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Roles of CYP3A4 and CYP2C19 in methyl hydroxylated and N-oxidized metabolite formation from voriconazole, a new anti-fungal agent, in human liver microsomes

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ABSTRACT

Involvement of cytochrome P450 (P450 or CYP) 2C19, 2C9, and 3A4 in *N*-oxidation of voriconazole, a new triazole antifungal agent, has been demonstrated using human liver microsomes. To confirm the precise roles of P450 isoforms in voriconazole clearance in individuals, we investigated the oxidative metabolism of voriconazole catalyzed by recombinant P450s as well as human liver microsomes genotyped for the *CYP2C19* gene. Among recombinant P450 isoforms using *Escherichia coli* expression systems, CYP2C19 and CYP3A4 had voriconazole *N*-oxidation activities, but not CYP2C9. Apparent K_m and V_{max} values of CYP2C19 and CYP3A4 for voriconazole *N*-oxidation were 14 ± 6 μM and 0.22 ± 0.02 nmol/min/nmol CYP2C19 and 16 ± 10 μM and 0.05 ± 0.01 nmol/min/nmol CYP3A4, respectively (mean ± S.E.). CYP3A4 produced a new methyl hydroxylated metabolite from voriconazole, detected by LC/UV and LC/MS/MS and confirmed by ^{1}H and ^{13}C NMR analyses, with K_m and V_{max} values of 11 ± 3 μM and 0.10 ± 0.01 nmol/min/nmol CYP3A4. The voriconazole 4-hydroxylation to *N*-oxidation metabolic ratios in liver microsomes from the wild-type *CYP2C19*1/*1* individuals (0.07) were lower than those observed in other genotypes (0.20–0.27) at a substrate concentration of 25 μM based on the reported clinical plasma level. These results suggest that the *CYP2C19* genotype, but not *CYP2C9* genotype, would be evaluated as a key factor in the pharmacokinetics of voriconazole and that 4-hydroxyvoriconazole formation may become an important pathway for voriconazole metabolism in individuals with poor CYP2C19 catalytic function.

1. Introduction

Voriconazole ((2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol) is a new antifungal agent developed as oral and intravenous formulations [1]. Primary mode of action of voriconazole is inhibition of fungal cytochrome P450 (P450 or CYP)-dependent 14-sterol demethylase by unmetabolized voriconazole [2]. Metabolic fate of ^{14}C-labeled voriconazole has been determined in mouse, rat, rabbit, guinea pig, dog, and human following intravenous and oral administration [3]. *In vivo* studies in humans have indicated that voriconazole is extensively metabolized with

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less than 2% of the dose excreted unchanged [3]. It has been reported that the major circulating metabolite of voriconazole results from fluoropyrimidine *N*-oxidation *in vivo* and that human CYP2C19, CYP2C9, and CYP3A4 forms responsible for the *N*-oxidation of voriconazole *in vitro* [4]. However, the precise roles of P450 isoforms in voriconazole clearance have not been examined by different recombinant P450 systems. Although a glucuronide of 4-hydroxyvoriconazole has been detected with 6% of the dose excreted in human urines, P450 isoforms involved in this methyl hydroxylation pathway (4-hydroxyvoriconazole formation) have not been clarified as yet.

In the present study, oxidative metabolism of voriconazole *in vitro* was investigated with recombinant P450 isoforms using *Escherichia coli* expression systems and human liver microsomes genotyped for the *CYP2C19* gene. We herein report that the methyl hydroxylation of voriconazole could be catalyzed by CYP3A in humans and that this metabolism may be an important alternative pathway for individuals who are genetically lacking the CYP2C19 catalytic function.

2. Materials and methods

2.1. Chemicals

Voriconazole and its fluoropyrimidine *N*-oxide (>99% purity) were supplied by Pfizer (Sandwich, UK). The other chemicals and reagents used were obtained in the highest grade available commercially.

2.2. Enzyme preparations

Liver microsomes from male Wistar rats (7 weeks old) untreated and intraperitoneally treated with dexamethasone (50 mg/kg) daily for 3 days were prepared as described previously [5]. Liver microsomes from individuals genotyped for the *CYP2C19* gene were used as described previously [6,7]. These studies were approved by the Committee on the Care and Use of Laboratory Animals and the Ethics Committee of Showa Pharmaceutical University, respectively. Recombinant human cytochrome P450 isoforms coexpressed with human NADPH-P450 reductase in *E. coli* membranes were prepared in this laboratory [8]. Pooled human liver microsomes, containing 19 pmol CYP2C19 and 108 pmol CYP3A4 per mg protein according to data sheet, were obtained from BD Gentest (Woburn, MA). Different preparations of recombinant CYP2C9 expressed in microsomes of insect cells were obtained from BD Gentest and Pan Vera (Madison, WI) to confirm undetectable activities of CYP2C9 in the voriconazole oxidation in contrast to reported findings [4].

2.3. Enzyme assays

Oxidative metabolism of voriconazole was determined according to the high-performance liquid chromatography method described previously [9] with minor modifications. Briefly, the typical incubation mixture of a total volume of 0.25 mL contained microsomal protein (0.125 mg) or recombinant P450 (10 pmol), 25 or 250 μM substrate, and an NADPH-generating system in 0.1 M potassium phosphate buffer (pH 7.4) unless otherwise specified. Voriconazole concentration of 25 μM was based on the reported clinical plasma level of total voriconazole in humans [3]. Voriconazole protein binding (40–50%) was not considered in our *in vitro* work. In the kinetic analyses, 2.5–2500 μM of voriconazole was incubated. Incubations were carried out for 30 min at 37 °C. The reaction was terminated by adding 2.0 mL of ethyl acetate. After vortex mixing, the tubes were centrifuged at 1000 × *g* for 10 min. The organic phase was transferred to a clean tube and evaporated to dryness at 40 °C under a gentle nitrogen stream. The residue was dissolved in 0.15 mL of mobile phase. The liquid chromatography system consisted of a pump and multi UV detector (Shimadzu, Kyoto, Japan) using an analytical C_{18} reversed-phase column (5 μm, 4.6 mm × 250 mm, Mightysil RP-18 GP, Kanto Chemicals, Tokyo, Japan). The mobile phase was 40% acetonitrile in 10 mM ammonium acetate (pH 4.0), at a flow rate of 1.0 mL/min. The UV detector was set at a wavelength of 255 nm unless otherwise specified. The HPLC apparatus was operated at room temperature.

To prepare oxidative products for identification, the product was isolated from a 100-mL incubation as mentioned above. The solvent extract was injected onto a semi-preparative C_{18} column (5 μm, 10 mm × 250 mm, Mightysil RP-18 GP, Kanto Chemicals) using the same solvent system employed in the analytical mode. The individual peaks were collected from the column and the volume was reduced *in vacuo*. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AV600-NMR instrument (Bruker, Billerica, MA), in $CDCl_3$ (99.96% D). The activities for formation of 4-hydroxyvoriconazole were presented on the basis of detector response using voriconazole as a standard. The standard curves (0.5–500 pmol/mL) for authentic *N*-hydroxyvoriconazole showed >0.99 of correction coefficients. The detection limit of 4- or *N*-hydroxyvoriconazole was <0.5 pmol/mL or <1 pmol product formation/min/nmol recombinant P450 under the present conditions.

Testosterone 6β-hydroxylation was determined as described previously [5]. The concentrations of total P450 [10] and NADPH-P450 reductase (EC 1.6.2.4) [11] were determined as described previously. The microsomal protein was determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

2.4. LC/MS/MS assay

A Quattro micro API mass analyzer was used for metabolites analyses (Waters, Tokyo, Japan). The instrument was operated in electrospray positive ionization mode and was directly coupled to the LC 2695 system with a C_{18} column (Atlantis, 3 μm, 2.1 mm × 150 mm) and MassLynx NT4.1 software for data acquisition (Waters). To tune the mass spectrometer, a solution of voriconazole (10 ppm in mobile phase) was infused into the ion source, and the cone voltage was optimized to maximize the intensity of the precursor ions for voriconazole, *m/z* 350. The collision energy was then adjusted to optimize the signal for one of the most abundant voriconazole product ions, *m/z* 224. Typical tuning conditions were as follows: electrospray capillary voltage, 3.2 kV; sample cone voltage, 30 V; and collision energy, 20 eV at a collision gas pressure 1.6×10^{-4} kPa argon.

2.5. Kinetic analysis

The kinetic analysis was done using a nonlinear regression analysis program (KaleidaGraph, Synergy Software, Reading, PA).

2.6. Docking simulation of voriconazole for CYP3A4

Docking calculation was carried out for voriconazole binding to the CYP3A4 corresponding to Protein Data Bank file designated 1W0G (http://www.rcsb.org/pdb/) using the MMFF94x force field distributed in the MOE software (ver. 2006.08, Chemical Computing Group, Montreal, Canada) with the Monte Carlo docking procedure of ASE-Dock 2005 (Ryoka Systems, Tokyo, Japan). Prior to docking, energy of CYP3A4 structure was minimized using the CHARM22 force field. Twenty-six solutions were generated for docking experiment and ranked according to total interaction energy ($U_{ele} + U_{vdw} + U_{ligand}$; i.e., summation of intermolecular electrostatic, van der Waals, and ligand energy).

3. Results

3.1. Metabolism of voriconazole

The metabolism of voriconazole was investigated using rat and human liver microsomes and recombinant human P450s. Typical chromatograms are shown in Fig. 1. After incubation of voriconazole (2.5 mM) with liver microsomes, the formation of two metabolites was observed (Fig. 1A and B). Peaks with retention times of 3.9 min (designated as peak 2) were assigned to voriconazole N-oxide with the authentic standard. The other peak at 3.5 min (designated as peak 1) was present both in the reaction mixtures with liver microsomes. The formation of voriconazole N-oxide and the peak 1 was observed after incubation of voriconazole with CYP3A4 (Fig. 1D), but CYP2C19 produced only voriconazole N-oxide (Fig. 1C) under the present conditions.

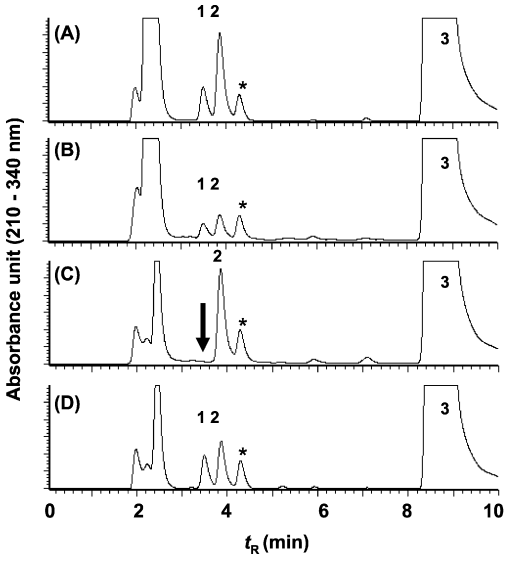

Fig. 1 – Representative LC chromatograms of voriconazole metabolites formed by liver microsomes and recombinant P450s. Voriconazole (2.5 mM) was incubated with liver microsomes from rats (A) and humans (B), CYP2C19 (C), and CYP3A4 (D), respectively. Peak 1, 4-hydroxyvoriconazole (confirmed by the data shown in Figs. 2 and 3); peak 2, voriconazole N-oxide; peak 3, voriconazole. *Peak at 4.3 min was an impurity peak associated with voriconazole.

3.2. Structural confirmation of the oxidative product, 4-hydroxyvoriconazole

Since voriconazole N-oxide has been reported as a sole metabolite *in vitro* in human liver microsomal P450 system [4], the novel peak 1 in this study (Fig. 1) was analyzed by UV and MS spectra in comparison with voriconazole and voriconazole N-oxide (Fig. 2). As shown in Fig. 2D and G, the authentic or formed N-oxide of the aromatic fluoropyrimidine ring resulted in the UV spectra change from maximum peak of 256 nm to 264 and 313 nm. However, the novel product had almost the same UV maximum peak at 257 nm (Fig. 2J) as that of voriconazole, suggesting unchanged aromatic moiety of the product. The parent ion of the product had a molecular weight of 366 (Fig. 2K), demonstrating an introduction of one oxygen atom to the voriconazole, similarly in the case of voriconazole N-oxide (Fig. 2E and H). In the analyses of collision-induced dissociation of the $[M + H]^+$ ions, voriconazole N-oxide and the novel product commonly produced 297 *m/z*, by losing the triazole group at C1-position; however, all of the voriconazole and its N-oxide, and the product indicated 224 *m/z* by losing the fluoropyrimidine ring. C3- or C4-position of the mainframe of butanol structure was suggested to be oxidized. As shown in Fig. 3E and F, ^1H and ^{13}C NMR spectra of the products revealed that the product possessed $–CH_2OH$ moiety, but not $–CH_3$ like N-oxide (Fig. 3C and D); namely the 4-hydroxylated group. Together with several lines of the present evidence and reported glucuronide metabolites in human urines [3], the novel product designated as peak 1 in human CYP3A system was assigned as 4-hydroxyvoriconazole.

3.3. Metabolites formation activities catalyzed by P450 isoforms

To determine which human P450 forms oxidize voriconazole, 25 and 250 μM voriconazole was incubated with recombinant human P450 enzymes expressed in *E. coli* membranes with NADPH-P450 reductase, based on reported clinical concentrations of 25 μM [3]. In the formation of N-oxide from voriconazole, CYP2C19 was highly active and CYP3A4 and CYP3A5 had moderate catalytic activities among eleven P450 isoforms tested (Table 1). In contrast, 4-hydroxylation of voriconazole was catalyzed by CYP3A forms. CYP3A4 expressed in *E. coli* membranes had higher 4-hydroxylation activities than N-oxidation. Apparent K_m and V_{max} values of CYP2C19 for voriconazole N-oxidation and of CYP3A4 for voriconazole N-oxidation and 4-hydroxylation were 14 ± 6 μM and 0.22 ± 0.02 nmol/min/nmol CYP2C19, 16 ± 10 μM and 0.05 ± 0.01 nmol/min/nmol CYP3A4, and 11 ± 3 μM and 0.10 ± 0.01 nmol/min/nmol CYP3A4, respectively (mean ± S.E.).

To investigate interindividual differences in voriconazole metabolism, voriconazole oxidation activities in human liver

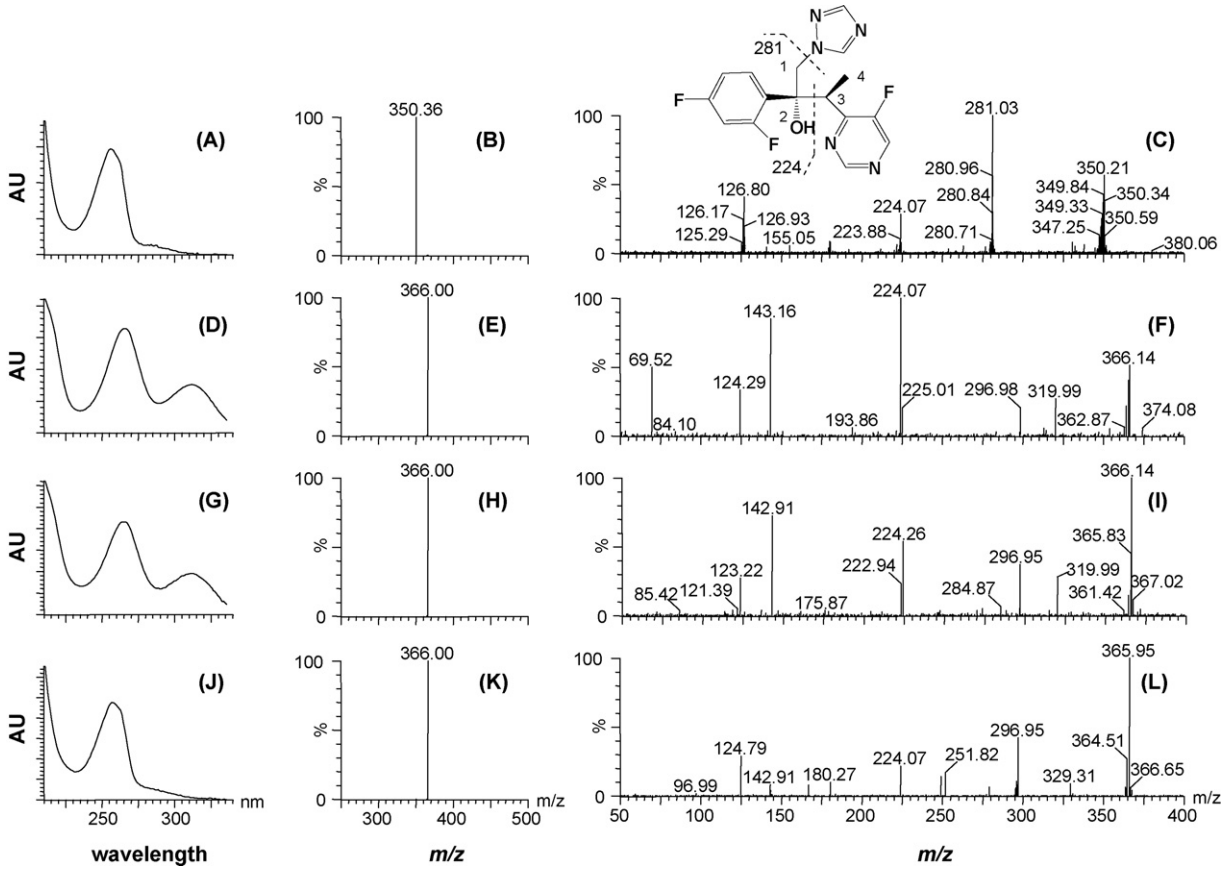

Fig. 2 – UV spectra (A, D, G and J), LC/MS spectra (B, E, H and K), and product ion spectra (C, F, I and L) obtained by collision-induced dissociation of the $[M + H]^+$ ion of voriconazole (A–C), of authentic (D–F) and formed (G–I) *N*-oxidized voriconazole, and of the novel metabolite, 4-hydroxyvoriconazole (J–L) in human CYP3A4 system.

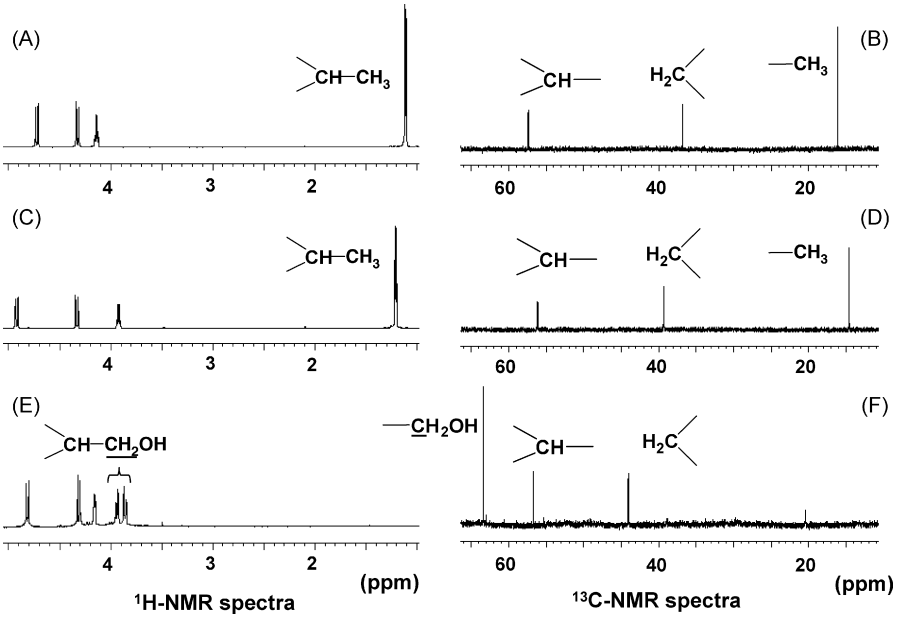

Fig. 3 – ^{1}H NMR (A, C and E) and ^{13}C NMR (B, D and F) spectra of voriconazole (A and B), formed *N*-oxidized voriconazole (C,D), and the novel metabolite, 4-hydroxyvoriconazole (E,F) in human CYP3A4 system. Underlines indicate the hydroxylated positions.

Table 1 – Voriconazole 4-hydroxylation and *N*-oxidation activities by recombinant human P450s

	pmol/min/nmol P450			
	25 μM		250 μM	
P450	4-Hydroxylation	*N*-Oxidation	4-Hydroxylation	*N*-Oxidation
1A2	–	–	–	–
2A6	–	–	–	–
2B6	–	–	–	–
2C8	–	–	–	–
2C9	–	–	–	–
2C19	–	130	–	210
2D6	–	–	–	–
2E1	–	–	–	–
3A4	71	30	90	44
3A5	16	7	16	12
3A7	4	2	3	2

Voriconazole (25 and 250 μM) was incubated at 37 °C for 30 min with each recombinant P450 (0.040 μM of P450) in the presence of an NADPH-generating system. Results are presented as means of duplicate determinations. –: not detected (<1 pmol/min/nmol P450).

microsomes were also determined at substrate concentrations of 25 and 250 μM (Fig. 4). The individual liver microsomes harboring the *CYP2C19*1*, *CYP2C19*2* or *CYP2C19*3* gene and pooled liver microsomes showed almost similar testosterone 6β-hydroxylation activities, as a marker for CYP3A levels (Fig. 4B). On the other hand, the product ratios of 4-hydroxylation to *N*-oxidation of voriconazole showed marked interindividual differences (Fig. 4A). Based on a genetic defect of the *CYP2C19* gene, *N*-oxidation of voriconazole was decreased; however, 4-hydroxylation activities were not likely to be affected in human liver microsomes, dependent on the individual CYP3A levels.

4. Discussion

Major metabolites of voriconazole, a new anti-fungal agent, following multiple administration of voriconazole have been proposed in animals and humans [3]. The main oxidative metabolite *in vivo* has been indicated to be voriconazole *N*-oxide in humans. In the present study, we demonstrated that 4-hydoxyvoriconazole as well as *N*-oxide was formed *in vitro*, especially produced by liver microsomal CYP3A isoforms in humans. Biotransformation of voriconazole into 4-hydroxylated metabolites by rat liver microsomes was also confirmed by pretreatment of dexamethazone with rats (Fig. 1). The *in vitro* formation of 4-hydroxyvoriconazole was confirmed by comparison using tandem mass spectrometry (Fig. 2), ^{1}H NMR, and ^{13}C NMR (Fig. 3) analyses. Importance of CYP3A4 in voriconazole metabolism has been suggested by drug interaction with ritonavir in individuals with poor CYP2C19 catalytic function [12].

Voriconazole adopted an orientation suitable for both *N*-oxidation and 4-hydroxylation for CYP3A4 in Fig. 5A and B, respectively, under the low energy docking solutions. The relative importance of the pathways in conjunction with the relative higher abundance of the CYP3A compared with low contents of CYP2C19 in human livers [13] may still translate to

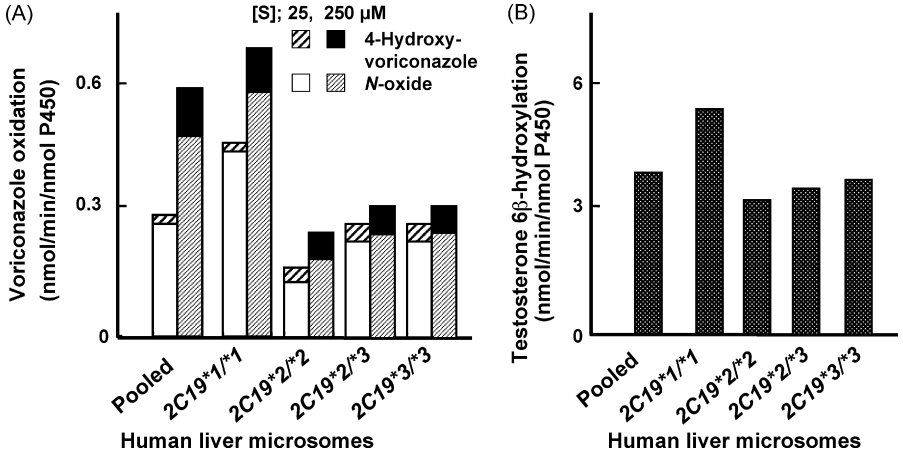

Fig. 4 – Oxidation of voriconazole (A) and testosterone (B) by liver microsomes from pooled samples and from individuals genotyped for the *CYP2C19* gene. In panel A, voriconazole (25 and 250 μM) was incubated with liver microsomes form pooled or individuals genotyped for the *CYP2C19* gene (*2C19*1*, *2C19*2* or *2C19*3*). The rates of voriconazole *N*-oxide and 4-hydroxyvoriconazole formation are indicated with open and filled boxes. In panel B, testosterone (100 μM) was incubated with liver microsomes for the comparison.

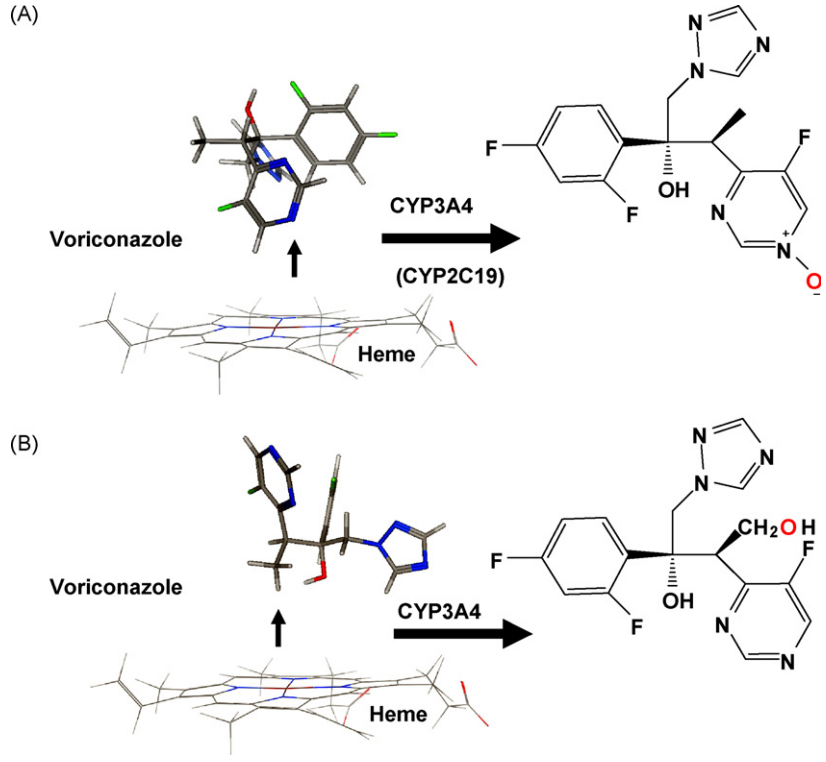

Fig. 5 – Proposed metabolic pathway of voriconazole in humans. Voriconazole adopted an orientation suitable for both *N*-oxidation (*U* = 21.4, A) and 4-hydroxylation (*U* = 24.8, B) for CYP3A4 (corresponding to 1W0G).

higher overall contribution of CYP3A, even having lower CYP3A activities compared with CYP2C19. The latter pathway should be an alternative route of the voriconazole primary metabolism that would be CYP2C19 poor metabolizers (Fig. 5), as supported by individual human liver microsomal experiments (Fig. 4). In consistent with the findings that crystallized CYP3A4 has a large substrate-binding pocket [14] different from a CYP2C19 homology model [15], both orientation of voriconazole to CYP3A4 would provide different oxidative metabolites. Glucuronide of 4-hydroxyvoriconazole has been detected in both animals and humans, especially accounting for 6% of the dose in humans, in contrast to detection of 4-hydroxyvoriconazole itself only in animals [3]. This apparent discrepancy between *in vivo* and *in vitro* research might be partly dependent on polymorphic CYP2C19 status and inducible CYP3A4 levels in the humans involved in these studies. There has been no information for 4-hydroxyvoriconazole in viewpoints of toxicity. In our preliminary experiments, 4-hydroxyvoriconazole did not apparently show any cytotoxicity in human HepG2 cells with or without exogenous CYP3A4 expression system for 48 h (data not shown).

In terms of contributions of human P450 isoforms to voriconazole oxidative metabolism, important roles of CYP2C19 and CYP3A4 in voriconazole *N*-oxidation were consistent with the previous report using a lymphoblastoid cell expression system [4]. In that report, high affinity and capacity of CYP2C19 toward voriconazole *N*-oxidation have been suggested [4], and a similar tendency in the kinetic characters was observed in the present study. However, a reported minor role of CYP2C9 were not seen in our present experiments using different enzyme sources of two commercial baculovirus expression systems and our *E. coli* membranes expressing CYP2C9. The reason was not clear for these phenomena concerning voriconazole using recombinant P450 proteins at present. In this context, we previously reported the case of troglitazone in which different sources of recombinant P450s yielded some negative results of its oxidative metabolism [16]. In accordance with the present results, safety of voriconazole in a patient with *CYP2C9*2/CYP2C9*2* genotype has been reported [17].

In conclusion, the present study suggested that the primary oxidative metabolism of voriconazole was efficiently catalyzed by polymorphic CYP2C19 and abundant CYP3A4 to form *N*-oxide of voriconazole in human liver microsomes. CYP3A could also mediate the methyl hydroxylation of voriconazole to yield the polar metabolite (4-hydroxyvoriconazole) more than its *N*-oxide. This may be an important alternative pathway of voriconazole clearance for individuals who are genetically lacking the CYP2C19 catalytic function. This information is worth understanding voriconazole disposition extensively metabolized in humans. Drug interactions with voriconazole *via* CYP3A4 should be paid more attention.

Acknowledgments

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