

# Ligand Diversity of Human and Chimpanzee CYP3A4: Activation of Human CYP3A4 by Lithocholic Acid Results from Positive Selection<sup>S</sup>

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## ABSTRACT:

For currently unknown reasons, the evolution of CYP3A4 underwent acceleration in the human lineage after the split from chimpanzee. We investigated the significance of this event by comparing *Escherichia coli*-expressed CYP3A4 from humans, chimpanzee, and their most recent common ancestor. The expression level of chimpanzee CYP3A4 was ~50% of the human CYP3A4, whereas ancestral CYP3A4 did not express in *E. coli*. Steady-state kinetic analysis with 7-benzoyloxyquinoline, 7-benzoyloxy-4-(trifluoromethyl)coumarin (7-BFC), and testosterone showed no significant differences between human and chimpanzee CYP3A4. Upon addition of  $\alpha$ -naphthoflavone (25  $\mu$ M), human CYP3A4 showed a slightly decreased substrate concentration at which 50% of the maximal rate  $V_{max}$  is reached for 7-BFC, whereas chimpanzee CYP3A4 showed a >2-fold increase. No significant differences in inhibition/activation were found for a panel of 43

drugs and endogenous compounds, suggesting that the wide substrate spectrum of human CYP3A4 precedes the human-chimpanzee split. A striking exception was the hepatotoxic secondary bile acid lithocholic acid, which at saturation caused a 5-fold increase in 7-BFC debenzoylation by human CYP3A4 but not by chimpanzee CYP3A4. Mutagenesis of human CYP3A4 revealed that at least four of the six amino acids positively selected in the human lineage contribute to the activating effect of lithocholic acid. In summary, the wide functional conservation between chimpanzee and human CYP3A4 raises the prospect that phylogenetically more distant primate species such as rhesus and squirrel monkey represent suitable models of the human counterpart. Positive selection on the human CYP3A4 may have been triggered by an increased load of dietary steroids, which led to a novel defense mechanism against cholestasis.

CYP3A4 is considered the most important drug-metabolizing enzyme in humans because of its abundant yet variable expression combined with an unusually wide substrate spectrum. CYP3A4 accounts, on average, for 30% of the total cytochrome P450 (P450) expression in liver and up to 80% in the small intestine (Shimada et al., 1994; Paine et al., 2006). The individual expression of CYP3A4 varies up to 90-fold (Lamba et al., 2002), which is, in part, determined by still unknown gene variants in the enzyme and its transcriptional regulators (Ozdemir et al., 2000). The inherited variability in CYP3A4

expression may be further enhanced by the transcriptional induction of the *CYP3A4* gene and by the inhibition of its protein product by many drugs and dietary constituents (Wilkinson, 2005).

The CYP3A4 substrate spectrum in humans is much wider and the catalytic activity frequently higher than the "minor" CYP3A isoforms CYP3A5 and CYP3A7 (Daly, 2006). CYP3A4 metabolizes an estimated 50% of the currently used drugs (Wilkinson, 2005). The enzyme is capable of accommodating large molecules such as cyclosporine and bromocriptine and exhibits non-Michaelis-Menten kinetics toward some substrates (Atkins, 2005). These characteristics are thought to be because of either multiple ligand-binding sites (Kenworthy et al., 2001; He et al., 2003) or multiple functionally distinct conformers (Davydov et al., 2003; Tsalkova et al., 2007; Fernando et al., 2008), or both. One of the consequences of CYP3A4 ligand promiscuity is its prominent involvement in clinically relevant drug-drug interactions (Wilkinson, 2005).

Using genomic sequences from 16 species, we recently investigated

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**ABBREVIATIONS:** P450, cytochrome P450; 7-BQ, 7-benzoyloxyquinoline; 7-BFC, 7-benzoyloxy-4-(trifluoromethyl)coumarin; ANF,  $\alpha$ -naphthoflavone; PCR, polymerase chain reaction;  $S_{50}$ , substrate concentration at which 50% of the maximal rate  $V_{max}$  is reached; LCA, lithocholic acid.

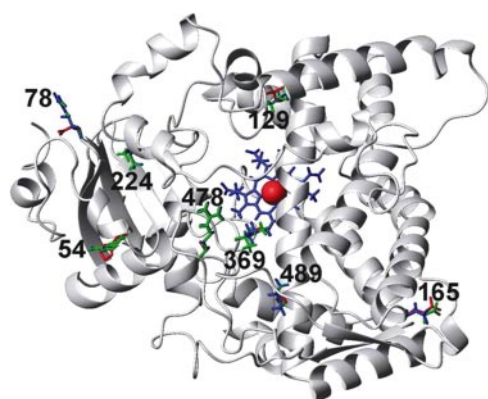


FIG. 1. Amino acid differences among the human (red), chimpanzee (blue), and ancestral (green) CYP3A4. The substrate-free human CYP3A4 structure (pdb code 1tqn; Yano et al., 2004) was used to model the other two proteins by energy minimization using AMBER. The root mean square deviation among the three models for the backbone was 0.3 Å. For clarity, only the backbone of the chimpanzee model is shown in gray ribbon representation. The heme moiety is shown in blue, and the iron moiety is shown in a red sphere.

the evolution of *Cyp3* genomic loci over a period of 450 million years (Qiu et al., 2008). We detected two noteworthy recent episodes of particularly strong positive selection acting on primate CYP3A protein coding sequence. CYP3A7 underwent changes early in the hominoid evolution, which was accompanied by a restriction of its hepatic expression to the fetal period. CYP3A4 acquired changes to six amino acids after the split of the chimpanzee and human lineages approximately 6 million years ago (Fig. 1; Supplemental Table 1). These findings suggested the acquisition of new, presently unknown catalytic functions of CYP3A7 and CYP3A4 especially important for the evolution of hominoids and humans, respectively.

In the present work, we investigated the cumulative effect of the human-specific changes to the CYP3A4 protein sequence after the human-chimpanzee split. We hypothesized that these changes may have widened the substrate spectrum of the human CYP3A4 isozyme. Alternatively, the wide substrate spectrum may have preceded the human-chimpanzee split. In this latter case, the acceleration of the CYP3A4 evolution in the human lineage would most likely reflect the adaptation to a change in the physiology or environment of our direct ancestors. To differentiate between these hypotheses, we inferred using a phylogenetic approach the sequence of CYP3A4 in the most recent common ancestor of humans and chimpanzees. We then set out to express all three enzymes in *Escherichia coli*, to compare their activities, including the inhibition and/or activation by a panel of exo- and endogenous CYP3A4 substrates. The uncovered differences in enzyme activities were further characterized using two chimpanzee and six human CYP3A4 mutants.

#### Materials and Methods

**Materials.** 7-Benzyloxyquinoline (7-BQ), 7-hydroxyquinoline, 7-benzyloxy-4-(trifluoromethyl)coumarin (7-BFC), and 7-hydroxy-4-(trifluoromethyl)coumarin were purchased from Invitrogen (Carlsbad, CA). NADPH,  $\alpha$ -naphthoflavone (ANF), and all the drugs and steroids were bought from Sigma-Aldrich (St. Louis, MO). Nickel-nitrilotriacetic acid affinity resin was purchased from QIAGEN (Valencia, CA). All other chemicals were of the highest grade available and were obtained from standard commercial sources. Recombinant NADPH-P450 reductase and cytochrome  $b_5$  from rat liver were prepared as described previously (Harlow and Halpert, 1997).

**Plasmid Construction.** Human CYP3A4 expression vector (pSE3A4HisTag) was double-digested with the restriction enzymes NcoI and KpnI (New England Biolabs, Frankfurt, Germany) at 37°C for 4 h followed by 5-min incubation at 90°C for enzyme inactivation. The digestion mixture was run on an agarose gel, and the vector band (5–6 kb) was extracted using gel

extraction kit (peQlab, Erlangen, Germany). cDNA derived from chimpanzee total liver RNA was from a previous study (Qiu et al., 2008). The CYP3A4 protein coding region was first amplified using a high-fidelity *Taq* polymerase (Fermentas, St. Leon-Rot, Germany) with primers 3A4e1F (5'-AAAGAGCAACACAGAGCTG-3') and 3A4e13R (5'-GTCCTTAGGAAAATTCAGG-3'). The products were diluted and used as templates for a second round of polymerase chain reaction (PCR) to amplify the coding region (codons 19–503). Modifications at the two ends of the open reading frame (i.e., truncation at the amino terminus and inclusion of four His-tags at the C terminus) were introduced by PCR reaction via the primers (3A4-F-NcoI, 5'-GGGGGCCATGGCTCTGTTATTAGCAGTTTTCTGGTGCT CCTCTATCTATATGGAAC-3' and 3A4-R-KpnI, 5'-CGGCGGGGTACCTCAGTGATGGT GATGGCTCCACTTACGGTGCC-3'). PCR products were digested with NcoI and KpnI and ligated with digested plasmid DNA at room temperature for 4 h using T4 ligase (New England Biolabs). The resulting expression construct containing the chimpanzee *CYP3A4* coding region was designated as pSE-pt-3A4-HisTag.

**Site-Directed Mutagenesis.** Since the human/chimpanzee divergence, six and two nonsynonymous mutations have accumulated in the human and chimpanzee CYP3A4, respectively (Fig. 1; Supplemental Table 1). The sequence of their most recent common ancestor was reconstructed as described previously (Qiu et al., 2008), i.e., using baseml included in the PAML package 3.15. As in-files, we used 1) an alignment made up of *Cyp3A4* orthologs of *Macaca mulatta*, *Papio anubis*, *Callithrix jacchus*, *Pongo pygmaeus*, *Homo sapiens*, and *Pan troglodytes*; and 2) an unrooted tree reflecting the commonly accepted phylogeny among these species. The calculations were conducted using the HKY85 model. Ambiguous codon positions were removed from the data set.

Chimpanzee CYP3A4 was used as a starting template to restore the CYP3A4 sequence predating the chimpanzee/human split, because only two artificial mutations (C495g and G1105a) were required. Two sequential rounds of site-directed mutagenesis were conducted on the above-described pSE-pt-3A4-HisTag plasmid using a QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Besides the putative ancestral CYP3A4, we also obtained plasmids separately expressing each of the two amino acid residues that differ from the chimpanzee enzyme (D165E and V369I). Site-directed mutagenesis was also used to revert amino acids 54, 78, 129, 224, 478, and 489 of human CYP3A4 into those present in the ancestral CYP3A4. Sense sequences of the oligonucleotides used in mutagenesis are given in Supplemental Table 2.

**Expression and Purification of CYP3A4.** CYP3A4 and mutants were expressed as His-tagged proteins in *E. coli* TOPP3 and purified using a nickel-affinity column as described previously (Domanski et al., 2001). Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA). The specific contents of the human and chimpanzee CYP3A4 were 14 and 8 nmol P450/mg protein, respectively.

**Characterizations of CYP3A4.** The standard NADPH-dependent assays with 7-BQ, 7-BFC, and testosterone were essentially carried out as described previously (Kumar et al., 2006). Enzyme inhibition or activation was measured using the 7-BFC *O*-deethylation assay in a final reaction volume of 100  $\mu$ l at the substrate concentration of 20  $\mu$ M as described previously (Domanski et al., 2001; Oezguen et al., 2008). Steady-state kinetic parameters were determined by regression analysis using SigmaPlot (SPSS, Inc., Chicago, IL). The  $S_{50}$  and  $n$  values were determined using the Hill equation. Each kinetic experiment included human CYP3A4 for more accurate comparison of the data. Thermal inactivation of P450 was carried out as described previously (Kumar et al., 2007). All data treatment and fitting of the titration curves for thermal inactivation were performed with our SpectraLab software as described previously (Davydov et al., 2003).

## Results

**P450 Expression and Stability.** The heterologous expression of chimpanzee CYP3A4 was ~50% of the human CYP3A4 (57 versus 120 nmol P450/l culture). Upon purification, chimpanzee CYP3A4 showed a higher amount of P420 (19 versus 4%) than human CYP3A4, suggesting lower protein stability. However, when tested for thermal stability (Supplemental Fig. 1), human and chimpanzee

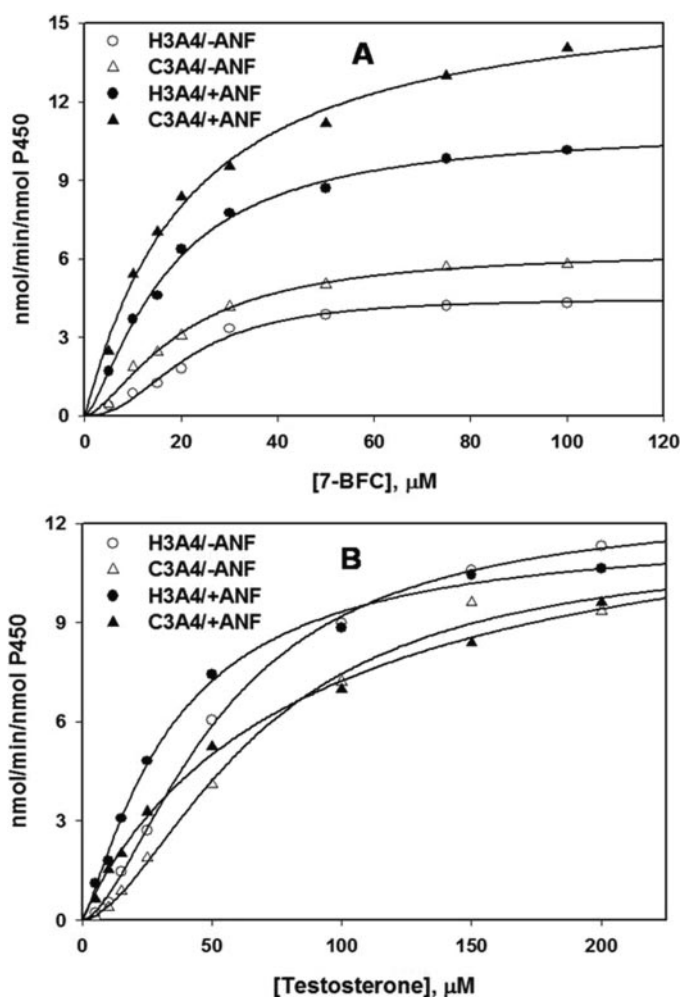


FIG. 2. Steady-state kinetic analysis of 7-BFC debenzoylation (A) and testosterone 6 $\beta$ -hydroxylation (B) using a standard reconstituted system in the absence and presence of ANF (25  $\mu$ M). The experiments were carried out as described under *Materials and Methods*. The kinetic parameters ( $k_{cat}$ ,  $S_{50}$ , and  $n$  values) were determined by fitting the curve to the Hill equation. H3A4, human CYP3A4; C3A4, chimpanzee CYP3A4.

CYP3A4 showed indistinguishable melting temperature values of  $53.8 \pm 0.64$  and  $54.4 \pm 0.67^\circ\text{C}$ , respectively. Ancestral CYP3A4 showed negligible heterologous P450 expression in *E. coli*. Because there are only two amino acid differences, E165D and I369V, between ancestral and chimpanzee CYP3A4 (Fig. 1; Supplemental Table 1), we introduced separately these two mutants into the chimpanzee enzyme. Both V369I (17 nmol P450/l culture) and D165E (6 nmol P450/l culture) showed greatly decreased expression.

**Steady-State Kinetic Studies Using Model Substrates and Activation by ANF.** Steady-state kinetics of human and chimpanzee CYP3A4 oxidation of the model substrates 7-BQ, 7-BFC, and testosterone were examined in the absence and presence of ANF. The results are presented in Fig. 2 and Table 1. Chimpanzee and human CYP3A4 showed no significant difference in  $k_{cat}$  with 7-BQ, 7-BFC, or testosterone. The only significant difference was that the chimpanzee enzyme showed >50% lower  $S_{50}$  with 7-BFC than the human. In addition, there was a slight difference in the Hill coefficient with 7-BFC and testosterone (Table 1). Similar to human CYP3A4, the chimpanzee enzyme also showed heterotropic activation by ANF, which is characterized by an increase in  $k_{cat}$  value and/or decrease in  $n$  value. The most striking difference was that the human CYP3A4 showed a slightly decreased  $S_{50}$  in the presence of ANF for 7-BFC

debenzoylation and testosterone hydroxylation, whereas chimpanzee CYP3A4 showed an increase in  $S_{50}$  in the presence of ANF (Table 1).

**Effects of Drugs and Steroids on Debenzoylation of 7-BFC by Human CYP3A4 and Chimpanzee CYP3A4.** To explore further whether a variety of xenobiotics and endogenous compounds that are known substrates, inhibitors, or activators of human CYP3A4 show different characteristics with chimpanzee CYP3A4, we performed activation/inhibition studies with both the enzymes using 43 compounds. The study was carried out using 7-BFC as substrate at 20  $\mu$ M and appropriate inhibitor/activator concentrations. The results are presented in Table 2. As expected, a majority of ligands either inhibit or activate the metabolism of 7-BFC by human CYP3A4. In most cases, no significant differences with regard to inhibition or activation were found between human and chimpanzee CYP3A4. The most striking difference was that LCA strongly activated human CYP3A4 (3-fold), whereas no activation was observed with chimpanzee CYP3A4 (Table 2). In contrast, the primary bile acids cholic and chenodeoxycholic acid showed comparable ( $\leq 2$ -fold) activation of either P450 (Table 2). Cholic and chenodeoxycholic acid are metabolized by human hepatic P450, especially by CYP3A4 (Deo and Bandiera, 2008). Furthermore, we determined the concentration dependence of the activation by LCA. The results are presented in Fig. 3. The LCA binding constant ( $K_E$ ) was  $216 \pm 20$  for human CYP3A4 and  $105 \pm 25$  for chimpanzee CYP3A4. The  $V_{max}$  for human CYP3A4 was >5-fold higher than the  $V_0$  ( $4.94 \pm 0.53$  versus  $27.3 \pm 0.88 \text{ min}^{-1}$ ). Chimpanzee CYP3A4 showed no significant activation ( $5.71 \pm 0.20$  versus  $6.70 \pm 0.49 \text{ min}^{-1}$ ).

**Activation of the Human CYP3A4 Mutants by LCA.** To identify the molecular determinants of the activation of human CYP3A4 by LCA, we reverted individually the six amino acid mutations acquired by human CYP3A4 into the respective ancestral ones. The P450 expression of these CYP3A4 mutants was similar to the wild-type enzyme, except for V489I (Supplemental Table 3). Because of low expression and poor binding to the affinity column, V489I could not be purified in a sufficient amount to assess enzyme activity. The activity of the remaining five mutants toward 7-BFC was similar to the activity of wild-type human and chimpanzee CYP3A4 (Table 3). Four mutants (H54R, Q78R, L129I, and T224I) showed a significant decrease in the activation of CYP3A4 activity by LCA compared with wild-type human CYP3A4. It is noteworthy that none of the mutations abolished the activity by LCA. S478R showed no significant decrease in the activation by LCA compared with wild-type human CYP3A4 (Table 3). Overall, the results suggest that most of the amino acid acquired by human CYP3A4 contribute to the activation by LCA.

## Discussion

The purpose of this work was to clarify the reasons for and effects of the unusually rapid changes to the protein sequence of human CYP3A4 after the human-chimpanzee split. Contrary to our original hypothesis, these changes are unlikely to be responsible for the wide substrate spectrum of human CYP3A4, which thus seems to have preceded the human-chimpanzee split. Instead, the amino acid substitutions may have been triggered by an increased load of dietary steroids, leading to a novel defense mechanism against cholestasis.

The above-mentioned conclusions can be reached despite our inability to express the ancestral CYP3A4 made by introducing Asp165 $\rightarrow$ Glu and Val369 $\rightarrow$ Ile mutations into chimpanzee CYP3A4. This failure may reflect the limitations of the heterologous P450 expression in *E. coli*, because of diminished protein stability, decreased level of heme incorporation, and/or increased level of P420 formation (Kumar et al., 2007). Alternatively, the prediction of the ancestral CYP3A4 sequence may have been erroneous, resulting in

TABLE 1

Steady-state kinetic analysis of the oxidation of model substrates by human and chimpanzee CYP3A4 in the absence and presence of 25  $\mu\text{M}$  ANFThe variation between two experiments is  $\leq 20\%$ . The experiments were carried out simultaneously for more accurate comparison between human (Hum) and chimpanzee (Chimp) CYP3A4 (+ or - ANF).

Sample	$k_{\text{cat}}$	$S_{50}$	$n$
	$\text{min}^{-1}$	$\mu\text{M}$	
7-BFC			
Hum 3A4/-ANF	4.48 $\pm$ 0.23 <sup>a</sup>	21.6 $\pm$ 1.8	2.2 $\pm$ 0.4
Chimp 3A4/-ANF	6.31 $\pm$ 0.27	9.90 $\pm$ 1.6	1.6 $\pm$ 0.1
Hum 3A4/+ANF	11.0 $\pm$ 0.46	17.0 $\pm$ 1.4	
Chimp 3A4/+ANF	16.3 $\pm$ 1.4	20.7 $\pm$ 4.0	1.0 $\pm$ 0.1
Testosterone			
Hum 3A4/-ANF	12.5 $\pm$ 0.4	54.1 $\pm$ 2.7	1.6 $\pm$ 0.1
Chimp 3A4/-ANF	11.3 $\pm$ 0.9	67.7 $\pm$ 10	1.7 $\pm$ 0.2
Hum 3A4/+ANF	11.8 $\pm$ 0.6	34.6 $\pm$ 4.2	1.2 $\pm$ 0.1
Chimp 3A4/+ANF	13.8 $\pm$ 1.7	90.9 $\pm$ 17	1.0 $\pm$ 0.1
7-BQ			
Hum 3A4	33.8 $\pm$ 8.4	152 $\pm$ 48	1.6 $\pm$ 0.3
Chimp 3A4	25.4 $\pm$ 3.0	127 $\pm$ 12	2.2 $\pm$ 0.4

<sup>a</sup> Standard errors for fit to the Hill equation.

TABLE 2

Effect of ligands on 7-BFC oxidation by human and chimpanzee CYP3A4

The 100% activities are 2.84  $\pm$  0.18 and 2.56  $\pm$  0.27 nmol/min/nmol P450 for the human and chimpanzee CYP3A4, respectively, for the debenylation of 7-BFC at 20  $\mu\text{M}$ . The data are the mean  $\pm$  S.D. of three independent experiments.

Ligand	Conc.	Activity	
		Human	Chimpanzee
	$\mu\text{M}$		%
None	0	100 $\pm$ 0	100 $\pm$ 0
Amiodarone HCl	100	61.1 $\pm$ 4.1	38.2 $\pm$ 3.1
Androstenedione	100	281 $\pm$ 72	320 $\pm$ 14
Benzphetamine	100	76.7 $\pm$ 6.3	57.1 $\pm$ 6.7
Bromocriptine	1	81.3 $\pm$ 11	56.8 $\pm$ 12
Chenodeoxycholic acid	100	200 $\pm$ 28	191 $\pm$ 23
Chloramphenicol	100	90.0 $\pm$ 26	90.6 $\pm$ 5.0
Cholesterol	100	110 $\pm$ 11	105 $\pm$ 5.6
Cholic acid	100	187 $\pm$ 11	150 $\pm$ 10
Cisapride monohydrate	0.1	70.4 $\pm$ 3.2	65.2 $\pm$ 5.8
Clotrimazole	0.01	73.8 $\pm$ 9.0	49.2 $\pm$ 2.3
Cyclosporin A	10	258 $\pm$ 9.3	312 $\pm$ 14.5
Cimetidine	100	97.7 $\pm$ 6.9	73.3 $\pm$ 5.7
Dexamethasone	100	107 $\pm$ 3.8	91.7 $\pm$ 10
Dehydroepiandrosterone	100	153 $\pm$ 2.8	166 $\pm$ 17
Diazepam	10	81.6 $\pm$ 17	84.2 $\pm$ 6.5
Diltiazem	100	80.4 $\pm$ 32	65.3 $\pm$ 22
Enalapril	10	75.6 $\pm$ 5.1	64.9 $\pm$ 8.7
Erythromycin	100	38.6 $\pm$ 15	66.2 $\pm$ 35
Estrone	100	84.0 $\pm$ 9.0	74.7 $\pm$ 13
Ethinylestradiol	100	45.2 $\pm$ 15	35.0 $\pm$ 22
Flunitrazepam	100	141 $\pm$ 5.0	155 $\pm$ 17
Hexobarbital	100	44.3 $\pm$ 7.4	53.3 $\pm$ 12
Ketoconazole	0.1	65.0 $\pm$ 1.5	52.4 $\pm$ 8.4
Lithocholic acid	100	304 $\pm$ 38	106 $\pm$ 5.3
Metronidazole	10	97.6 $\pm$ 3.8	69.1 $\pm$ 5.0
Miconazole	100	26.8 $\pm$ 2.9	9.89 $\pm$ 1.8
Mifepristone	100	57.2 $\pm$ 24	44.6 $\pm$ 16
Midazolam	10	90.9 $\pm$ 8.5	105 $\pm$ 11
Nifedipine	10	70.3 $\pm$ 4.0	59.4 $\pm$ 5.5
Omeprazole	100	75.5 $\pm$ 7.0	50.9 $\pm$ 4.2
Progesterone	100	196 $\pm$ 29	199 $\pm$ 28
Quinidine	10	80.9 $\pm$ 23	63.2 $\pm$ 20
Raloxifene	1	54.7 $\pm$ 31	63.8 $\pm$ 17
Secobarbital	100	70.4 $\pm$ 1.8	89.1 $\pm$ 5.0
Sertraline	10	106 $\pm$ 19	101 $\pm$ 14
Temazepam	100	145 $\pm$ 46	149 $\pm$ 52
Terfenadine	10	140 $\pm$ 20	127 $\pm$ 25
Testosterone	100	99.8 $\pm$ 15	98.0 $\pm$ 7.2
Ticlopidine	1	125 $\pm$ 41	104 $\pm$ 35
Triazolam	100	79.8 $\pm$ 4.8	79.8 $\pm$ 14
Troleandomycin	10	86.7 $\pm$ 7.8	76.2 $\pm$ 10
Tamoxifen	10	92.6 $\pm$ 11	112 $\pm$ 7.0
Verapamil	100	84.8 $\pm$ 11	77.1 $\pm$ 9.6

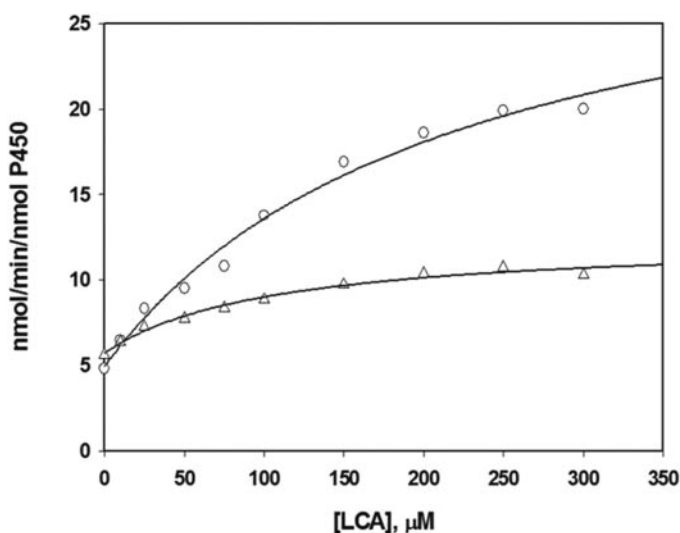


FIG. 3. Effect of LCA on 7-BFC debenzylation by human CYP3A4 (circles) and chimpanzee CYP3A4 (triangles). Varying amounts of LCA were included in assays containing 50  $\mu\text{M}$  7-BFC. The equation  $V = V_0 + V_{\text{max}}[E](K_E + [E])^{-1}$  was used to analyze the data, where  $V$ ,  $V_0$ , and  $V_{\text{max}}$  represent the oxidation rate at effector [E] concentration, the oxidation rate in the absence of effector, and the maximum rate, respectively.

the inclusion of an amino acid incompatible with expression. Because this latter possibility is extremely unlikely considering the small number of differing amino acids, we investigated whether Asp165→Glu or Val369→Ile substitutions disrupted salt bridges and/or hydrogen bonds. To this end, we created models of chimpanzee and ancestral CYP3A4 based on the crystal structure of human CYP3A4 as shown in Fig. 1. Detailed *in silico* analysis suggested no apparent change in electrostatic interactions and/or hydrogen bonds except that Val489 of human CYP3A4 has an additional H-bond with Gly480 (data not shown). This is in contrast to recent results with CYP2B4dH in which an Asp192→Ala substitution abolished hydrogen bonds and decreased melting temperature by  $>10^\circ\text{C}$  (Oezguen et al., 2008).

Although the ancestral CYP3A4 was not available for activity analysis, the wide substrate spectrum of human CYP3A4 probably precedes the human-chimpanzee split. This conclusion is supported by the paucity of major activity differences toward a large effector panel between human and chimpanzee CYP3A4 that differs from the ancestral CYP3A4 in only two amino acids. The absence of activity differences is consistent with the sympatric evolution of human and chimpanzee lineages in East Africa until approximately 100,000 years ago, because any major changes in the exposure to environmental chemicals would be expected to affect either lineage.

The most striking differences between human and chimpanzee CYP3A4 include the responses to ANF and LCA. ANF had opposite effects on the  $S_{50}$  values for 7-BFC debenzylation or testosterone hydroxylation by the human compared with the chimpanzee enzyme. Of the eight residues that differ between chimpanzee and human CYP3A4, Ile369 in the human enzyme is in the active site, as evidenced by X-ray crystallography (Yano et al., 2004). In addition, a Val substitution at that position, as in the chimpanzee enzyme, alters progesterone hydroxylation and sensitivity to activation by ANF (He et al., 1997). Otherwise, a comparison of models of the human and chimpanzee enzymes suggests no significant difference in their structures, with a root mean square value of 0.3 Å (Fig. 1). Accordingly, the structural basis for the opposite effects of ANF on the  $S_{50}$  values for 7-BFC and testosterone oxidation by the human as opposed to chimpanzee enzyme remains unclear.

The other difference was the strong activation of human (but not chimpanzee) CYP3A4 by LCA, a very hepatotoxic secondary bile acid generated by intestinal bacteria. LCA is a rare example of a toxic endobiotic, and several mechanisms have evolved to solve the problem of its efficient detoxification. The efficient sulfation of LCA, exclusive for humans and chimpanzees, has reduced its toxicity in these species (Hofmann, 2004). The conversion of LCA by CYP3A4 to several hydroxylated metabolites (Bodin et al., 2005) further facilitates their conjugation and excretion. Both LCA and its major metabolite, 3-dehydro-LCA (Bodin et al., 2005), are activators of the pregnane X receptor (Staudinger et al., 2001) and vitamin D receptor (Makishima et al., 2002). In fact, the increasing complexity of the bile acid composition during vertebrate evolution may have affected the ligand specificity of pregnane X receptor (Krasowski et al., 2005), although the resulting induction of CYP3A4 is surprisingly modest (Stedman et al., 2004).

The activation of human CYP3A4 by LCA reported in our present work would be expected to increase the detoxification of this and other bile acids metabolized by the enzyme, although this remains to be formally demonstrated. In contrast to LCA, no activation differences were detected in response to the less toxic LCA precursor chenodeoxycholic acid and the other major primary bile acid, cholic acid. This suggested a previously unknown defense mechanism against LCA-mediated cholestasis, which evolved after the split of the common human-chimpanzee lineage. The physiological necessity of such a mechanism may be related to our ancestors, beginning with *Homo erectus* some 1.8 million years ago, having adapted to an energy-dense, meat-based diet. Contemporary human foraging populations derive more than half of their dietary energy from animal foods, in comparison with 5 to 10% observed in chimpanzees. This adaptation may have been a prerequisite for the subsequent dramatic increase in the brain size in the human lineage (Leonard et al., 2007). It is noteworthy that meat-based diet increased the load of animal steroids and thus the risk of cholestasis.

Alternatively to the increased load of animal steroids, the activation of human CYP3A4 by LCA may represent a side effect of the adaptive response to another selecting stimulus. In the former case, most of the amino acid differences between human and ancestral CYP3A4 would be expected to affect the magnitude of CYP3A4 activation by LCA. In the latter case, most of the LCA responsiveness would be expected to be a chance effect of only one amino acid substitution (Zhang et al., 2002). To distinguish between these possibilities, we reverted individually the six amino acid mutations acquired by the human CYP3A4 after the split from the chimpanzee. Mutating amino acid 478 from Ser to the ancestral Glu had no effect on activation by LCA, suggesting that Ser478 was selected by a factor different from LCA. This interpretation is consistent with this residue having undergone positive selection not only in the human lineage but also across the whole primate CYP3A phylogeny (Qiu et al., 2008). The selecting factor may have been related to the overall activity and stereoselectivity, because mutating residue 478 reduced the activity of CYP3A4 by 80 to 90% and changed its regioselectivity toward aflatoxin B1 metabolites (Wang et al., 1998).

All four remaining expressing mutants displayed reduced activation in response to LCA, clearly supporting this substance as a selecting agent. The individual effects of the other four mutations on the activation by LCA were smaller than the difference between the human and chimpanzee. This suggests the collective effect of all sequence differences, although this would need to be verified by expressing combinations of these mutants as well as of the two amino acid differences (165 and 369) exclusive for human and chimpanzee. The importance to LCA activation of the latter two residues, which differ

TABLE 3

Activation of 7-BFC-debenzoylation by LCA in human and chimpanzee wild-type (wt) CYP3A4, and in five human CYP3A4 mutants

The activity was determined using the standard NADPH system at 20  $\mu$ M 7-BFC. The data are the mean  $\pm$  S.D. of three independent experiments.

P450	Activity		-Fold Activation
	No LCA	100 $\mu$ M LCA	
	<i>nmol/min/nmol P450</i>		
Chimpanzee wt	2.05 $\pm$ 0.46	2.5 $\pm$ 0.43	1.22 $\pm$ 0.19
Human wt	2.22 $\pm$ 0.15	6.23 $\pm$ 0.68	2.80 $\pm$ 0.31
H54R	2.41 $\pm$ 0.28	4.65 $\pm$ 0.73	1.93 $\pm$ 0.33
Q78R	2.37 $\pm$ 0.26	5.01 $\pm$ 0.75	2.11 $\pm$ 0.34
L129I	2.53 $\pm$ 0.28	4.85 $\pm$ 0.78	1.85 $\pm$ 0.35
T224I	2.61 $\pm$ 0.34	4.42 $\pm$ 0.63	1.69 $\pm$ 0.29
S478R	2.05 $\pm$ 0.22	5.15 $\pm$ 0.82	2.51 $\pm$ 0.37

between human and chimpanzee but not between human and ancestral sequences, was not investigated, because of their apparently complex and species-specific effect on the CYP3A4 protein expression level. Indeed, Ile369 and Glu165 greatly decreased the expression of the chimpanzee CYP3A4, whereas they were compatible with high expression levels of human CYP3A4. Taken together, our results strongly suggest that LCA may have driven the evolution of CYP3A4 in the human lineage. This may constitute a rare example of incremental adaptive changes in response to a selective agent (Zhang et al., 2002).

Because of the importance of CYP3A4 in human drug metabolism, it is of substantial interest to find its appropriate animal model. Transgenic *CYP3A4* mice (Cheung and Gonzalez, 2008) have their limitations, because of differences between rodents and primates in the regulation of *CYP3A* gene expression, as well as in the expression and activity of many other genes important to drug response. Our data indicate that chimpanzee CYP3A4 is an excellent model of its human counterpart. For ethical, logistical, and economical reasons, the use of chimpanzee in drug discovery should generally be avoided. However, the strong similarity between the chimpanzee and human CYP3A4 raises the possibility of function conservation between CYP3A4 of humans and of a primate more widely used in medical research, such as rhesus or squirrel monkey.

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