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In Vitro Metabolism of Quinidine: The (3*S*)-3-Hydroxylation of Quinidine Is a Specific Marker Reaction for Cytochrome P-4503A4 Activity in Human Liver Microsomes

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ABSTRACT

The aim of this study was to evaluate the (3*S*)-3-hydroxylation and the *N*-oxidation of quinidine as biomarkers for cytochrome P-450 (CYP)3A4 activity in human liver microsome preparations. An HPLC method was developed to assay the metabolites (3*S*)-3-hydroxyquinidine (**3-OH-Q**) and quinidine *N*-oxide (**Q-*N*-OX**) formed during incubation with microsomes from human liver and from *Saccharomyces cerevisiae* strains expressing 10 human CYPs. **3-OH-Q** formation complied with Michaelis-Menten kinetics (mean values of V_{max} and K_m: 74.4 nmol/mg/h and 74.2 μM, respectively). **Q-*N*-OX** formation followed two-site kinetics with mean values of V_{max}, K_m and V_{max}/K_m for the low affinity isozyme of 15.9 nmol/mg/h, 76.1 μM and 0.03 ml/mg/h, respectively. **3-OH-Q** and **Q-*N*-OX** formations were potently inhibited by ketoconazole, itraconazole, and triacetyloleandomycin. Isozyme specific inhibitors of CYP1A2, -2C9, -2C19, -2D6, and -2E1 did not inhibit **3-OH-Q** or **Q-*N*-OX** formation, with K_i values comparable with previously reported values. Statistically significant correlations were observed between CYP3A4 content and formations of **3-OH-Q** and **Q-*N*-OX** in 12 human liver microsome preparations. Studies with yeast-expressed isozymes revealed that only CYP3A4 actively catalyzed the (3*S*)-3-hydroxylation. CYP3A4 was the most active enzyme in **Q-*N*-OX** formation, but CYP2C9 and 2E1 also catalyzed minor proportions of the *N*-oxidation. In conclusion, our studies demonstrate that only CYP3A4 is actively involved in the formation of **3-OH-Q**. Hence, the (3*S*)-3-hydroxylation of quinidine is a specific probe for CYP3A4 activity in human liver microsome preparations, whereas the *N*-oxidation of quinidine is a somewhat less specific marker reaction for CYP3A4 activity, because the presence of a low affinity enzyme is demonstrated by different approaches.

Hepatic biotransformation by the cytochrome P-450 (CYP) enzymes is the most important pathway of drug elimination, and CYP3A4 accounts for 30–40% of the total amount of identified drug-metabolizing cytochromes. Considerable variation in clearance of CYP3A4 substrates among relatively homogenous groups of volunteers exists. Even larger are the variations within heterogeneous populations (von Moltke et al., 1995). The variation in presence and activity (due to inhibition, induction, and, perhaps, activation) results in 10-fold variations in dosing requirements for some CYP3A4-metabolized drugs (Lown et al., 1994).

Quinidine has been used clinically for more than 200 years and is still important for the treatment of atrial flutter and fibrillation. Quinidine is metabolized to the main metabolite (3*S*)-3-hydroxy-quinidine (**3-OH-Q**), quinidine-*N*-oxide (**Q-*N*-OX**), and a few other quantitatively negligible metabolites. The in vivo metabolic clearance is 15 times faster for the **3-OH-Q** pathway than for the **Q-*N*-OX** pathway (Nielsen et al., 1995). In vitro, an anti-CYP3A4 antibody has been shown to inhibit more than 95% and 85% of the formations of **3-OH-Q** and **Q-*N*-OX**, respectively (Guengerich et al., 1986). Heterologously expressed CYP3A4 has been shown to actively metabolize quinidine, whereas CYP3A5 did not (Wrighton et al., 1990). It is likely that quinidine is more specific for CYP3A4 activity than drugs like nifedipine, cortisol, and others, because these drugs were shown to be substrates for both CYP3A4 and CYP3A5 in the same study (Wrighton et al., 1990). An in vivo pharmacokinetic interaction between quinidine and the effective CYP3A4 inhibitor erythromycin has been reported by Spinler et al. (1995).

The putative involvement of P-450s other than CYP2D6, -3A4, and -3A5 in quinidine metabolism has not been inves-

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ABBREVIATIONS: CYP, cytochrome P-450; HL, human liver; **3-OH-Q**, (3*S*)-3-hydroxyquinidine; **Q-*N*-OX**, quinidine *N*-oxide; 4-CPBG, 4-chlorophenylbiguanide; [S], concentration of substrate; [I], concentration of inhibitor; IC_{50}, inhibitor concentration causing a 50% reduction in velocity of a reaction; n, Hill coefficient describing sigmoidicity of cooperative binding of an inhibitor; r_s, correlation coefficient using Spearman's rank correlation.

tigated so far. The present study takes CYP1A2, -2C8, -2C9, -2C19, -2D6, -2E1, and -3A4 in human liver microsomes into account, as well as CYP1A1, -2C18, and -3A5 expressed by yeast cells. The aim of the study was to find further support that quinidine metabolism may serve as a specific marker reaction for CYP3A4 activity in vitro.

Materials and Methods

Chemicals. The following drugs and metabolites were kindly donated by the following companies: erythromycin (Abbott Scandinavia, Stockholm, Sweden); felodipine and terfenadine (Astra A/S, Copenhagen, Denmark); bifonazole, nifedipine, and nitrendipine (Bayer A/S, Copenhagen, Denmark); nefazodone HCl and paclitaxel (Bristol-Myers Squibb, Princeton, NJ); dapsone (Christian Friis & Co., Copenhagen, Denmark); fluvoxamine maleate (Duphar B.V., Weesp, Holland); fluoxetine HCl and norfluoxetine maleate (Eli Lilly A/S, Copenhagen, Denmark); vinblastine H_2SO_4 and vincristine H_2SO_4 (Eli Lilly Research Laboratories, Indianapolis, IN); astemizole, itraconazole and ketoconazole (Janssen Pharmaceuticals, Beerse, Belgium); **3-OH-Q** (Laboratoire Nativelle, Paris, France); citalopram hydrogene bromide and desmethyl-citalopram HCl (Lundbeck Pharma A/S, Copenhagen, Denmark); paroxetine (Novo-Nordisk Farmaka A/S, Copenhagen, Denmark); ethinyl-estradiol, 8-methoxypsoralen, prednisolone, and prednisone (Nycomed DAK A/S, Copenhagen, Denmark); sertraline (Pfizer A/S, Copenhagen, Denmark); methyl-prednisolone and triazolam (Pharmacia & Upjohn A/S, Copenhagen, Denmark); flucytosine, midazolam, and moclobemide (Roche A/S, Copenhagen, Denmark); bromocriptine mesilate, cyclosporin A, and dihydroergotamine mesilate (Sandoz LTD, Basel, Switzerland); and gestodene and testosterone (Schering A/S, Copenhagen, Denmark). The following drugs and compounds were purchased from: metronidazole, cocaine, and estradiol-hemihydrate (FAS centralapoteket, Odense, Denmark); fluconazole (Pfizer A/S); and α-naphtoflavone, cortisol, diethyldithiocarbamate, quinidine anhydrous, quinine HCl, naringenin, naringin, sulfaphenazole, and triacetyloleandomycin: Sigma Chemical Co. (St. Louis, MO). **Q-*N*-OX** was synthesized from purified quinidine (Nielsen et al., 1994). Chemicals were of analytical grade, and solvents were of HPLC grade. Quinidine anhydrous was dissolved in 0.1 M NaH_2PO_4 (pH, 7.4) and 2.5 mM HCl (not giving rise to changes in microsome activity).

Human Liver Microsomes. Kinetic and inhibition studies were carried out in one laboratory with microsomes from three human livers (HL1, HL2, and HL3) prepared by a standard technique (Meier et al., 1983). Protein concentrations were measured according to Lowry et al. (1951). Approval was obtained from the regional ethics committee. Correlation studies and immunoinhibition studies, as well as studies with yeast-expressed isozymes, were carried out in a second laboratory. Correlation studies were carried out with microsomes from 13 human livers prepared as previously described by Kremers et al. (1981). Immunoinhibition studies were carried out with microsomes from 2 of the above 13 livers selected because of their high content of CYP3A4. Protein concentrations were determined by biscinchonic acid assay according to the supplier's (Pierce, Rockford, IL) procedure. The P-450 concentration of individual CYPs in microsomes used for correlation studies and in yeast-expressed isozymes were determined according to Omura and Sato (1964).

Immunochemical Determination and Immunoinhibition. Microsomes from 13 preparations were separated by electrophoresis and transferred to nitrocellulose sheets as previously described (Laemmli, 1970; Towbin et al., 1979). Proteins were probed with polyclonal antibodies and the relative amounts of CYP1A2, -2C8, -2C9, -2D6, -2E1, and -3A4 with reference to the contents in one preparation were quantified by densiometry as described by Belloc et al. (1996). Anti-CYP3A4 antibodies were also used for immunoinhibition studies and have previously been shown to reduce erythromycin-*N*-demethylase activity by between 60 and 95% (Belloc et al., 1996).

Yeast-Expressing Human P-450s. Single-stranded cDNAs were obtained by reverse transcription of human liver mRNAs from *CYP1A1, -1A2, -2C8, -2C18, -2C19, -2D6, -2E1, -3A4,* and *-3A5* and subjected to polymerase chain reaction amplification (Gautier et al., 1996). Coding sequences of human *CYP2C9* were amplified by polymerase chain reaction from plasmids described by Srivastava et al. (1991). The sequences were inserted into the yeast expression vector pYeDP60 using classical cloning procedures (Urban et al., 1990), except for the *CYP3A4* sequence, which was inserted by gap repair (Peyronneau et al., 1992). The plasmids were transferred to the *Saccharomyces cerevisiae* strain W(R), except for the plasmid-expressing CYP3A4, which was inserted into the *S. cerevisiae* strain W(hR). The latter strain was specifically used for CYP3A4, because it has been observed that with some substrates CYP3A4 was poorly active in the W(R) strain (Pompon et al., 1996). This was usually not observed with other CYPs expressed in W(R) yeast. By using this system we usually have good correlation between results obtained with human liver microsomes and results obtained with the yeast-expressing systems (Lemoine et al., 1993; Nielsen et al., 1996; Gautier et al., 1996). Expression of NADPH CYP-450 reductase was galactose-inducible in both W(R) and W(hR) strains, the former overexpressing the yeast reductase, whereas the latter expressed the human reductase. Culture media, selection, growth, harvesting, and preparation of microsomes have previously been described by Cullin and Pompon (1988) and Urban et al. (1990).

Incubation Conditions. Microsomes were incubated in a final volume of 200 μl in a NaH_2PO_4 buffer (0.1 M, pH 7.4) using 100 μg of microsomal protein. The reactions were initiated by adding 20 μl of a NADPH-generating system (final concentrations: isocitrate dehydrogenase, 1 U/ml; NADP-Na_2, 1 mM; DL-isocitrate-Na_3, 5 mM; and $MgCl_2$, 5 mM). Incubations were carried out at 37°C in a shaking water bath in air. The reactions were terminated after 20 min by adding 1 ml of K_2CO_3 (0.6 M) followed by 10 μl of an internal standard (quinine, 250 μM). All incubations were run in duplicate and less than 5% of the substrate was consumed during the incubations. A single-step, liquid-liquid extraction was carried out by addition of 3 ml of an extraction solution (heptane, 47.9%; tert butylmethyl ether, 47.9%; *n*-butanol, 4.2%) followed by shaking for 10 min and centrifugation for another 10 min at 2,500 rpm. The tubes were placed in −30°C for 1 to 2 min, and the organic phase then transferred into conical glass tubes and subsequently evaporated to dryness under a nitrogen steam for 30 min at 55°C. Reconstitution and analysis of metabolites formed during incubation were performed according to a previously described HPLC method (Nielsen et al., 1994). Linearity was observed between the formation rates of both metabolites and increasing concentrations of protein in the incubations (up to 1.0 mg/ml), as well as between the formation rates of both metabolites and increasing incubation time (up to 30 min).

Microsomes from HL1, HL2, and HL3 and from yeasts expressing CYP3A4 were incubated with quinidine solutions in 12 final concentrations of substrate ([S]) ranging from 10 to 500 μM. These incubations were performed six times. Equations describing both one- and two-site models were fitted to the formations of **3-OH-Q** and **Q-*N*-OX** by means of an iterative curve-fitting program based on nonlinear regression analysis (N. Holford; MK Model, version 4. Biosoft, Cambridge, England, 1990):

$$\text{one-site model:} \quad V = \frac{V_{max} \times [S]}{K_m + [S]} \tag{1}$$

$$\text{two-site model:} \quad V = \frac{V_{max} \times [S]}{K_m + [S]} + L \times [S] \tag{2}$$

V_{max} is the maximal formation rate by a high-affinity enzyme, K_m is the Michaelis constant, and L is the ratio between V_{max} and K_m for a low-affinity enzyme.

Forty-six drugs in final concentrations of inhibitor ([I]) of 0, 1, 10 and 100 μM were screened for their ability to alter quinidine metabolism. Those able to reduce the formation of either **3-OH-Q** or **Q-*N*-OX** by more than 30% at [I] = 100 μM were incubated at 10 different inhibitor concentrations around their apparent K_i. Except for gestodene, this was performed three times at different quinidine concentrations (50, 75, and 150 μM). For each of the three data sets, the data were fitted by nonlinear regression analysis to equations describing the inhibition by both one- and two-site models:

$$\text{one-site model:} \quad V = \frac{V_{max} \times [S]}{[S] + K_m\left(\frac{[I]}{K_i} + 1\right)} \tag{3}$$

$$\text{two-site model:} \quad V = \frac{V_{max} \times [S]}{[S] + K_m\left(\frac{[I]}{K_i} + 1\right) + L \times [S]} \tag{4}$$

K_i is the inhibitor constant for inhibition of the high- affinity site. The K_i values given in *Results* are the averages of the three K_i values obtained for each inhibitor. Inhibition by gestodene was analyzed from the intersection of two Lineweaver-Burk plots obtained from data from two sets of incubations: One set with increasing concentrations of quinidine ranging from 10 to 500 μM and a similar set after preincubation with 150 μM gestodene. The inhibition by ketoconazole was also analyzed by nonlinear regression fitting data to the Hill equation (eq. 5).

$$\text{Hill equation:} \quad Y[I] = [I]^n/([I]^n + \mathrm{IC}_{50}{}^n) \tag{5}$$

The equation describes the inhibition (Y) ranging from 0 (no inhibition) to 1 (maximum inhibition) as a function of [I].The Hill coefficient, n, describes the sigmoidicity of the cooperative binding of the inhibitor.

Twenty picomoles of total P-450 enzyme was used for correlation and immunoinhibition studies and for experiments with yeast-expressed P-450s. For these studies the NADPH-regenerating system consisted of: glucose 6-phosphate dehydrogenase, 5 U/ml; NADP, 0.15 mM; glucose 6-phosphate, 2.5 mM; $MgCl_2$, 5 mM; and glycerol, 10%. Yeast-expressed P-450s were incubated at 28°C and the activities were multiplied by the content (CYP1A1, 1 pmol; 1A2, 25 pmol; 2C8, 10 pmol; 2C9, 100 pmol; 2C18, 10 pmol; 2C19, 10 pmol; 2D6, 25 pmol; 2E1, 100 pmol; 3A4, 250 pmol; and 3A5, 50 pmol) in pmoles of the isozymes generally found per mg of microsomal protein (Iribarne et al., 1996; P. Beaune, personal communication). The correlation studies were tested with Spearman's rank correlation considering a $p < 0.05$ to be statistically significant.

Results

Enzyme Kinetics. 3-OH-Q was the dominant metabolite formed during incubation with quinidine in liver microsomes (Table 1) and its formation could be described according to Michaelis-Menten kinetics. Eadie-Hofstee plots (Fig. 1) were linear and the apparent K_m values estimated from experiments with human liver microsomes (74.0 μM) and yeast-expressed CYP3A4 (70.9 μM) were in concordance. The formation of **Q-*N*-OX** (Table 1) was ideally described by the two-site model and Eadie-Hofstee plots were biphasic (Fig. 1).

Inhibition Studies. Inhibition of the (3*S*)-3-hydroxylation could be described with the one-site model. Noncompetitive inhibition of the (3*S*)-3-hydroxylation was observed from Lineweaver-Burk plots following preincubation with

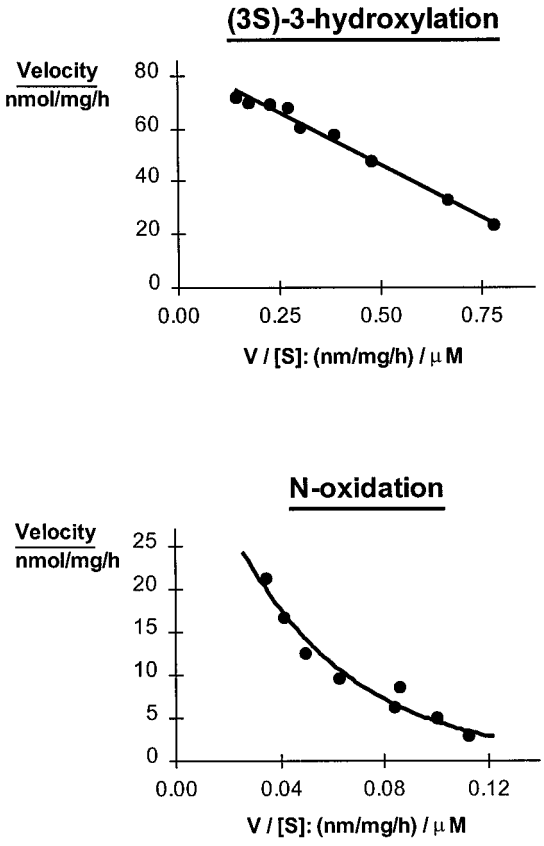

Fig. 1. Eadie-Hofstee plots for (3*S*)-3-hydroxylation (top) using microsomes from HL1 and *N*-oxidation (bottom) using microsomes from HL3.

the mechanism-based CYP3A4-inactivator gestodene. Inhibitors with K_i values of less than 5 μM (Table 2: ketoconazole, bifonazole, itraconazole, bromocriptine, dihydroergotamine, nefazodone, triacetyloleandomycin, cyclosporin A, midazolam, and vinblastine) were able to reduce the formation of **3-OH-Q** by 90 to 95%. The concomitant formation of **Q-*N*-OX** was only reduced by 60 to 80%. Inhibition of the *N*-oxidation was ideally described by the two-site model. Table 2 lists the K_i values for 22 compounds capable of inhibiting the formation of **3-OH-Q** and **Q-*N*-OX**. The following 11 compounds did not possess any inhibitory potential toward the two reactions when introduced in concentrations up to 100 μM: prednisone, prednisolone, estradiol, citalopram, desmethylcitalopram, dapsone, flucytosine, cocaine, metronidazole, naringin, and triazolam. None of the 10 following isozyme specific inhibitors were able to inhibit either reaction, with K_i values in a range suggesting involvement of the enzymes in question: fluvoxamine and 8-methoxypsoralen (CYP1A2); sulfaphenazole (CYP2C9); moclobemide (CYP2C19); paroxetine, fluoxetine, norfluoxetine and sertraline (CYP2D6); and ethanol and diethyldithiocarbamate (CYP2E1).

At quinidine concentrations of 50 and 75 μM, as well as 150 μM, log likelihood values computed by the regression model indicated that the Hill equation described the inhibition of the (3*S*)-3-hydroxylation by ketoconazole 2 to 5 times as well as the one- or two-site models (Fig. 2). The inhibitor concentration causing a 50% reduction in velocity of a reaction (IC_{50}) was estimated to 0.85 and n was estimated to 3.1.

A strong statistically significant correlation was observed between the 10 lowest K_i values for inhibition of **3-OH-Q**

TABLE 1

Kinetic parameters for quinidine metabolism

Values are estimated by nonlinear regression analysis, using one-enzyme model for (3*S*)-3-hydroxylation and two-enzyme model for the *N*-oxidation. L is ratio V_{max}/K_m for low-affinity enzyme involved in *N*-oxidation.

Liver	3-OH-Q			Q-*N*-OX			
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	L
	nmol/mg/h	*μM*	*ml/mg/h*	*nmol/mg/h*	*μM*	*ml/mg/h*	*ml/mg/h*
HL1	85.5	77.6	1.1	23.9	131.6	0.2	0.009
HL2	41.1	75.1	0.5	9.2	69.2	0.1	0.024
HL3	70.9	69.3	1.0	14.9	77.9	0.2	0.036
Mean	65.8	74.0	0.9	16.0	92.9	0.2	0.023
S.D.	22.6	4.3	0.3	7.4	33.8	0.1	0.014

TABLE 2

K_i values for inhibition of (3*S*)-3-hydroxylation and *N*-oxidation

Values for inhibition by 22 drugs/compounds. Values represent mean of values from three inhibition studies.

Drug/compound	K_i	
	3-OH-Q	Q-*N*-OX
	μM	*μM*
Ketoconazole	0.15	0.23
Bifonazole	0.48	0.59
Itraconazole	0.49	1.0[a]
Fluconazole	14.9	19.8[a]
Astemizole	5.7	33.9
Terfenadine	10.1	21.6
Bromocriptine	0.8	1.6
Dihydroergotamine	1.2	1.0
Vinblastine	4.7	5.9
Vincristine	24.8	27.2
Paclitaxel	58.5[a]	54.8[a]
Nitrendipine	20.2	14.1[b]
Nifedipine	28.2[b]	[c]
Felodipine	83.8[b]	[c]
Erythromycin	28.4	35.2
Triacetyloleandomycin	2.3	2.9
Ciclosporin A	2.3	3.2
Midazolam	4.3	7.2
Gestodene	33.4	73.5
Ethinylestradiol	107.6	[c]
Naringenin	109.8	103.2[b]
Nefazodone	1.9	1.9

[a] K_i value is mean of K_i values from two inhibition studies.
[b] K_i value from one inhibition study.
[c] No inhibition with inhibitor concentrations up to 125 μM.

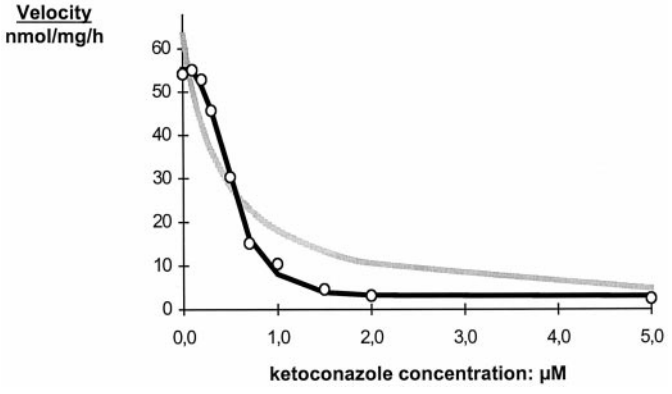

Fig. 2. Inhibition of the (3*S*)-3-hydroxylation by ketoconazole at a quinidine concentration of 150 μM quinidine. The same pattern evolved with quinidine concentrations of 50 and 75 μM. Curves are fitted to data by regression analysis using the Hill equation (sigmoid curve) and one-site inhibition kinetics.

formation versus inhibition of **Q-*N*-OX** formation (r_s=.948, $p < .002$ using Spearman's rank correlation). Screening of the influence of methylprednisolone (up to 150 μM), cortisol (up to 200 μM), and testosterone (up to 250 μM) on quinidine metabolism revealed activation rather than inhibition of the *N*-oxidation. The formation of **3-OH-Q** was not altered by these three steroids. With the above concentrations, the *N*-oxidation was increased to 200% with methylprednisolone, 170% with cortisol, and 150% with testosterone. This activation was concentration dependent (Fig. 3). The CYP3A4 in vitro activator α-naphtoflavone (Shou et al., 1994) did not increase the velocity of either reactions.

Correlation Studies. Statistically significant correlations were observed between the immunoquantified content of CYP3A4 versus formation of both **3-OH-Q** (r_s=.806, $p < .01$) and **Q-*N*-OX** (r_s=.698, $p < .02$) in 12 human liver microsome preparations when the quinidine concentration was 75 μM. At 5 μM quinidine, only the **3-OH-Q** formation correlated with the immunoquantified content of CYP3A4 (r_s=.722, $p < .02$). Upon inhibition with ketoconazole, the formation of **3-OH-Q** did not correlate with the immunoquantified content of CYP1A2, -2C8, -2C9, -2D6, -2E1, and -3A4 or with proguanil metabolism. **Q-*N*-OX** formation, however, did correlate with formation of 4-chlorophenylbiguanide (4-CPBG) from proguanil when 0.75 μM ketoconazole was present (r_s=.860, $p < .002$).

Yeast-Expressed Enzymes. At a quinidine concentration of 75 μM, studies with 10 yeast-expressed isozymes (CYP1A1, -1A2, -2C8, -2C9, -2C18, -2C19, -2D6, -2E1, -3A4, and -3A5) showed that the contribution of CYP3A4 to total (3*S*)-3-hydroxylation was 88% (Fig. 4). This percentage increased to 97% when the quinidine concentration was lowered to 5 μM. An important finding was the negligible 1% catalyzed by CYP3A5. CYP3A4 was also the most active component in **Q-*N*-OX** formation: At a quinidine concentration of 75 μM, the CYP3A4 contribution to the *N*-oxidation was approximately 65%, but CYP2C9 and CYP2E1 also cat-

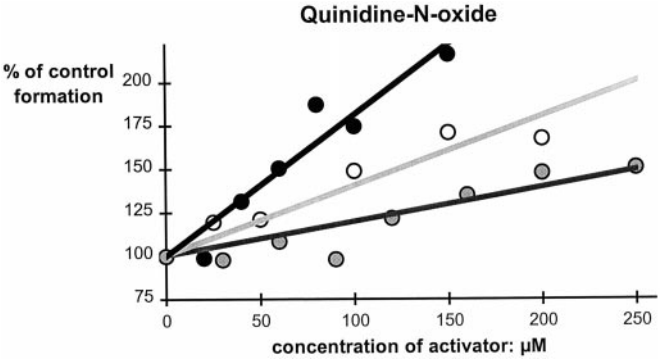

Fig. 3. Activation of *N*-oxidation by methylprednisolone (black circle), cortisol (white circle), and testosterone (gray circle).

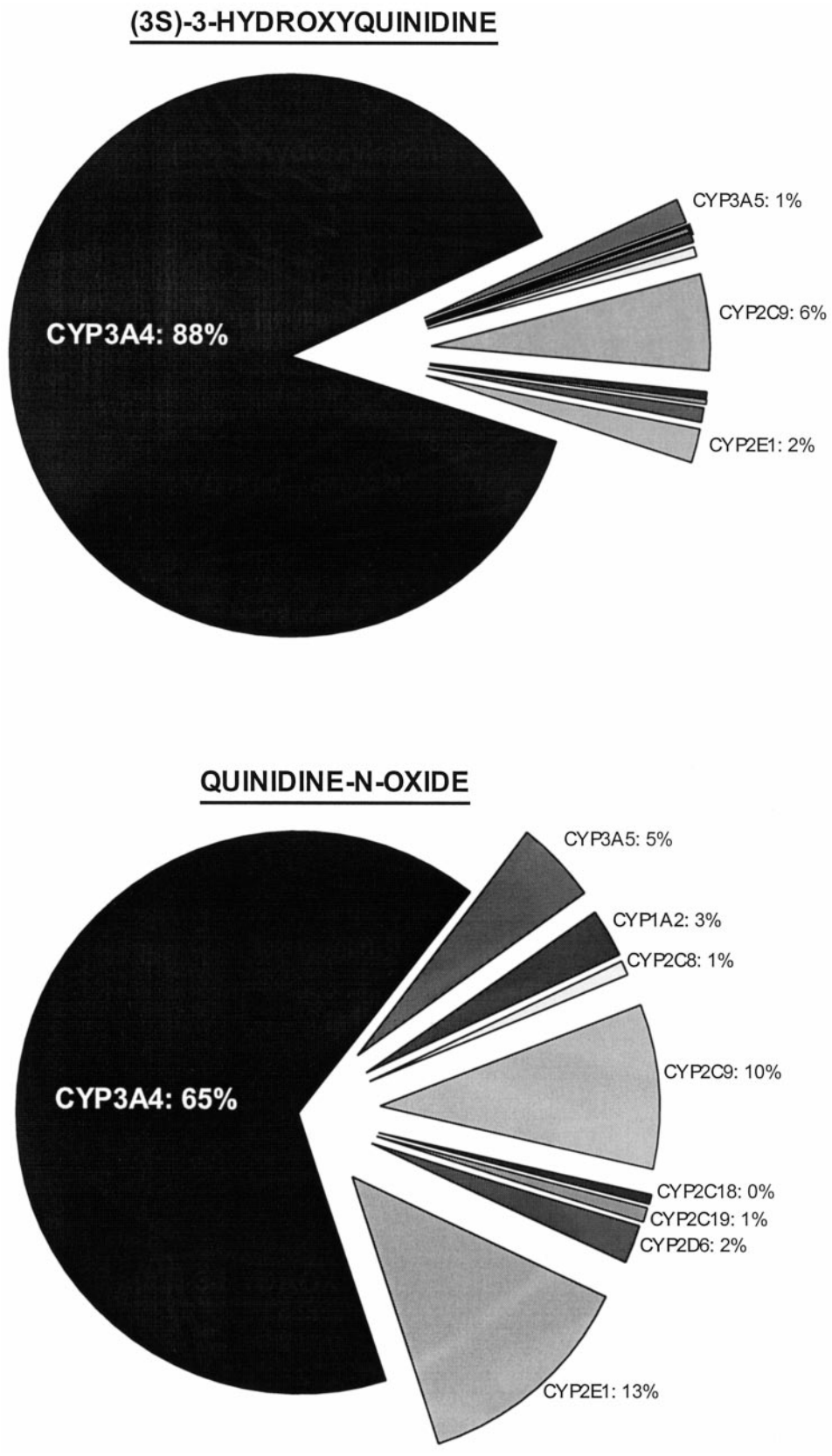

Fig. 4. Contribution of different yeast-expressed CYPs to (3*S*)-3-hydroxylation (top) and *N*-oxidation (bottom) at a quinidine concentration of 75 μM. Chart shows percentage that each isozyme contributed to overall metabolism. Content of individual isozymes in microsomal protein is taken into account as is detailed in *Materials and Methods*.

alyzed approximately 10% and 13%, respectively (Fig. 4). Other isozymes catalyzed minor proportions.

Immunoinhibition. These studies demonstrated that the formation of **3-OH-Q** was reduced by approximately 95% upon addition of 2.5 μg anti-CYP3A4 antibody per pmole of P-450 enzyme. As a control, preimmune serum reduced the formation of **3-OH-Q** by 40% when the same final concentration (2.5 μg/pmol) of immunoglobulin G from preimmune

serum was present. Studies were carried out with a quinidine concentration of 75 μM and the results were quite similar for the *N*-oxidation, with reductions by 87% and 40% when adding anti-CYP3A4 antibody (2.5 μg/pmol) and preimmune serum (2.5 μg immunoglobulin G/pmol), respectively.

Discussion

The data obtained leave no doubt that the (3*S*)-3-hydroxylation of quinidine is catalyzed by CYP3A4. All other P-450 isozymes (including CYP3A5) have very low activities toward catalyzing this reaction and the results are in line with a previous study by Zhao et al. (1996) showing that CYP3A4 catalyzed the 3-hydroxylation of quinine (the enantiomer of quinidine). Enzyme kinetics and inhibition kinetics reveal the existence of one and only one enzyme catalyzing the reaction, both when analyzed with nonlinear regression and when evaluated graphically from Eadie-Hofstee plots. Additionally, the inhibition of the (3*S*)-3-hydroxylation by 90 to 95% by potent CYP3A4 inhibitors like ketoconazole, triacetyloleandomycin, and itraconazole have K_i values comparable with previously reported values. The K_i values are out of range of previously reported values when using isozyme-specific inhibitors of other enzymes. Thus participation of these isozymes are ruled out. The noncompetitive inhibition by gestodene is in concordance with the inhibition mode of this mechanism-based inactivator of CYP3A4 (Guengerich, 1990). The correlation studies and studies with yeast-expressed isozymes are also strongly addressing the importance of CYP3A4, both at quinidine concentrations of 5 μM, which is close to the free unbound plasma concentration of quinidine when used as a therapeutic agent, and at a concentration of 75 μM, which is close to the K_m of the (3*S*)-3-hydroxylation in vitro. At the latter concentration, the studies with yeast showed a 6% contribution from CYP2C9, which apparently is too small to influence the kinetic or inhibition studies in such a manner that two-site kinetics evolves. All other isozymes contribute with percentages that can be considered negligible. At 5 μM, the percentages from all isozymes but CYP3A4 were negligible. Finally, CYP3A4 involvement is depicted by the reduction in the formation of **3-OH-Q** by 95% upon addition of 2.5 μg anti-CYP3A4 antibody per pmole of P-450 enzyme. This must be compared with a reduction by 40% when using preimmune serum in the same concentration. The results from the immunoinhibition studies cannot stand alone, because one would not expect significant inhibition by preimmune serum.

Neither the one- nor the two-site model gave satisfactory descriptions of ketoconazole inhibition of the (3*S*)-3-hydroxylation. Rather, the data were described by the Hill equation. Previously, CYP3A4-mediated metabolism of diazepam, aflatoxin B1, amitriptyline, 17β-estradiol, and testosterone have been shown to comply to the Hill equation, suggesting cooperativity in drug metabolism (Andersson et al., 1994; Gallagher et al., 1996; Schmider et al., 1996; Ueng et al., 1997). To our knowledge, this study for the first time describes inhibition of metabolism as sigmoid.

The *N*-oxidation of quinidine is catalyzed mainly by CYP3A4, but with the participation of one or more low-affinity isozymes catalyzing approximately 30% of the *N*-oxidation. Enzyme kinetics, as well as inhibition kinetics, reveal the existence of a high- and low- affinity enzyme catalyzing the reaction, both when analyzed with nonlinear regression and when evaluated graphically from Eadie-Hofstee plots. An 80% inhibition of the *N*-oxidation was observed using potent CYP3A4 inhibitors like ketoconazole, triacetyloleandomycin, and itraconazole, and the correspondent K_i values were in consonance with previously reported values when two-site inhibition kinetics were applied. The strong statistically significant correlation between the K_i values for the 10 most potent inhibitors of the (3*S*)-3-hydroxylation versus the K_i values for the concomitant inhibition of the *N*-oxidation emphasizes the importance of CYP3A4 toward the latter reaction. The activation of the *N*-oxidation by methylprednisolone, cortisol, and testosterone is probably due to activation of CYP3A4, because in vitro activation of CYP3A4 by organic compounds with appropriately placed ketones has previously been shown by Shou et al. (1994). Methyl-prednisolone, cortisol, and testosterone all possess a ketone functional group and testosterone has been shown to activate CYP3A4-catalyzed reactions (Kerlan et al., 1992). To our knowledge, in vitro activation has only been demonstrated for CYP3A4 substrates. The correlation studies and studies with yeast- expressed isozymes are again addressing the importance of CYP3A4. The reduction in the formation of **Q-*N*-OX** by 87% upon addition of 2.5 μg anti-CYP3A4 antibody per pmole of enzyme also suggests CYP3A4 involvement, again when compared with a 40% reduction by preimmune serum in the same concentration. The correlation between formation of **Q-*N*-OX** during ketoconazole inhibition of CYP3A4 versus formation of 4-CPBG indicates that one low-affinity enzyme could be CYP2C19, because the metabolism of proguanil to 4-CPBG is catalyzed by CYP2C19 (Jeppesen et al., 1997). This finding could not be confirmed by the incubations with yeast-expressed enzymes, suggesting that approximately 13% of the **Q-*N*-OX** formation is attributable to CYP2E1, 10% to CYP2C9, and smaller proportions to other enzymes (Fig. 4). This study does not identify with sufficient evidence the low-affinity enzyme or enzymes catalyzing the *N*-oxidation.

CYP3A4 inhibition by ketoconazole is well established. A recent placebo-controlled study confirmed an in vivo interaction between quinidine and itraconazole (Kaukonen et al., 1997). K_i values observed in this study for drugs like nefazodone, vinblastine, cyclosporin A, midazolam, and fluconazole suggest that these drugs could alter the in vivo biotransformation of drugs metabolized by CYP3A4. In fact, in vivo interactions have already been demonstrated with inhibition by nefazodone (Kroboth et al., 1995), vinblastine (Zhou et al., 1993), and fluconazole (Back and Tjia, 1991; Varhe et al., 1996). The ability of several drugs different in size and type to alter the in vitro metabolism of quinidine underline the immense importance of CYP3A4 in drug metabolism and the necessity to gain further insight into isozyme-specific drug metabolism to avoid harmful or even fatal drug combinations. Extra care should especially be taken during polypharmacy with antineoplastics, immunosuppressants, some antibiotics and antimycotics, antiarythmics, calcium antagonists, some antihistamines, steroids, benzodiazepines, and some anticonvulsants.

Being a specific marker reaction for CYP3A4 activity in human liver microsomes, the (3*S*)-3-hydroxylation of quini-

dine can serve as a method to screen new drugs for their potential to inhibit CYP3A4-catalyzed biotransformation. The reaction can also be used for correlation studies identifying other CYP3A4 substrates. Finally, the reaction might prove useful as an in vivo marker reaction for CYP3A4 activity. However, the existence of CYP3A4 in the gastrointestinal tract should be considered (Lown et al., 1994).

References

Andersson T, Miners JO, Veronese ME and Birkett DJ (1994) Diazepam metabolism by human liver microsomes is mediated by both *S*-mephenytoin hydroxylase and CYP3A4 isoforms. *Br J Clin Pharmacol* **38**:131–137.

Back DJ and Tjia JF (1991) Comparative effects of the antimycotic drugs ketoconazole, fluconazole, itraconazole and terbinafine on the metabolism of cyclosporin by human liver microsomes. *Br J Clin Pharmacol* **32:**624–626.

Belloc C, Baird S, Cosme J, Lecoeur S, Gautier JC, Challine D, de Waziers I, Flinois JP and Beaune PH (1996) Human cytochromes P450 expressed in *Escherichia coli*: Production of specific antibodies. *Toxicology* **106:**207–219.

Cullin C and Pompon D (1988) Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3–1 in the yeast *Saccharomyces cerevisiae*. *Gene* **65:**203–217.

Gallagher EP, Kunze KL, Stapleton PL and Eaton DL (1996) The kinetics of aflatoxin B1 oxidation by human cDNA-expressed and human liver microsomal cytochromes P450 1A2 and 3A4. *Toxicol Appl Pharmacol* **141:**595–606.

Gautier JC, Lecoeur S, Cosme J, Perret A, Urban P, Beaune P and Pompon D (1996) Contribution of human cytochrome P450 to benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol metabolism, as predicted from heterologous expression in yeast. *Pharmacogenetics* **6:**489–499.

Guengerich FP (1990) Mechanism-based inactivation of human liver microsomal cytochrome P450 IIIA4 by gestodene. *Chem Res Toxicol* **3:**363–371.

Guengerich FP, Müller-Enoch D and Blair IA (1986) Oxidation of quinidine by human liver cytochrome P-450. *Mol Pharmacol* **30:**287–295.

Iribarne C, Berthou F, Baird S, Dréano Y, Picart D, Bail JP, Beaune PH and Ménez JF (1996) Involvement of cytochrome P450 3A4 enzyme in the *N*-demethylation of methadone in human liver microsomes. *Chem Res Toxicol* **9:**365–373.

Jeppesen U, Rasmussen BB and Brøsen K (1997) Fluvoxamine inhibits the CYP2C19-catalyzed bioactivation of chloroguanide. *Clin Pharmacol Ther* **62:**279–286.

Kaukonen KM, Olkkola KT and Neuvonen PJ (1997) Itraconazole increases plasma concentrations of quinidine. *Clin Pharmacol Ther* **62:**510–517.

Kerlan V, Dreano Y, Bercovici JP, Beaune P, Floch HH and Berthou F (1992) Nature of cytochromes P450 involved in the 2-/4-hydroxylations of estradiol in human liver microsomes. *Biochem Pharmacol* **44:**1745–1756.

Kremers P, Beaune P, Cresteil T, de Graeve J, Columelli S, Leroux JP and Gielen JE (1981) Cytochrome P-450 monooxygenase activities in human and rat liver microsomes. *Eur J Biochem* **118:**599–606.

Kroboth PD, Folan MM, Lush RM, Chaikin PC, Shukla UA, Barbhaiya R and Salazar DE (1995) Coadministration of nefazodone and benzodiazepines: I. Pharmacodynamic assessment. *J Clin Psychopharmacol* **15:**306–319.

Laemmli, UK (1970) Change of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685.

Lemoine A, Gautier JC, Azoulay D, Kiffel L, Belloc C, Guengerich FP, Maurel P, Beaune P and Leroux JP (1993) Major pathway of imipramine metabolism is catalyzed by cytochrome P-450 1A2 and P-450 3A4 in human liver. *Mol Pharmacol* **43:**827–832.

Lown KS, Kolars JC, Thummel KE, Barnett JL, Kunze KL, Wrighton SA and Watkins PB (1994) Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. *Drug Metab Dispos* **22:**947–955.

Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ (1951) Protein measurement with pholin phenol reagent. *J Biol Chem* **193:**265–275.

Meier PJ, Mueller HK, Dick B and Meyer UA (1983) Hepatic monooxygenase activities in subjects with a genetic defect in drug oxidation. *Gastroenterology* **85:**682–692.

Nielsen F, Nielsen KK and Brøsen K (1994) Determination of quinidine, dihydroquinidine, (3*S*)-3-hydroxyquinidine and quinidine *N*-oxide in plasma and urine by high-performance liquid chromatography. *J Chromat B* **660:**103–110.

Nielsen F, Rosholm J-U and Brøsen K (1995) Lack of relationship between quinidine pharmacokinetics and the sparteine oxidation polymorphism. *Eur J Clin Pharmacol* **48:**501–504.

Nielsen KK, Flinois JP, Beaune P and Brøsen K (1996) The biotransformation of clomipramine in vitro, identification of the cytochrome P450s responsible for the separate metabolic pathways. *J Pharmacol Exp Ther* **277:**1659–1664.

Omura T and Sato R (1964) The carbon monoxide binding pigment of liver microsomes. *J Biol Chem* **239:**2370–2385.

Peyronneau M, Renaud JP, Truan G, Urban P, Pompon D and Mansuy D (1992) Optimization of yeast-expressed human liver cytochrome P-450 3A4 catalytic activities by coexpressing NADPH-cytochrome P-450 and cytochrome b5. *Eur J Biochem* **207:**109–116.

Pompon D, Louerat B, Bronine A and Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. *Methods Enzymol* **272:**51–64.

Schmider J, Greenblatt DJ, Harmatz JS and Shader RI (1996) Enzyme kinetic modelling as a tool to analyse the behaviour of cytochrome P450 catalysed reactions: Application to amitriptyline *N*-demethylation. *Br J Clin Pharmacol* **41:**593–604.

Shou M, Grogan J, Mancewicz JA, Krause KW, Gonzalez FJ, Gelboin HV and Korzekwa KR (1994) Activation of CYP3A4: Evidence for the simultaneous binding of two substrates in a cytochrome P450 active site. *Biochemistry* **33:**6450–6455.

Srivastava PK, Beaune PH, Ged C and Guengerich FP (1991) Separation of human liver microsomal tolbutamide hydroxylase and (*S*)-mephenytoin 4′-hydroxylase cytochrome P450 enzymes. *Mol Pharmacol* **40:**69–79.

Spinler SA, Cheng JWM, Kindwall KE and Charland SL (1995) Possible inhibition of hepatic metabolism of quinidine by erythromycin. *Clin Pharmacol Ther* **57:**89–94.

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76:**4350–4354.

Ueng YF, Kuwabara T, Chun YJ and Guengerich FP (1997) Cooperativity in oxidations catalyzed by cytochrome P450 3A4. *Biochemistry* **36:**370–381.

Urban P, Cullin C and Pompon D (1990) Maximizing the expression of mammalian cytochromes P-450 monooxygenase activities in yeast cells. *Biochimie* **72:**463–472.

Varhe A, Olkkola KT and Neuvonen P (1996) Fluconazole, but not terbinafine, enhances the effects of triazolam by inhibiting its metabolism. *Br J Clin Pharmacol* **41:**319–323.

von Moltke LL, Greenblatt DJ, Schmider J, Harmatz JS and Shader RI (1995) Metabolism of drugs by cytochrome P450 3A isoforms. *Clin Pharmacokinet* **29:**33–44.

Wrighton SA, Brian WR, Sari MA, Iwasaki M, Guengerich FP, Raucy JL, Molawa DT and Vandenbranden M (1990) Studies on the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (Hlp3). *Mol Pharmacol* **38:**207–213.

Zhao XJ, Yokoyama H, Chiba K, Wanwimolruk S and Ishizaki T (1996) Identification of human cytochrome P450 isoforms involved in the 3-hydroxylation of quinine by human liver microsomes and nine recombinant human cytochromes P450. *J Pharmacol Exp Ther* **279:**1327–1334.

Zhou XJ, Zhou-Pan XR, Gauthier T, Placidi M, Maurel P and Rahmani R (1993) Human liver microsomal cytochrome P450 3A isozymes mediated vindesine biotransformation. *Biochem Pharmacol* **45:**853–861.

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