidation was characterized by a $K_m$ value of 235 $\mu$M and a $V_{max}$ value of 0.14 pmol of UK-121,265/min/pmol of P450 (Fig. 7). These $K_m$ values are consistent with CYP2C9 and CYP2C19 being the high-affinity components and CYP3A4 the low-affinity component in human liver microsomes.

Metabolism by CYP2C19 Poor Metabolizer Liver Microsomes. The effects of ketoconazole and sulfaphenazole on voriconazole metabolism in five normal and two CYP2C19 poor metabolizer microsomes indicated a minor role for CYP2C19 in the $N$-oxidation of voriconazole. Inhibition was observed on coincubation with sulfaphenazole (Fig. 8), despite demonstrating that this concentration of sulfaphenazole inhibits greater than 85% of CYP2C9 activity (data not shown). Inhibition by ketoconazole varied from 80% to no inhibition in the microsomes prepared from normal donors, with the extent of inhibition reflecting the relative CYP3A4 activities in these livers. The most extensive inhibition with ketoconazole was observed in the two CYP2C19 poor metabolizer livers, suggesting that in the absence of CYP2C19 there is an increased role for CYP3A4.

Discussion
The $N$-oxidation of voriconazole to form UK-121,265 has been examined using human liver microsomes as well as microsomes expressing recombinant human cytochrome P450 enzymes to identify the P450 enzyme(s) involved in this metabolic pathway. Voriconazole is $N$-oxidized in human liver microsomes to produce UK-121,265, which is the major circulating metabolite of voriconazole in humans. This pathway is mediated by at least two cytochrome P450 enzymes, evidenced by biphasic Eadie-Hofstee plots. The mean kinetic parameters determined for this reaction in three human livers are $K_m = 8.1 \pm 2.9 \mu$M, $V_{max} = 835 \pm 182 \mu$M, $V_{max1} = 9.3 \pm 11$, and $V_{max2} = 32.2 \pm 28.4 \mu$M, suggesting this to be the low-affinity, high-capacity enzyme. However, the results of inhibition studies suggest a somewhat greater role for CYP3A4 at all concentrations. This is also supported by the studies using the CYP2C19 poor metabolizer Supermix, where CYP2C19 involvement was estimated at only 62% at 1 $\mu$M voriconazole.

Of the various specific P450 inhibitors examined in human liver microsomes, ketoconazole (2.5 and 25 $\mu$M) strongly inhibited metabolism at both voriconazole concentrations. Although high concentrations of ketoconazole inhibit reactions mediated by several P450 enzymes, lower concentrations specifically inhibit CYP3A4 (Baldwin et al., 1995; Newton et al., 1995). These data would therefore suggest a specific involvement of CYP3A4 as the low-affinity, high $K_m$ enzyme, with the effect of ketoconazole at the low voriconazole concentration being attributed to the CYP3A4 contribution at this level. Inhibition at 25 $\mu$M voriconazole was also observed with the CYP2C19 inhibitor sulfaphenazole (Baldwin et al., 1995), suggesting this enzyme to be a high-affinity enzyme in addition to CYP2C19.

At 2500 $\mu$M voriconazole, correlation analysis across a bank of 23 individual human liver microsomes showed a strong correlation with CYP3A4 activity, suggesting this to be the low-affinity, high-capacity enzyme. At 25 $\mu$M voriconazole, metabolic activity also correlated with CYP3A4 activity, in addition to CYP2C19. This would indicate that CYP2C19 is a high-affinity, low-capacity enzyme in human liver microsomes.
microsomes responsible for the N-oxidation of voriconazole but that at this concentration there is still a role for CYP3A4.

Of the individually expressed human cytochrome P450 enzymes investigated, CYP2C9, CYP2C19, and CYP3A4 exhibited substantial voriconazole oxidase activity. These three enzymes were characterized by $K_m$ values of 21, 4, and 235 μM for CYP2C9, CYP2C19, and CYP3A4, respectively. These values are in keeping with CYP2C9 and CYP2C19 both being high-affinity enzymes and CYP3A4, the low-affinity enzyme in human liver. To investigate this further, studies using the chemical inhibitors sulfaphenazole (CYP2C9 inhibitor) and ketoconazole (CYP3A4 inhibitor) were performed in human liver microsomes prepared from individual donors, including two CYP2C19 poor metabolizers. The involvement of CYP2C9 has been shown to be minimal in human liver microsomes, with sulfaphenazole having no effect on voriconazole metabolism in these seven individual livers. In contrast, ketoconazole was most potent as an inhibitor in the CYP2C19 poor metabolizer livers, suggesting an increased role for CYP3A4 in those individuals lacking the CYP2C9 enzyme.

Our studies have shown that voriconazole N-oxidation is mediated via CYP2C9, CYP2C19, and CYP3A4, and drugs that modulate these activities could have the potential to effect voriconazole plasma levels. Of these enzymes, the greatest effect is likely to be observed with modulators of CYP3A4, which is also the major P450 enzyme present in human liver (Shimada et al., 1994). In clinical studies, the enzyme-inducer phenytoin significantly decrease plasma voriconazole concentrations, such that an increase in voriconazole dose is recommended.

Another factor effecting voriconazole exposure will be genotype status for CYP2C19. CYP2C19 is a polymorphic enzyme which in 2 to 5% of Caucasians and 15 to 20% of Japanese subjects are functionally deficient or absent (Kubota et al., 1996; Xie et al., 1999). Analysis of healthy volunteer data has demonstrated that CYP2C19 genotype is the most important covariate determining plasma levels of voriconazole; however, there is considerable overlap in voriconazole exposure across the genotypes. Furthermore, the influence of genotype on voriconazole exposure is confounded by drug-drug and drug-disease interactions in the population, thus no dose adjustment based on genotype is recommended.

Considerable interindividual variability in the expression levels of CYP3A is also well documented. The range in hepatic protein expression differs considerably between individuals, with variability as high as 40-fold having been reported previously (DeWaziers et al., 1990; Shimada et al., 1994). Large interindividual differences in CYP3A4-dependent in vitro intrinsic clearances are also evident. The interindividual variability in voriconazole pharmacokinetics is high (EPAR for Vfend, 2002). In analysis of healthy volunteer data, the
three covariates CYP2C19 “status”, age, and sex could only account for 55% of the observed area under the curve variability after 200 mg every 12 h for 7 days (P. A. Milligan and M. O. Karlsson, unpublished data). It is reasonable to conclude that were information on CYP3A4 status available the level of unexplained variability would have been significantly lower.

References
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