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Diclofenac hydroxylation in monkeys: Efficiency, regioselectivity, and response to inhibitors

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ABSTRACT

The catalytic efficiency, regioselectivity, and response to chemical inhibitors of diclofenac (DF) hydroxylation in three Old World monkey liver microsomes (rhesus, cynomolgus, and African green monkey) are different from those determined with human liver microsomes. In contrast to the high affinity–high capacity (low K_m–high V_{max}) characteristics of DF 4′-hydroxylation in humans, this reaction proceeded in all monkey species with catalytic efficiencies >20-fold lower. However, DF 5-hydroxylation, a negligible reaction in human liver microsomes, was kinetically favored in monkeys mainly due to the increased V_{max} values. Chemical inhibitors (reversible or mechanism-based) selective to human CYP3A4 and CYP2C9 failed to differentiate monkey orthologs involved in DF hydroxylation. Immunoinhibition studies with monoclonal antibodies against human CYPs revealed the major contribution of CYP2C and CYP3A to 4′- and to 5-hydroxylation, respectively, in rhesus and cynomolgus liver microsomes. However, in African green monkeys, in addition to CYP2C, CYP3A also appeared to be involved in 4′-hydroxylation. Further studies with recombinant rhesus and African green monkey CYP2C and CYP3A enzymes (rhesus CYP2C75, 2C74, and 3A64; African green monkey CYP2C9agm and CYP3A4agm) confirmed the major role of CYP enzymes of these two subfamilies in DF 4′- and 5-hydroxylation. Clearly, while monkey CYP2C and 3A enzymes retain the same substrate selectivity towards DF hydroxylation as their human orthologs, their altered catalytic efficiency and response to chemical inhibitors may indicate different structural features of active sites as opposed to human orthologs.

1. Introduction

Cytochrome P450 2C9 (CYP2C9) accounts for approximately 18% of the CYP protein content in human liver microsomes and catalyzes approximately 20% of the CYP-mediated metabolic reactions of drugs currently on the market [1,2]. It is also genetically polymorphic with a number of variants showing reduced catalytic activity [3]. As a result, drug interactions associated with CYP2C9 and altered pharmacokinetics in CYP2C9 polymorphic subjects have been an important theme in both academic fields and the pharmaceutical industry. It is well known that the success of these two

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Abbreviations: DF, diclofenac; 4′-OHD, 4′-hydroxy diclofenac; 5-OHD, 5-hydroxy diclofenac; CYPs, cytochrome P450s; K_m, apparent Michaelis constant; K_s, substrate inhibition constant; V_{max}, maximal velocity; HLM, human liver microsomes; Rh-MLM, rhesus monkey liver microsomes; Cyno-MLM, cynomolgus monkey liver microsomes; AG-MLM, African green monkey liver microsomes; KTZ, ketoconazole; SLF, sulfaphenazole; TA, tienilic acid

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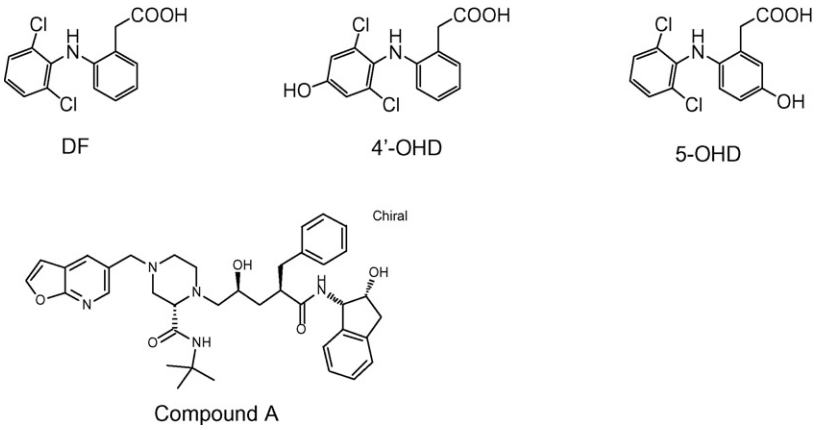

Fig. 1 – Structures of DF, 4′-OHD, 5-OHD, and Compound A.

types of studies relies heavily on the selection of the probe substrates. Selectivity is the most important criterion for in vitro studies, while clinical relevance and safety have to be considered for in vivo studies. Diclofenac (DF), a non-steroidal anti-inflammatory drug, is considered a preferred and acceptable in vitro and in vivo probe [4].

DF can be oxidized at both the 4′- and 5-positions (Fig. 1), but it is selectively metabolized to 4′-hydroxyl DF (4′-OHD) by CYP2C9 with a high catalytic efficiency. Interestingly, the other members of the human CYP2C subfamily, CYP2C8, 2C18, and 2C19, also are able to catalyze DF hydroxylation, but they lead to simultaneous formation of 4′-OHD and 5-hydroxy DF (5-OHD) with a much lower efficiency. Studies of the CYP2C9 active site employing DF and its derivatives [5], homology modeling [6], and site-directed mutagenesis [7] suggest that DF is strictly positioned in the CYP2C9 active site via its COO^- functionality in favor of position 4′, which is not chemically preferred. Thus, DF 4′-hydroxylase activity has been measured in many in vitro and in vivo drug interaction and genotyping studies to indicate the function of human CYP2C9.

Ethical constraints and cost-effective considerations have led to rigorous endeavors in searching for animal models to predict the potentials of drug interactions in humans. While mice and rats have been widely used for this purpose, differences among species in many physiological and biochemical functions quite often make it difficult to extrapolate rodent data to humans. Hence, nonhuman primates, which are genetically closer to humans than are rodents, are considered to be better species in this regard. Rhesus monkeys (*Macaca mulatta*) and cynomologus monkeys (*Macaca fascicularis*) are commonly used throughout the pharmaceutical industry as preclinical safety species. They have successfully mimicked drug interactions by inhibitors or inducers verified in humans, although mainly for CYP3A [8–12]. Recently, African green monkeys (*Cercopithecus aethiops*) have been reported to be a good model for studying induction of brain and liver CYP2E1 by nicotine [13,14] and CYP2B6 by Phenobarbital [15]. However, similar cases with monkey CYP2C enzymes are lacking. In contrast to human livers where CYP2C9 accounts for the major component of CYP2C members [16], a recent report [17] describes the identification of a novel major member CYP2C76 in cynomolgus monkey liver. This new CYP2C member was also detected in rhesus monkey liver but not in humans or the great apes. Although its functional characteristics are unknown, the emergence of this new CYP2C member may underscore the importance of a better understanding of substrate selectivity of monkey CYP2C enzymes, if a drug interaction study involving monkey CYP2C enzymes is investigated.

Although DF has been used to characterize heterologously expressed monkey CYP2C enzymes [18], its suitability as a probe for monkey CYP2C enzymes orthologous to human CYP2C9 has not been adequately characterized. Instead, 5-hydroxylation catalyzed by monkey CYP3A has drawn more attention, presumably due to bioactivation and quinidine stimulation concerning this pathway [19,20]. Involvement of CYP2C enzymes in DF 4′-hydroxylation was deduced mainly based on cross-inhibition by antibodies raised against human or rat CYP2C enzymes [17,21,22]. Little effort has been reported comparing this reaction in humans and monkeys in terms of the substrate specificity, kinetic properties, and responses to inhibitors. When a discrepancy was observed between in vitro and in vivo rifampin induction of monkey DF 4′-hydroxylase activity, the suitability of DF as a CYP2C probe substrate was questioned [23]. In this report, we disclose our efforts in evaluating DF as a probe substrate for monkey CYP2C and 3A with the aid of monoclonal antibodies against human CYP3A and 2C enzymes, and recombinant human (CYP2C9 and 3A4), rhesus (CYP2C74, 2C75, and 3A64), and African green monkey (CYP2C9agm and 3A4agm) CYPs. We also investigated whether common human CYP2C9 and 3A4 inhibitors were effective in inhibiting respective monkey orthologs. The results of these studies indicate that rhesus and cynomolgus monkeys are closer to humans than African green monkeys in the regioselectivity of DF hydroxylation, but kinetic parameters and response to chemical inhibitors are significantly different between humans and all monkey species.

2. Materials and methods

2.1. Chemicals and biological materials

DF, 4′-OHD, sulfaphenazole (SLF), ketoconazole (KTZ), and tienilic acid (TA) were purchased from Sigma Chemicals (St.

Louis, MO). The procedures for in-house preparations of human CYP2C9 and CYP3A4 and rhesus CYP3A64 have been described elsewhere [24,25]. Rhesus CYP2C74 (GenBank accession no. AY635462) and CYP2C75 (GenBank accession no. AY635463), and African green monkey CYP2C9agm (GenBank accession no. DQ022201) and CYP3A4agm (GenBank accession no. DQ022197) have been cloned and expressed in Sf21, and the respective microsomes prepared by the same method for human CYP2C9 and CYP3A4 [24]. Preparations of male human, rhesus, and cynomolgus monkey liver microsomes, abbreviated as HLM, Rh-MLM, and Cyno-MLM (pooled from 10, 5, and 5 subjects, respectively), were obtained from Xenotech LLC (Lenexa, KS). Liver microsomes of nine African green monkeys (AG-MLM, eight males [002,003, 085, 096, 119, 120, 126, and 130] and one female [001]) were prepared by differential centrifugation (9000 *g* and 105,000 *g*), as described previously [43], and were reconstituted in a buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, 0.5 mM dithiothreitol, 1.15% KCl, and 0.1 M potassium phosphate and stored at −80 °C until used. Equal amounts of microsomal protein from six male individual preparations (085, 096, 119, 120, 126, and 130) were pooled to provide a representative average preparation. 5-OHD was prepared following the method by Kenny et al. [26]. Compound A (Fig. 1, *N*-[2-hydroxy-1-indanyl]-5-[2-[((1,1-dimethylethyl)amino)carbonyl]-4-[(furo[2,3-b]pyridin-5-yl)methyl]piperazin-1-yl]-4-hydroxy-2-(phenylmethyl)pentanamide), a mechanism-based inhibitor of human CYP3A4, was synthesized at Merck Research Laboratories. Mouse ascites containing monoclonal antibodies raised against CYP2C9 (MAb 30-12-1) and CYP3A4 (MAb 10-1-1) were prepared in-house. The antibodies have been characterized with respect to their CYP selectivity [24]. NADPH, $MgCl_2$, EDTA, acetic acid, ammonia hydroxide, and acetonitrile were purchased from Sigma Chemicals.

2.2. DF hydroxylation assay

For studies on kinetics, chemical inhibition or immunoinhibition, formation of 4′-OHD and 5-OHD in liver microsomes was determined in incubations (0.25 ml final volume) consisting of liver microsomes (0.1 mg protein/ml) or recombinant CYPs (10 pmol/ml), $MgCl_2$ (10 mM), NADPH (1 mM), and DF (0.25–250 μM) in potassium phosphate buffer (100 mM, pH 7.4). The reactions were initiated by the addition of NADPH and terminated with 200 μl of acetonitrile after a 20-min incubation at 37 °C, followed by the addition of 100 μl of tolbutamide (internal standard, 5 μM in 50% acetonitrile aqueous solution). The resultant supernatant following centrifugation was subject to LC–MS/MS analysis.

Co-incubation inhibition studies with SLF (0.1–10 μM for HLM and human recombinant CYPs; 2.5–100 μM for MLM and monkey recombinant CYPs) and KTZ (0.1–10 μM for all enzyme sources) were carried out at a final DF concentration close to the K_m for each species (details described in the respective results). Both SLF and KTZ were dissolved in 50% (v/v) acetonitrile as stock solutions (50-fold). Control incubations contained the same concentration of acetonitrile but no inhibitor (≤1%, v/v, final concentration).

Time-dependent inhibition by TA and Compound A was determined by measuring the rates of DF 4′- and 5-hydroxylation obtained following pre-incubation of enzymes with the inhibitors. Specifically, TA (10 μM) and Compound A (1 and 10 μM) were incubated with liver microsomes (1 mg/ml) or recombinant CYPs (100 pmol/ml) in the presence of NADPH. Then, an aliquot (25 μl) of such pre-incubated mixture was added to a tube containing NADPH and DF (250 μM) in a volume of 225 μl.

In the immunoinhibition studies, mouse ascites (fluid containing antibodies against human CYP2C and CYP3A) were diluted with potassium phosphate buffer (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 dilutions) to define the amount required for the maximal effect. An aliquot (5 μl) of each diluted ascites solution was incubated with 25 μg of liver microsome protein or 2.5 pmol respective recombinant CYPs at room temperature for 20 min before the addition of $MgCl_2$, DF and phosphate buffer. In the subsequent experiments, diluted ascites (1:4) was applied to estimate contributions of CYP2Cs and CYP3As to 4′- and 5-hydroxylation in different species or individual liver microsomal preparations, giving a final ratio of 50 μl ascites/mg protein and 0.5 μl ascites/pmol CYP.

For HPLC-UV analysis, incubations (1 ml final volume) consisted of liver microsomes (1 mg/ml), $MgCl_2$ (10 mM), NADPH (1 mM), and potassium phosphate buffer (100 mM, pH 7.4). After incubation for 120 min, 50 μl of acetic acid was added to terminate the reaction, followed by extraction with ethyl acetate (2 ml × 2 ml). Following evaporation under a stream of nitrogen, the residues were reconstituted with 150 μl of 30% acetonitrile aqueous solution.

2.3. Sample analysis

The separation of 4′-OHD, 5-OHD, and DF, and internal standard was accomplished on a Synergi Fusion-RP column (2.1 mm × 50 mm, 4 μm, 80 Å, Phenomenex, Torrance, CA) using PE 200 binary pumps (Perkin-Elmer Life And Analytical Sciences, Inc., Wellesley, MA). Solvent A consisted of 0.02% aqueous acetic acid, pH adjusted to 4.5 with NH_4OH, and acetonitrile (90:10), and solvent B consisted of acetonitrile and water (90:10). The mobile phase was delivered at a flow rate of 0.5 ml/min with a linear increase of solvent B from 10 to 25% over a period of 0.5 min, to 53% over 3.5 min, then finally to 80% over 0.1 min. This value was held for 0.4 min before returning to 10% over 0.1 min. Equilibration was allowed for an additional 1.4 min, giving a total chromatographic run time of 6.0 min. Under these conditions, 5-OHD, 4′-OHD, and DF eluted at 3.15, 3.40, and 4.5 min, respectively.

A tandem mass experiment was performed on a Sciex (Concord, Ontario, Canada) Model API 3000 triple quadrupole mass spectrometer interfaced to the column eluant via a Sciex turbospray probe operating at 350 °C. Operating conditions were optimized by infusion of 4′-OHD and tolbutamide along with the LC flow (500 μl/min, solvent A/B = 50/50). Selected reaction monitoring (SRM) experiments in the positive ionization mode were performed using a dwell time of 200 ms per transition to detect ion pairs at *m/z* 296/215 (DF), 312/231 (4′-OHD and 5-OHD), and 271/155 (tolbutamide). The lower limit of quantitation for 4′- and 5-OHD was 50 nM and the recovery was greater than 95%. The calibration curves for both metabolites were linear up to 5000 nM.

HPLC-UV analysis was conducted to illustrate the regioselectivity of DF hydroxylation in liver microsomal incubations. DF and its metabolites were separated on a Synergi polar-RP column (4.6 mm × 150 mm, 5 μm, 80 Å; Phenomenex) using a Shimadzu LC-10AS HPLC system (Shimadzu Scientific Instrument, Columbia, MD). The same mobile phases as described above were delivered at a constant flow rate of 1.0 ml/min. The initial mobile phase consisted of 20% of solvent B, which increased linearly to 35% over a period of 15 min, then to 80% over 3 min. This value was held for 2.0 min before returning to 20% over 1.0 min. Equilibration was allowed for an additional 4.0 min, giving a total chromatographic run time of 25 min. The elution of 4′-OHD (15.0 min), 5-OHD (13.4 min), and DF (19.8 min) was monitored by UV detection (280 nm).

2.4. Data analysis

Estimates of apparent K_m, V_{max}, K_s, and IC_{50} were obtained using the methods described previously [28].

3. Results

3.1. Catalytic profiles of DF hydroxylation in human, rhesus, cynomolgus, and African green monkey liver microsomes

LC–MS/MS analysis of extracts of all liver microsomal incubations containing DF (250 μM) and NADPH revealed the presence of two major oxidative metabolites with an increase of the protonated molecular weights (MH^+) by 16 Da. They were confirmed to be 4′- and 5-hydroxy DF by comparison of retention times and product spectra with authentic standards (data not shown). Since these two metabolites possess similar UV characteristics [5], their regioselective formation in different species is demonstrated by the HPLC-UV chromatograms (λ at 280 nm, Fig. 2). While human liver microsomes highly favored 4′-hydroxylation, 5-OHD formation in rhesus and cynomolgus monkeys was appreciably higher than in humans, although oxidation still was in favor of position 4′. Interestingly, a reverse regioselectivity was observed in African green monkey liver microsomes where 5-hydroxylation exceeded the oxidation at position 4′.

The kinetic characteristics of 4′- and 5-hydroxylation in human and monkey liver microsomes were evaluated by measuring reaction rates as a function of DF concentration (0.5–250 μM). The derived kinetic parameters (K_m, V_{max}, and V_{max}/K_m) are listed in Table 1.

In HLM, 4′-hydroxylation was characterized as a high affinity and high capacity reaction as indicated by the low K_m (7.2 μM) and high V_{max} (2560 pmol/min/mg protein). On the contrary, 5-hydroxylation was described as a low affinity (K_m of 92 μM) and low capacity (V_{max} of 187 pmol/min/mg protein) reaction. The 5-OHD product was detectable only at high DF concentrations (>10 μM) under our conditions. As observed previously [29], substrate inhibition of 4′-hydroxylation was evident in the current study with a substrate inhibition constant (K_s) of 368 μM.

Similar to the regioselectivity in humans, the rate of 4′-OHD formation in Rh-MLM and Cyno-MLM exceeded that of 5-OHD,

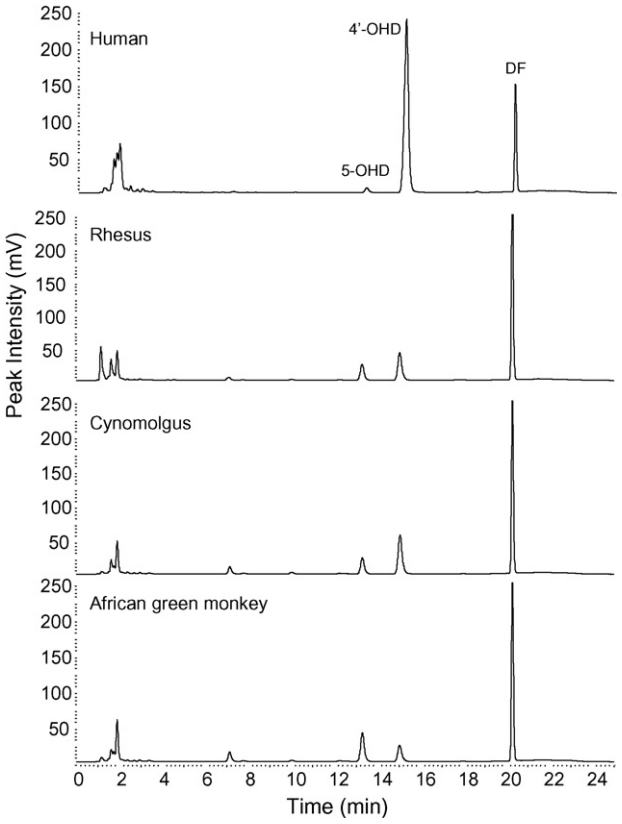

Fig. 2 – HPLC-UV chromatograms of 4′-OHD and 5-OHD formed in human and monkey liver microsomal incubations in the presence of NADPH. The incubations contained 1 mg/ml microsomal proteins and 250 μM DF in the total volume of 1 ml. Following liquid–liquid extraction, the dried residues were reconstituted with 150 μl of 30% acetonitrile aqueous solution and 25 μl of the reconstituted samples were injected.

but kinetic analysis revealed increased K_m (~39 μM) and decreased V_{max} (460–580 pmol/min/mg protein) for the oxidation at position 4′. However, 5-hydroxylation in these two monkey species became more efficient than in humans, reflected primarily by the increased V_{max} (380–430 pmol/min/mg protein). Because of the kinetic characteristics, the ratio of 5-OHD to 4′-OHD increased from 0.35 to 0.70 in the range of DF concentration tested (2.5–250 μM). This finding highlights the necessity for proper separation of these two isomers in sample analyses, especially those with LC–MS methods, where fast elution is usually employed.

The regioselectivity reversed in AG-MLM such that 5-hydroxylation surpassed 4′-hydroxylation as a result of the increase in both the affinity and catalytic capacity for the former reaction (Table 1). With respect to the catalytic efficiency (V_{max}/K_m), the ratio of 5- to 4′-OHD ranged from 1.4 to 7 in nine preparations. Of special interest are the preparations AGM-001 and AGM-003, which gave rise to the highest and lowest ratios (7.2 and 1.4, respectively). Results of immunoinhibition studies (see below) suggest the predominant activities of CYP3A and CYP2C in these two subjects, respectively.

Table 1 – Kinetic parameters of DF 4′- and 5-hydroxylation in human and three monkey liver microsomal preparations

	Parameters[a]	K_m	K_s	V_{max}	V_{max}/K_m
4′-OHD	HLM[b]	7.2 ± 0.7	368 ± 54	2561 ± 99	356
	Rh-MLM[b]	39 ± 3		460 ± 9	12
	Cyno-MLM[b]	40 ± 5		583 ± 23	16
	AG-MLM[c]	59 ± 18		416 ± 87	7.6 ± 2.8
5-OHD	HLM[b]	92 ± 16		187 ± 14	1.9
	Rh-MLM[b]	144 ± 5		434 ± 7	3.0
	Cyno-MLM[b]	113 ± 10		384 ± 16	3.3
	AG-MLM[c]	37 ± 27		829 ± 97	32 ± 16

[a] Units are μM for K_m and K_s, pmol/min/mg protein for V_{max}, and μl/min/mg protein for V_{max}/K_m, respectively.
[b] Data for HLM, Rh-MLM, and Cyno-MLM are expressed as mean ± S.E. from three determinations of pooled preparations with the number of subjects of 10, 5, and 5, respectively.
[c] Data for AG-MLM are expressed as mean ± S.D. from nine individual preparations.

3.2. *Kinetic properties of diclofenac hydroxylation in stably expressed rhesus and African green monkey CYP3A and CYP2C*

Five recombinant monkey CYP enzymes, including rhesus CYP3A64, CYP2C74, and CYP2C75, and African green monkey CYP3A4agm, and CYP2C9agm, were cloned, expressed, and characterized in-house. DF 4′- and 5-hydroxylations by these recombinant CYPs followed Michaelis-Menten kinetics and the kinetic parameters are summarized in Table 2. In general, these monkey CYP3A and CYP2C enzymes functioned largely as human CYP3A4 and CYP2C9 in regioselectivity in that these CYP2C enzymes greatly favored 4′-hydroxylation, while the CYP3A enzymes were more selective towards 5-hydroxylation; however, differences in kinetics were evident. In contrast to human CYP2C9, which is the most efficient in catalyzing 4′-hydroxylation (V_{max}/K_m of 15 μl/min/pmol CYP), CYP2C75 efficiency was reduced by ~7-fold (2.4 μl/min/pmol CYP) due to a lower affinity and lower capacity. The change was even more dramatic in CYP2C9agm, which only showed 1% efficiency of human CYP2C9 (0.15 μl/min/pmol CYP). Substrate inhibition was evident for both human CYP2C9 and rhesus CYP2C75 while the K_s is ~3-fold greater for the latter. Interestingly, at high DF concentrations, all CYP2C enzymes generated 5-OHD. The catalytic efficiency, as expected, was very low in CYP2C enzymes (only 0.011–0.017 μl/min/pmol CYP). The selectivity decreased from CYP2C9, CYP2C75 to CYP2C9agm as indicated by the ratio of 4′-to 5-hydroxylation efficiency (>1000, 144, and 14, respectively). It is worth noting that CYP2C74 showed a regioselectivity favoring hydroxylation at position 5 over 4′ (V_{max}/K_m of 0.11 μl/min/pmol CYP versus 0.002 μl/min/pmol CYP) mainly owing to the significant difference in binding affinity (Table 2). This behavior mirrored what has been reported for human CYP2C8 in DF hydroxylation [5].

Although CYP3A has been reported to be responsible for DF 5-hydroxylation [30,31], DF appeared to be a poor substrate for this reaction by human CYP3A4 and rhesus CYP3A64 with values of catalytic efficiency being only 0.015 and 0.078 μl/min/pmol CYP, respectively. These values are significantly lower than those for 4′-hydroxylation by their CYP2C counterparts (Table 2). On the contrary, 5-hydroxylation by CYP3A4agm (V_{max}/K_m of 0.38 μl/min/pmol CYP) not only appreciably surpassed those by the other two CYP3A enzymes, but also

Table 2 – Kinetic parameters of DF 4′- and 5-hydroxylation in stably expressed human and monkey CYP2C and CYP3A enzymes

	Enzymes	K_m	K_s	V_{max}	V_{max}/K_m
4′-OHD	CYP2C9	5.5 ± 0.8	162 ± 27	83 ± 5	15
	CYP3A4	278 ± 68		1.7 ± 0.3	0.006
	CYP2C74	288 ± 37		0.69 ± 0.06	0.002
	CYP2C75	15.8 ± 0.8	466 ± 45	38.6 ± 1.0	2.4
	CYP3A64	414 ± 70		4.7 ± 0.6	0.01
	CYP2C9agm	45.4 ± 4.4		6.6 ± 0.2	0.15
	CYP3A4agm	53.1 ± 16.4		1.07 ± 0.18	0.02
5-OHD	CYP2C9	616 ± 148		8.5 ± 1.6	0.014
	CYP3A4	78.9 ± 10.2		1.20 ± 0.06	0.015
	CYP2C74	4.7 ± 0.6		0.50 ± 0.01	0.106
	CYP2C75	128 ± 25		2.18 ± 0.21	0.017
	CYP3A64	33.0 ± 4.5		2.58 ± 0.11	0.078
	CYP2C9agm	248 ± 73		2.64 ± 0.47	0.011
	CYP3A4agm	29.0 ± 3.8	497 ± 122	11.0 ± 0.8	0.379

Units are μM for K_m and K_s, pmol/min/pmol CYP for V_{max}, and μl/min/pmol CYP for V_{max}/K_m, respectively. Data are expressed as mean ± S.E. from three determinations.

exceeded that of 4′-hydroxylation by the counterpart CYP2C9agm (V_{max}/K_m of 0.15 μl/min/pmol CYP). Interestingly, 5-hydroxylation efficiency increased from human, rhesus to African green monkey CYP3A, as opposed to the decrease in 4′-hydroxylation efficiency by CYP2C in the same order. Similarly, all three CYP3A enzymes exhibited 4′-hydroxylation activity at high DF concentrations, but CYP3A4agm appeared to be more selective than human CYP3A4 and rhesus CYP3A64 based on their respective ratios of 5- to 4′-hydroxylation efficiency (2.3, 7.0, and 25 for CYP3A4, CYP3A64, and CYP3A4agm, respectively).

3.3. *Effects of chemical inhibitors selective to human CYP3A4 and CYP2C9*

Up to 100 μM, SLF, a potent, selective human CYP2C9 inhibitor [32,33], failed to inhibit DF 4′-hydroxylation catalyzed by either recombinant monkey CYP2C enzymes (CYP2C75 and CYP2C9agm) or monkey liver microsomes of the three species, but it strongly inhibited this reaction in HLM with the IC_{50} of 0.4 μM (Table 3). In addition, TA, a mechanism-based human CYP2C9 inhibitor [7,34,35], failed to inhibit monkey 4′-hydroxylation in a time-dependent manner at 10 μM. At this concentration, TA significantly reduced human 4′-hydroxylation (Fig. 3). On the contrary, KTZ, a potent, selective human CYP3A4 inhibitor [32], significantly inhibited monkey 4′-hydroxylation with IC_{50} values ≤3.4 μM, although it was more potent towards monkey 5-hydroxylation (IC_{50} <0.1 μM). However, Compound A, a known potent mechanism-based inhibitor selective to human CYP3A4 [27], was unable to inhibit monkey 5-hydroxylation in a time-dependent manner, but appeared to inhibit the reaction in a non-pre-incubation-dependent manner, as indicated by the appreciable activity drop by ~40% before pre-incubation was initiated (Fig. 4). Nevertheless, Compound A showed no impact on monkey 4′-hydroxylation either in co-incubation or pre-incubation experiments (data not shown).

Table 3 – Effects of SLF and KTZ on DF 4′- and 5-hydroxylation in human and monkey liver microsomes and recombinant CYP2Cs and CYP3As

Reaction	Enzyme source	IC_{50} (μM)	
		SLF	KTZ
4′-OHD	HLM	0.43 ± 0.02	>50
	Rh-MLM	>100	3.4 ± 0.5
	Cyno-MLM	>100	2.3 ± 0.4
	AG-MLM	>100	0.42 ± 0.11
	CYP2C9	0.85 ± 0.08	12.9 ± 0.3
	CYP2C75	>100	1.2 ± 0.06
	CYP2C9agm	>100	2.2 ± 0.3
5-OHD	HLM	>100	0.020 ± 0.006
	Rh-MLM	>100	0.017 ± 0.004
	Cyno-MLM	>100	0.040 ± 0.033
	AG-MLM	>100	0.062 ± 0.039
	CYP3A4	>100	0.065 ± 0.023
	CYP3A64	>100	0.063 ± 0.025
	CYP3A4agm	>100	0.082 ± 0.014

DF concentration varied according to the K_m values determined in respective enzyme sources. For 4′-hydroxylase it was 10 μM for HLM and CYP2C9, and 50 μM for Rh-MLM, Cyno-MLM, CYP2C75, and CYP2C9agm. For 5-hydroxylase, it was 250 μM for all preparations. Experiments were conducted with duplicates for each concentration. Data are expressed as IC_{50} ± S.E. from curve fitting.

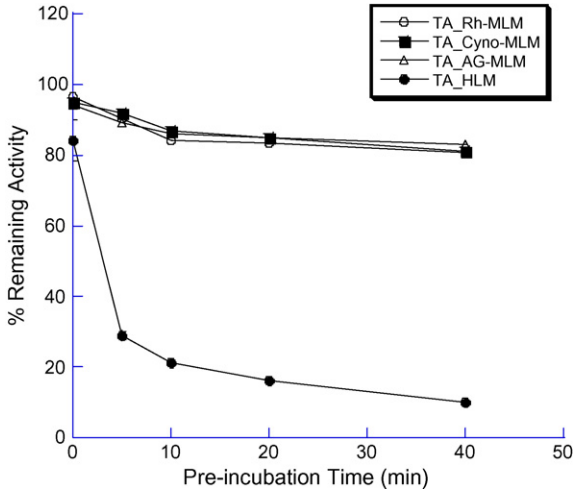

Fig. 3 – Time-dependent inhibition of DF 4′-hydroxlyase in human and monkey liver microsomes by TA. The first incubations contained 10 μM TA and 1 mg/ml microsomal proteins. After incubation for the desired length, aliquots of 25 μl incubate were transferred to 225 μl incubates consisting of 250 μM DF. Solvent control is not shown since the result is similar in all incubations. Each data point is the mean value of two determinations.

3.4. *Inhibition of monkey DF hydroxylation by monoclonal antibodies against human CYP3A and CYP2C*

The effects of monoclonal antibodies selective to human CYP2C (Mab 30-12-2) and CYP3A (Mab 10-1-1) were first evaluated with recombinant CYPs. The maximal inhibition of 4′- and 5-hydroxylation by both antibodies (≥80 and >90% for anti-CYP2C and anti-CYP3A) was achieved with 0.5 μl of mouse ascites containing the respective antibodies for 1 pmol CYP, as illustrated by the results of the titration study using recombinant CYP2C9agm and CYP3A4agm (Fig. 5). Similarly, under these conditions, anti-CYP2C antibodies significantly reduced 4′-hydroxylation by human CYP2C9 and rhesus CYP2C75 (by ≥80%), and anti-CYP3A antibodies strongly inhibited DF 5-hydroxylation by recombinant human CYP3A4 and rhesus CYP3A64 (by >90%). These results demonstrated the potent cross-inhibitory activity of anti-human CYP3A and CYP2C antibodies towards the orthologous rhesus and African green monkey isozymes, which facilitated the subsequent phenotyping of DF metabolism in monkey liver microsomes.

A titration study with liver microsomal preparations revealed the maximal inhibition of 4′-OHD and 5-OHD formation in the presence of 25 μl of antibody-containing ascites for 1 mg of microsomal protein (data not shown). To ensure the maximal inhibition, the amount of antibodies was increased to 50 μl/mg protein in the following studies. Anti-human CYP2C antibodies strongly inhibited 4′-hydroxylation in rhesus and cynomolgus liver microsomes with the maximal

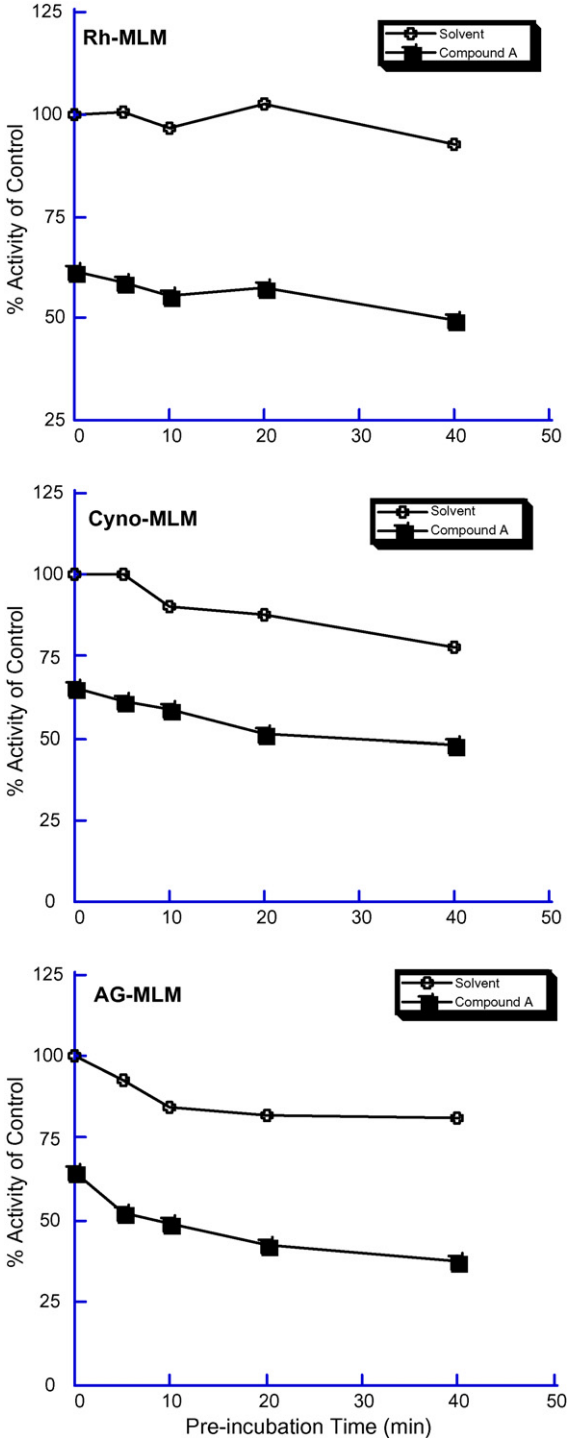

Fig. 4 – Time-dependent inhibition DF 5-hydroxylase in monkey liver microsomes. The experimental conditions are the same as described in Fig. 3.

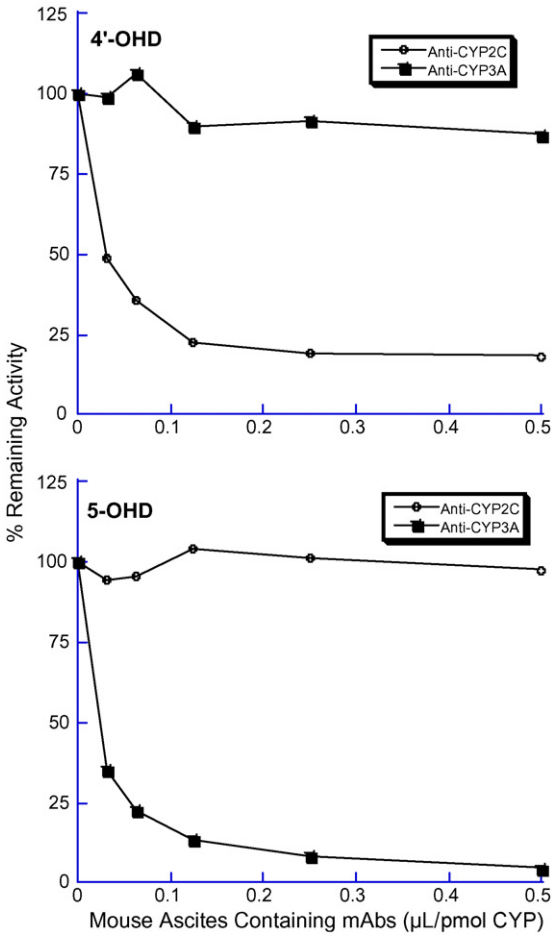

Fig. 5 – Concentration-dependent inhibition by monoclonal antibodies against human CYP2C and CYP3A of 4′-OHD and 5-OHD formation catalyzed by African green monkey CYP2C9agm and 3A4agm, respectively. Each data point is the mean value of two determinations.

inhibition of ≥75% in both species, as illustrated in Fig. 6. Similarly, anti-human CYP3A antibodies significantly reduced 5-hydroxylation in these two monkey species (by ~75%). However, anti-CYP3A antibodies only partially inhibited human 5-hydroxylation (by ~55%), while human CYP2Cs appeared to contribute to this reaction. This result may be explained by the fact that, apart from CYP3A4, other CYP2C members are also capable of catalyzing 5-OHD formation [36].

The results obtained from the pooled AG-MLM were intriguing (Fig. 6). While anti-CYP3A antibodies strongly reduced 5-hydroxylation (by >80%), anti-CYP2C antibodies only gave rise to ~50% inhibition of 4′-hydroxylation. Surprisingly, additional inhibition was attained when anti-human CYP3A antibodies were included in the incubation, leading to a total inhibition of ~80% of 4′-hydroxylation (Fig. 6). This finding elicited further investigation of the relative contribution of CYP2C and CYP3A in DF hydroxylation in nine individual AG-MLM preparations. It was found that contributions of CYP2C and CYP3A enzymes to 4′-hydroxylation varied considerably in these nine subjects (Fig. 7). For instance, CYP2C enzymes appeared to be the primary contributors to this reaction in subjects AGM-003 and AGM-119, while they failed to show appreciable impact in subject AGM-001, where this reaction appeared to be principally mediated by CYP3A enzymes. On the other hand, the majority of 5-hydroxylation activity was inhibited (≥80%) by the anti-human CYP3A antibodies, while the anti-human CYP2C antibody did not lead to further inhibition (Fig. 7).

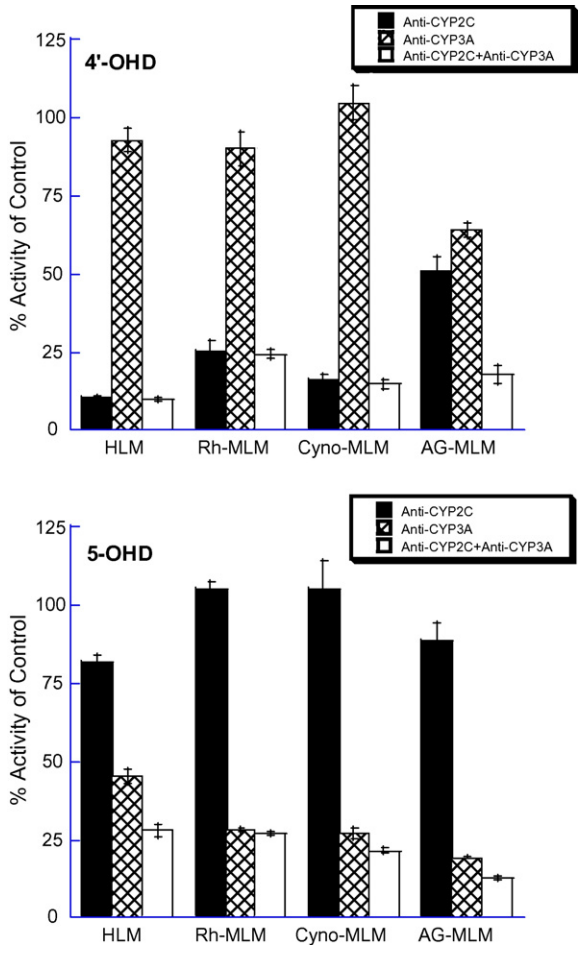

Fig. 6 – Inhibition of DF 4′- and 5-hydroxylase activities in human and monkey liver microsomes by monoclonal antibodies against human CYP2C and CYP3A. The total ascites volume was 50 μl for per mg microsomal protein. Data are expressed as mean ± S.D. from three determinations.

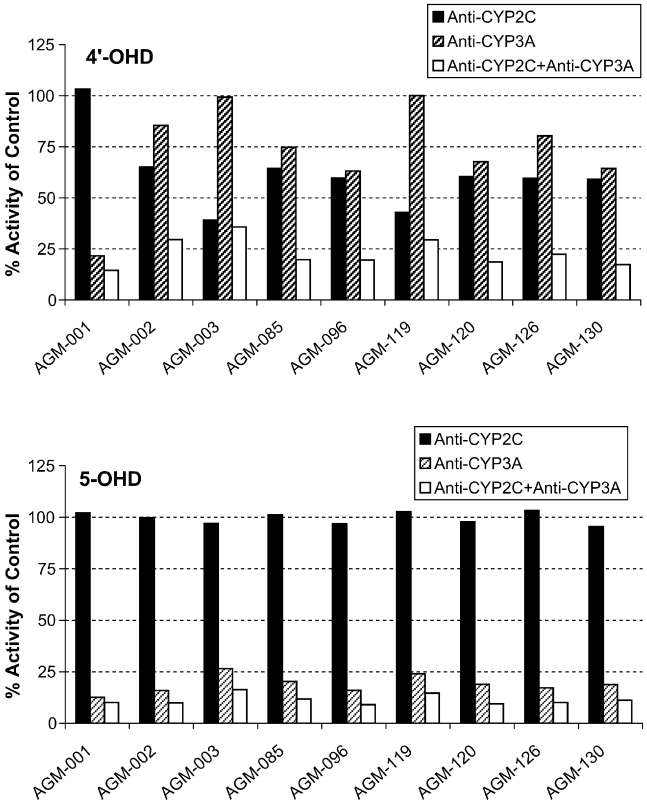

Fig. 7 – Inhibition of DF 4′- and 5-hydroxylase activities in nine AG-MLM preparations by monoclonal antibodies against human CYP2C and CYP3A. The total ascites volume was 50 μl for per mg microsomal protein. Data are expressed as mean values from two determinations.

4. Discussion

The present study demonstrated that monkeys differed functionally from humans in DF hydroxylation, as reflected in catalytic efficiency, regioselectivity, and responses to chemical inhibitors. Relative to humans, the overall catalytic efficiency (V_{max}/K_m) of DF oxidation in monkey liver microsomes of all three species was lower in catalyzing DF oxidation (>10-fold). While 4′-hydroxylation was similarly favored over 5-hydroxylation in rhesus and cynomolgus monkeys, the relative level of 5-hydroxylation was considerably higher than in humans. Interestingly, a reversed regioselectivity was observed in African green monkeys. Moreover, the lack of response of 4′-hydroxylation in monkeys to SLF and TA contradicted the effects of these potent inhibitors towards the same reaction in humans, for which CYP2C9 is responsible.

The reduced catalytic efficiency of DF oxidation in monkeys, as opposed to in humans, was mainly attributed to the decreased 4′-hydroxylation. This reaction proceeded in monkeys with lower affinity and lower capacity. Studies with monoclonal antibodies against human CYP2Cs strongly suggested that this reaction was primarily catalyzed by monkey CYP2C orthologs. Efforts were then devoted to clone and express rhesus CYP2C75 and African green monkey CYP2C9agm, which bear high amino acid (94 and 92%) and nucleotide (96 and 95%) homology to human CYP2C9. As expected, these two enzymes are effective in catalyzing DF 4′-hydroxylation but have significantly lower affinities and capacities than those of human CYP2C9, which is consistent with the observations of the aforementioned liver microsomal preparations. On the basis of the almost identical amino acid sequence between rhesus and cynomolgus monkey CYP2C75 (GenBank accession no. DQ074805), coupled with very comparable kinetic profiles of DF-4′-hydroxylation in Rh-MLM and Cyno-MLM, it is conceivable that rhesus and cynomolgus CYP2C75s should be identical in function as well. Thus, monkey CYP2C orthologs from all three species are less efficient than human CYP2C9 in catalyzing DF 4′-hydroxylation, resulting in the decreased overall oxidation of DF in monkey liver microsomes.

This reduced 4′-hydroxylation, combined with enhanced 5-hydroxylation, explains the lower or reversed regioselectivity in MLM relative to the case in HLM. DF oxidation at position 5 was negligible in HLM, but it became more significant in Rh-MLM and Cyno-MLM primarily due to the increased V_{max} (Table 1). It even exceeded 4-hydroxylation in AG-MLM. These

changes in regioselectivity cannot be attributed to an altered substrate selectivity of the enzyme since monkey CYP2C and 3A enzymes are still the respective major contributors to 4′- and 5-hydroxylation in monkeys, based on studies with selective antibodies and respective recombinant monkey CYPs (Fig. 6; Table 2). Along with the reduced efficiency of monkey CYP2C orthologs for DF 4′-hydroxylation, CYP3A64 and, in particular, CYP3A4agm catalyzed DF 5-hydroxylation more efficiently than did human CYP3A4. As a result, the relative level of 5-OHD significantly increased in monkeys.

Two unexpected results were observed with AG-MLM. One is the apparent involvement of CYP3A, in addition to CYP2C, in the formation of 4′-OHD as suggested by the immunoinhibition result (Figs. 6 and 7). In light of DF 4′-hydroxylase activity (in terms of V_{max}/K_m) of CYP3A4agm, which accounted for 13% of that of CYP2C9agm (Table 2), it is possible that CYP3A4agm generates 4′-OHD at an appreciable level if CYP3A4agm is more abundant than CYP2C9agm in AG-MLM, as in the case of HLM. This scenario would not occur in HLM or Rh-MLM because the 4′-hydroxylase activity derived from CYP3A4 or CYP3A64 is too small to make any significant contribution in comparison with their CYP2C counterparts (Table 2), albeit these CYP3A enzymes might be present in liver microsomes in much excess over the respective CYP2Cs. Another possibility is that other CYP3A isoforms in AG-MLM are capable of mediating DF 4′-hydroxylation. Supporting this notion is a recent finding that human CYP3A5 converted DF to both 4′-OHD and 5-OHD at a comparable level (data not shown). While the definite mechanism needs further investigation, the involvement of both CYP2C and 3A in 4′-hydroxylation may limit DF as a CYP2C probe substrate for studies in African green monkeys.

Another unexpected finding is the highly variable contributions of CYP2C and 3A to 4′-OHD formation in individual liver microsomal preparations from nine African green monkeys (Fig. 7). For instance, the vast majority of 4′-hydroxylase activity in subject 001 was from CYP3A enzymes which, however, did not contribute to this activity in subject 003 and 019. Furthermore, these two opposite cases coincided with the highest and lowest values of 5-OHD/4′-OHD ratios in terms of V_{max}/K_m (7.2 and 1.4). It appeared that CYP2C enzymes in subject 001 were not operative as DF 4′-hydroxylase, and the same can be postulated about CYP3A enzymes in subjects 003 and 019. It is not clear at the present time what caused this phenotypic variability. Additional studies are in progress to quantify CYP2C9agm and CYP3A4agm in those preparations. Nevertheless, this finding indicates that African green monkeys, like humans, could have variable CYP levels due to either genetic or environmental factors.

An additional respect in which monkeys and humans differed in DF hydroxylation was the response to selective chemical inhibitors. With either monkey liver microsomes or recombinant monkey CYP2C enzymes, SLF failed to inhibit DF 4′-hydroxylase even at a concentration up to 100 μM. Similarly, TA lost its potency to inactivate DF 4′-hydroxylase. It has been established that TA is metabolically activated by CYP2C9 to form a highly electrophilic intermediate (thiophene sulfoxide), which either reacts with water to form 5-hydoxy TA as a major stable end-product or covalently binds to a critical residue in the active side to inactivate the enzyme [37]. TA underwent the same pathway in monkeys as suggested by the formation of 5-hydroxy TA in liver microsomes of the three monkey species as well as by the recombinant CYP2C75 and CYP2C9agm (data not shown). Apparently, its failure to inactivate the enzyme cannot be accounted for by an altered metabolism. On the contrary, monkey DF 4′-hydroxylation became more susceptible to KTZ, a selective inhibitor towards human CYP3A4 (Table 3). The IC_{50} values decreased more than 15- and 5-fold with liver microsomes and recombinant CYP2C enzymes, respectively, relative to the values for humans. Hence, despite the comparable inhibitory potency for both human and monkey DF 5-hydroxylation, it would be difficult for KTZ to function as a selective CYP3A inhibitor in monkeys. In addition, Compound A, a mechanism-based human CYP3A4 inhibitor, did not inhibit monkey DF 5-hydroxylation in a pre-incubation-dependent fashion, although it appeared to competitively inhibit the reaction. This finding surprisingly contradicted a recent report that Compound A operated as a time-dependent inhibitor towards midazolam 1′-hydroxylation mediated by rhesus CYP3A64 [23]. Whether this discrepancy originated from substrate dependency would be an interesting subject for future studies. It appears that application of chemical inhibitors for drug interactions associated with monkey CYP2C enzymes is unfortunately limited. In fact, chemical inhibitors selective to human CYP isozymes do not always work for animals due to the lack of potency or the lack of selectivity [38–41]. Unlike a variety of potent human CYP3A4 inhibitors, SLF appeared to be the only one for human CYP2C9 until the recent emergence of benzbromarone and derivatives as a new class with inhibitory potency greatly exceeding SLF [42]. Endeavors to characterize their selectivity and to explore their utility in other species would facilitate research in CYP2C enzyme-involved drug interactions within and beyond humans.

The molecular mechanisms whereby DF and SLF bind in human CYP2C9 active site and TA inactivates the enzyme have been extensively investigated. It is believed that at least two major interactions are involved: an ionic interaction via DF COO^- or SLF SO_2N^- anionic site with cationic residues, and pi-stacking via DF or SLF aromatic rings with the aromatic region of the enzyme active site. Site-directed mutagenesis studies have revealed the central roles of Phe467 and Phe114 in the efficiency and regioselectivity of DF hydroxylation as well as SLF inhibition, and confirmed Ser365 as the target nucleophile adducted by the electrophilic intermediate of TA [7]. It seems that the structural features determined by these critical amino acid residues have changed in orthologous monkey CYP2C enzyme active sites, likely resulting in different interactions with DF and inhibitors.

This detailed characterization of DF hydroxylation in monkeys revealed the functional differences between monkey CYP2C orthologs and human CYP2C9. In general, DF could still serve as a good in vitro CYP2C probe substrate for rhesus and cynomolgus monkeys as well as for humans, but may be limited in African green monkeys. In contrast, the increased 5-hydroxylase may be advantageous for monitoring CYP3A activity in monkeys. However, selective chemical inhibitors for phenotyping studies are not available so far. It appears that studies on drug interactions in monkeys would heavily rely on antibodies with potent cross-reactivity and recombinant

monkey CYPs. Undoubtedly, advances in these areas would facilitate identification of good probes for phenotyping and drug interactions in monkeys.

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