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CYTOCHROME P450IID SUBFAMILY IN NON-HUMAN PRIMATES

CATALYTICAL AND IMMUNOLOGICAL CHARACTERIZATION

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Abstract—Interindividual variations of debrisoquine metabolism was recently identified in non-human primates tested *in vivo*. The catalytical and immunological characterization of cytochrome P450IID subfamily was undertaken in hepatic microsomes from extensive metabolizer primates. The NADPH/O_2 mediated metabolism of debrisoquine, dextromethorphan and bufuralol was similar to the kinetics reported in humans. The CuOOH mediated metabolism of bufuralol suggested that at least two enzymes are responsible for bufuralol 1′-hydroxylation. Eleven compounds were tested for their capacity to modify P450IID function *in vitro*. Eight competitive inhibitors of P450IID6 in man were all and exclusively competitive inhibitors of P450IID subfamily in non-human primates. Quinidine, which is the strongest competitive inhibitor in man, exhibited the higher inhibitory potency in monkey (K_i = 0.75 μM). Anti-LKM antibody against P450IID subfamily cross-reacted with two proteins of 49 and 47 kDa, and sera containing anti-LKM antibody against these two proteins inhibited dextrorphan formation *in vitro*. These data provide evidence for catalytical and immunological similarities between human and monkey microsomes and indicate that the primate system could be a model for enzymatic studies of P450IID.

Cytochrome P450 (P450‡) multiple enzymes play a major role in the metabolism of xenobiotics [1] and several individual forms exhibit a polymorphic activity under genetic control [2]. The most widely investigated polymorphism is related to debrisoquine metabolism. From 3 to 9% of the caucasian population, because of a specific debrisoquine P450 (P450IID6) defect, inherited as an autosomal recessive trait, are poor metabolizers [3, 4]. The metabolism of an increasing number of drugs is dependent upon P450IID6. The pharmacokinetic consequences of the enzymatic defect are significant if P450IID6 contributes predominantly to the elimination of the drug, but the clinical impact is important for drugs with a small therapeutic margin as tricyclic antidepressants and antiarrhythmics. With these drugs, interindividual variability in the plasma concentrations after standard dose regimen leads to toxic effects or therapeutic failures [5].

The relevance of genetic polymorphism in drug metabolism in the development of new drugs has been recently discussed [6]. *In vitro* metabolic studies are performed early in their development in order to determine their main metabolic pathways and a possible linkage with a known genetic polymorphism. *In vivo* pharmacokinetic studies should be performed in volunteers phenotyped as extensive and poor metabolizers to define the pharmacokinetic and clinical significance of *in vitro* findings.

Until now, because of interspecies differences in hepatic metabolism, *in vitro* and *in vivo* studies have been mostly conducted in humans, but the availability of human liver samples, particularly from subjects of defined phenotypes is limited.

Recently, we reported *in vivo* interindividual variations of debrisoquine metabolism in non-human primates [7]. Because an animal model would provide distinct advantages over human studies for the screening of new drugs, the present study was undertaken to characterize P450IID subfamily in microsomes from extensive metabolizer primates. The metabolism of debrisoquine, dextromethorphan and bufuralol, which are prototype substrates of P450IID6 in man [8], were studied in the presence of an NADPH regenerating system (NADPH/O_2). We also compared the hydroxylation of (+)-bufuralol using the classical NADPH/O_2 assay system and the cumene hyperoxide (CuOOH) assay system. Various compounds, which are known competitive inhibitors of P450IID6, were tested for their ability to modify P450IID function. We also conducted *in vitro* immunological studies using anti-liver kidney microsome antibody (LKMA) against P450 from the IID subfamily. It was recently demonstrated that this auto-antibody found in autoimmune hepatitis specifically recognize a microsomal protein of 48 kDa in human liver, identified as P450IID6 [9].

MATERIALS AND METHODS

Chemicals and equipment

Debrisoquine hemisulfate and 4-hydroxydebrisoquine hemisulfate, dextromethorphan hydrobromide and dextrorphan tartrate, (+)-bufuralol

‡ Abbreviations: P450, cytochrome P450; anti-LKMA, anti-liver kidney microsome antibody; anti-SMA, anti-smooth muscle antibody; DEM, dextromethorphan; DOR, dextrorphan; NADPH/O_2, NADPH/O_2 regenerating system; CuOOH, cumene hyperoxide system.

and 1′-hydroxybufuralol were generously provided by Hoffman-La Roche (Basle, Switzerland). Mephenytoin was kindly supplied by Professor Branch (Nashville, TN, U.S.A.), phenobarbital by Professor Bourdon (Paris, France), metoprolol by Dr J. P. Thenot (LERS, Meudon, France) and flecainide acetate by Riker 3M (Rueschlikon, Switzerland). NADPH, isocitrate, isocitrate deshydrogenase and quinidine sulfate were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals and solvents were analytical grade.

The extraction procedures, chromatographic conditions and equipment have been previously reported [9, 10]. Stainless steel columns were packed with Nucleosil 5-C-18 (dp 5 μm, Macherey-Nagel, Duren, F.R.G.) for debrisoquine and dextromethorphan, or with Sherisorb ODS (dp 5 μM, Phase separations, Norwolk, CT, U.S.A.) for bufuralol.

Metabolic assays

Liver samples—preparation of microsomes. Primate livers were obtained from two extensive metabolizer monkeys (*Macaca fascicularis*) with debrisoquine/4-hydroxydebrisoquine metabolic ratio of 0.12 and 0.06, respectively. Human, primate and rat liver microsomes were prepared as described [11]. Protein concentrations in microsomal preparations were determined according to the method of Lowry *et al.* [12].

Assay of debrisoquine 4-hydroxylase activity. Microsomal protein (1000 μg) were preincubated for 10 min at 37° with 100 μL of the NADPH regenerating system (NADP: 10 mM, isocitrate: 50 mM, isocitrate deshydrogenase: 10 units/mL, $MgCl_2$: 50 mM) in 0.1 M sodium phosphate buffer, pH 7.4. The final volume of incubation was 1 mL. The reaction was started by addition of debrisoquine (5–1000 μM). The reaction was stopped after 30 min by the addition of a citric acid buffer (pH 3). Denaturated protein was precipitated by centrifugation. The production of 4-hydroxydebrisoquine was determined by HPLC with fluorescence detection.

Assay of dextromethorphan O-demethylase activity. Microsomal protein (250 μg) and dextromethorphan (1–500 μM) were incubated for 30 min, as described above. The production of dextrorphan was determined by HPLC with fluorescence detection.

In inhibitory experiments, all drugs but mephenytoin were added as 100 μL of an aqueous solution of 10 times the desired concentration with the substrate. Mephenytoin was added as a 10 μL solution in propylene glycol in an incubation volume of 1 mL. For the screening of drugs, the production of dextrorphan was measured at dextromethorphan concentrations of 5, 50 and 500 μM in control incubations (without inhibitor) and in incubations containing 100 μM of five drugs: debrisoquine, metoprolol, quinidine, mephenytoin and phenobarbital.

In addition, dextromethorphan *O*-demethylase activity was determined over the range of 1.25 to 100 μM of dextromethorphan, in the presence of 5, 20, 50, 100 and 200 μM debrisoquine, and over the range of 0.5 to 50 μM of dextromethorphan in the presence of 0.5, 1, 5 and 10 μM of quinidine.

Assay of bufuralol 1′-hydroxylation activity. Microsomal protein (1–100 μg) was incubated in a final volume of 100 μL containing an NADPH-regenerating system and (+)-bufuralol (1–1000 μM) in 0.1 M sodium phosphate buffer, pH 7.4. The reaction was stopped after 15 min by the addition of 10 μL of 60% $HClO_4$ (w/v). When the NADPH regenerating system was replaced by CuOOH system, 10 μL of a CuOOH solution was added to the microsomal suspension together with (+)-bufuralol (1–1000 μM). Incubation time was between 5 and 10 min. The production of 1′-hydroxybufuralol was determined in $HClO_4$ supernatants by HLPC with fluorescence detection.

In inhibitory experiments, the inhibitor was added as 100 μL of an aqueous solution of 10 times the desired concentration. For the screening of drugs, the production of 1′-hydroxybufuralol was measured at (+)-bufuralol concentrations of 5 and 1000 μM in control incubations (without inhibitor) and in incubations containing 100 μM of eight drugs: dextromethorphan, flecainide, sparteine, propranolol, debrisoquine, quinidine, phenacetin and lidocaine.

Immunological studies

Human sera. Sera were obtained from eight patients at the onset of autoimmune hepatitis. One patient had anti-smooth muscle antibody (SMA), six had anti-LKMA and one had anti-cytosol antibody [13] by immunofluorescence studies. In immunoblot analysis of rat microsomes, four LKMA positive sera showed anti-50 kDa antibody [14] and two anti-66 kDa reactivity [15].

Immunoblot. Proteins from 20 μg of rat liver microsomes, 10 μg of primate liver microsomes and 60 μg of human liver microsomes were separated by electrophoresis in a 10% SDS–polyacrylamide gel. Immunoblot technique was done essentially as described [16]. Serum from a child with autoimmune hepatitis (patient 1), that recognizes the rat liver microsome 50 kDa protein, was used as the first antibody at a dilution of 1 : 1000. The second antibody was a peroxidase conjugated goat anti-human IgG (Biosys, Compiègne, France) used at a dilution of 1 : 1000. The peroxidase activity was detected using diaminobenzidine 50 mg in 100 mL of 50 mM Tris–HCl pH 7.4 and H_2O_2 at a final concentration of 0.01%.

Immunoinhibition studies. The effects of the various sera were studied by preincubation (1 hr at 4°) of primate liver microsomes with various dilutions of patients' sera or control serum (100 μL), followed by the standard incubation procedure. Serum from patient 1 with a high titer of anti-LKMA (anti-50 kDa) was used to determine the complete inhibition kinetics of dextrorphan formation. The concentrations of dextrorphan ranged from 5 to 500 μM, and the titers of the serum ranged from 1:20 to 1:10,000. For all the other patients, the inhibition studies were carried out at two titers (1:100 and 1:500) using a dextromethorphan concentration of 50 μM. Control incubation studies

Table 1. Kinetic parameters of P450IID activity mediated by NADPH/O_2: comparison of monkey and human values

Substrates	Monkey No. 1	Monkey No. 2	Man	
Dextromethorphan				
V_{max}	23.1	36.5	16.7 ± 0.7	10.2 ± 5.3
K_m	4	11	3.9 ± 0.6 (Ref. 8)	3.4 ± 1.04 (Ref. 18)
Debrisoquine				
V_{max}	9.5	11.2	3.8 ± 1.1	4.2 ± 0.9
K_m	120	126	57.5 ± 2.8 (Ref. 8)	120 ± 24 (Ref. 19)
(+)-Bufuralol				
V_{max} 1	58		10.0 ± 2.6	Monophasic
K_m 1	4.3		4.7 ± 2.2	9.2 ± 0.5
V_{max} 2	66		10.9 ± 2.3	18.7 ± 2.8
K_m 2	38		83.5 ± 39.4 (Ref. 8)	(Ref. 19)

K_m is in μM, V_{max} is in nmol (DOR)/mg protein/hr.

were carried with 100 μL of serum obtained from a volunteer blood donor.

Data analysis

All data points were at least assayed in duplicate. Untransformed data of kinetic studies were analysed by an iterative non-linear least-square fitting program (SIPHAR, Société SIMED, France and McIntosh for bufuralol [17]. The estimated kinetic parameters were the maximum velocity (V_{max}) and the apparent affinity constant (K_m). The equations assuming one or two independent (additive) enzymatic activities were tested. The apparent dissociation constant (K_i) of the enzyme–inhibitor complex was determined from Dixon plots of the data.

RESULTS

Kinetic studies of prototype substrates

Debrisoquine 4-hydroxylation kinetics. The formation of 4-hydroxydebrisoquine was linear with respect to protein (125–500 μg/assay) and time (15–60 min). Formation of metabolite, in the range of debrisoquine concentrations tested was best described by the assumption of monophasicity. The K_m were 120 and 126 μM and the V_{max} were 9.5 and 11.2 nmol/mg protein/hr for the two liver microsome preparations.

Dextromethorphan O-demethylation kinetics. The formation of dextrorphan was linear with respect to protein (250–1000 μg/assay) and time (15–60 min). Formation of dextrorphan, in the range of dextromethorphan concentrations tested, was best described by the assumption of monophasicity. The K_m were 4 and 11 μM and the V_{max} were 23 and 37 nmol/mg protein/hr for the two liver microsome preparations.

Bufuralol 1′-hydroxylation kinetics. The formation of 1′-hydroxybufuralol was linear with respect to protein (50–400 μg/assay) and time (5–120 min). The kinetic characteristics of (+)-bufuralol 1′-hydroxylation were biphasic and identified two components of the enzymatic reaction with the NADPH/O_2 assay system with K_m values of 4.3 and 38 μM. With the CuOOH assay system, a biphasic reaction was also detected. The K_m values of the high and low affinity components of enzyme activity were 5.5 and 875 μM, respectively.

The results are presented in Tables 1 and 2 and are compared with the data derived from the different human studies reported in the literature, as indicated by the references numbers.

Table 2. Kinetic parameters of microsomal bufuralol 1′-hydroxylation mediated by NADPH/O_2 or CuOOH: comparison of monkey and human values

Assay system	Monkey K_m	Monkey V_{max}	Man (Ref. 20) K_m	Man (Ref. 20) V_{max}
NADPH/O_2				
Enzyme 1	4.3	58	5.3 ± 0.4	12.0 ± 1.2
Enzyme 2	38	66	121.0 ± 0.4	24.7 ± 1.0
CuOOH				
Enzyme 1	5.5	73	7.3 ± 0.1	86.2 ± 1.0
Enzyme 2	875	194	ND	ND

Results were obtained with (+)-bufuralol as substrate: K_m is in μM (+)-bufuralol, V_{max} is in nmol (1′-OH-bufuralol)/mg protein/hr.
ND, not detected.

Inhibition experiments

A number of compounds were tested for inhibitory effects on dextromethorphan O-demethylation and/or bufuralol 1′-hydroxylation. In man, dextromethorphan [18, 21], propranolol [22], flecainide [23], sparteine [24, 25] metoprolol [22] and debrisoquine [3, 25] are substrates of P450IID6. Quinidine is a competitive inhibitor of P450IID6 activity but is not a substrate of this enzyme [26, 27]. Mephenytoin [28, 29], phenobarbital [22],

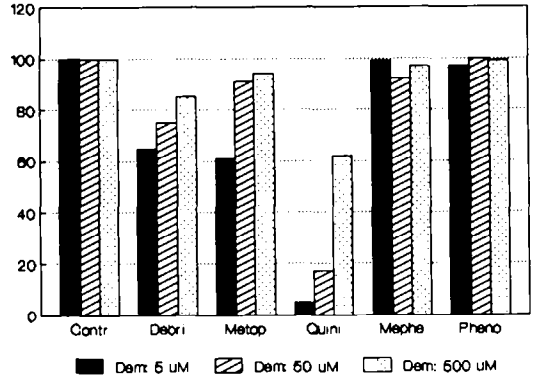

Fig. 1. Inhibition of dextromethorphan O-demethylation by various compounds. Incubation conditions: dextromethorphan 5, 50 and 500 μM; inhibitors 100 μM. From left to right: debrisoquine (debri), metoprololol (metop), quinidine (quini), mephenytoin (mephe) and phenobarbital (pheno). Results are expressed as percentages of control activity (in the absence of inhibitor).

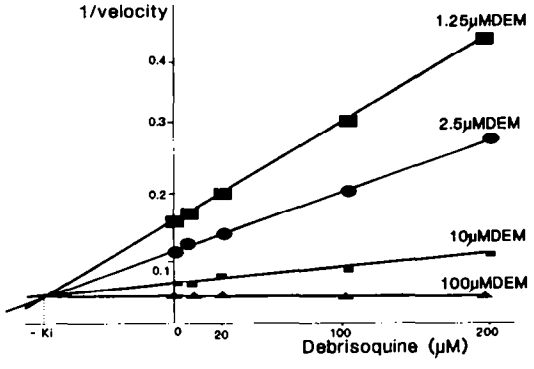

Fig. 2. Competitive inhibition of dextromethorphan O-demethylation by debrisoquine (Dixon plot). Velocity is in nmol (DOR)/mg protein/hr.

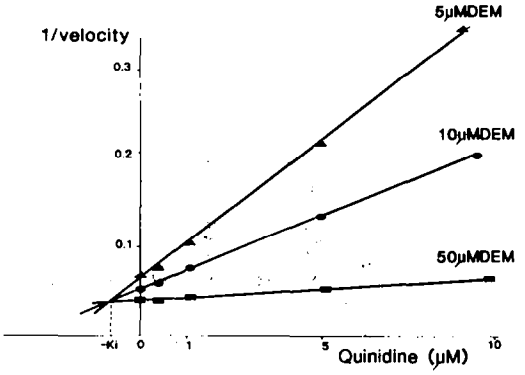

Fig. 3. Competitive inhibition of dextromethorphan O-demethylation by quinidine (Dixon plot). Velocity is in nmol (DOR)/mg protein/hr.

phenacetin [30] and lidocaine [31] are not substrates of P450IID6.

Inhibition of dextromethorphan O-demethylation by various substrates. Debrisoquine, metoprolol and quinidine inhibited dextrorphan formation, while mephenytoin and phenobarbital had no effect on dextromethorphan metabolism (Fig. 1).

In addition, kinetics of inhibition were determined with debrisoquine and quinidine. The K_i for debrisoquine was 90 μM (Fig. 2) and it was 0.75 μM for quinidine (Fig. 3).

Inhibition of bufuralol 1'-hydroxylation by various compounds. Eight drugs were tested in the presence of either NADPH/O_2 or CuOOH (Fig. 4A and B). Dextromethorphan, propranolol, flecainide, sparteine, debrisoquine and quinidine inhibited 1'-bufuralol hydroxylation. The inhibitory potency was higher for all inhibitors in kinetic reactions mediated by CuOOH system when compared to NADPH/O_2 system. Lidocaine and phenacetin had no effect on bufuralol 1'-hydroxylation.

Immunological studies

Immunoblot analysis (Fig. 5). Serum No. 1 showing a reactivity with a protein of 50 kDa (P450 db1 and db2) in rat liver microsomes (Fig. 5, lane a), and a protein of 48 kDa (P450 db1) in human liver microsomes (Fig. 5, lane b) was used to identify P450s from the IID subfamily in primate liver microsomes. Two proteins of 49 and 47 kDa, respectively (Fig. 5, lane c) were recognized by this specific antibody. Apparently, these proteins are present in similar amounts.

Immunoinhibition studies. The kinetics of dextrorphan formation were analysed in the presence of various titers of a serum that specifically recognized 49 and 47 kDa proteins in primate liver microsomes (serum No. 1). A strong inhibitory effect was observed at serum dilutions of 1:1000 or lower, when compared to controls, and resulted in a decrease in V_{max} and an increase in K_m. The maximum inhibitory potency occurred at dextromethorphan concentration of 50 μM. In contrast, the dextrorphan formation was not inhibited in incubations containing this serum at titers of 1:5000 and 1:10,000 or containing control serum.

Each of the sera was then blindly assayed for its ability to inhibit dextromethorphan metabolism *in vitro*. Dextromethorphan (50 μM) was incubated in the presence of two titers of each serum (1:100 and 1:500). Sera containing anti-49 kDa and 47 kDa antibodies reduced DOR formation when compared to control serum or to sera containing other types of autoantibodies. Results are presented in Table 3.

DISCUSSION

We previously reported that non-human primates phenotyped *in vivo* with debrisoquine could be separated between extensive and poor metabolizers using individual metabolic ratios [7]. *In vitro* catalytical and immunological studies were undertaken to characterize cytochrome P450IID function in non-human primates.

In humans, P450IID6 activity *in vitro* has been extensively investigated using prototype substrates:

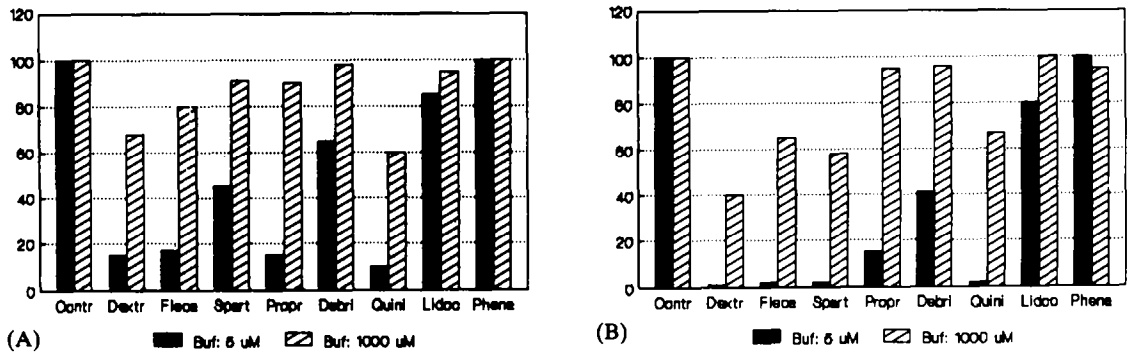

Fig. 4. Inhibition of (+)-bufuralol 1'-hydroxylation in the presence of NADPH (A) or CuOOH (B). Incubation conditions: (+)-bufuralol 5 and 1000 μM; inhibitors 100 μM. From left to right: dextromethorphan (dextr), flecainide (fleca), sparteine (spart), propranolol (propr), debrisoquine (debri), quinidine (quini), lidocaine (lidoc) and phenacetin (phena). Results are expressed in percentages of control activity (in the absence of inhibitor).

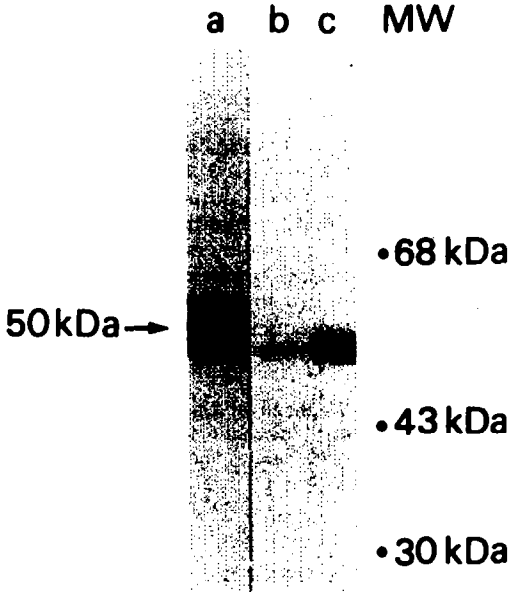

Fig. 5. LKMA recognizes P450 from the IID subfamily in immunoblot analysis of rat (lane a), human (lane b) and primate (lane c) liver microsomes.

debrisoquine [8, 19], dextromethorphan [8, 18] and bufuralol [8, 19]. We conducted kinetic studies in hepatic microsomes using the same compounds. No differences were found between humans and monkeys in the classical NADPH/O_2 mediated metabolism of these three substrates. With debrisoquine and dextromethorphan, the pattern of metabolite formation appeared monophasic in the range of concentrations tested and the apparent Michaelis–Menten constants (K_m) were similar to the value of the high affinity component of enzyme activity reported in humans [8, 18, 19]. An additional low affinity component of enzyme activity characterized by a high K_m was detected in humans with the two substrates at concentrations over 1 mM. With (+)-bufuralol, the pattern of metabolite formation appeared biphasic and was also multiphasic in human microsomal preparations. The exact number of enzymes involved in the measured enzymatic activity is always difficult to determine with kinetic studies at the microsomal level. However, in humans the CuOOH-mediated bufuralol 1'-hydroxylation appeared exclusively catalysed by P450bufl (P450IID6), this specific isozyme being absent in poor metabolizers of debrisoquine [32]. Therefore, we compared NADPH/O_2 and CuOOH-mediated bufuralol metabolism in non-human primate microsomes. As previously stated, the kinetics of bufuralol 1'-hydroxylation appeared biphasic with NADPH/O_2, but in contrast to human results, it was also biphasic with the CuOOH assay system. This could suggest that monkey microsomes contain at least two active enzymes of P450IID subfamily.

Inhibition experiments were conducted to study the substrate specificity of P450IID subfamily in non-human primates. Seven known substrates of P450IID6 and one competitive inhibitor (quinidine) were tested for their ability to inhibit dextromethorphan or (+)-bufuralol metabolism. They were all and exclusively competitive inhibitors of P450IID function in non-human primates. When debrisoquine was studied, it demonstrated a competitive inhibition of dextromethorphan metabolism. In addition, the K_i for inhibition was similar to the K_m for metabolism suggesting that the same P450 was involved in the oxidation of debrisoquine and dextromethorphan [25]. In contrast, four drugs known not to be dependent upon P450IID6 for their metabolism had no effect on P450IID activity in primates. Among the drugs studied, quinidine is of particular interest. In humans, this drug is not a substrate but the most potent competitive inhibitor of P450IID6 activity *in vitro*. *In vivo*, the elimination of drugs via P450IID6 was impaired by the co-administration of quinidine at low doses [26]. Such an inhibition test may be useful to determine if the

Table 3. Relative dextrorphan formation in incubations containing primate microsomal proteins (250 μg) and dextromethorphan (50 μM) in the presence of control serum or anti-50 kDa, anti-66 kDa or anticytosol antibodies

Patients	Type of antibodies (ELISA)	Serum titer	DOR formation nmol/mg protein/hr	Relative DOR formation (%)
Control	0	0	55.2	100
1	Anti-50 kDa	1/100	15.1	27.3
		1/500	17.4	31.5
2	Anti-50 kDa	1/100	13.4	24.2
		1/500	36.6	66.2
3	Anti-50 kDa	1/100	26.5	48.0
		1/500	42.6	77.1
4	Anti-50 kDa	1/100	26.7	48.3
		1/500	40.2	72.8
5	Anti-66 kDa	1/100	53.8	97.3
		1/500	53.5	96.8
6	Anti-66 kDa	1/100	42.7	77.2
		1/500	53.8	97.3
7	Anticytosol	1/100	51.4	93.0
		1/500	54.8	99.2
8	Anti-SMA	1/100	54.8	99.2
		1/500	54.9	99.4

metabolism of new drugs is linked with the genetically controlled routes of metabolism, as previously reported [33]. The K_i value of quinidine in monkey microsomes was 0.75 μM demonstrating the strong inhibitory potency of this compound and indicating that it may be used for *in vitro* and *in vivo* inhibition tests of P450IID function in non-human primates.

The antibody against the P450IID subfamily allowed further characterization of this primate system. This auto-antibody found in autoimmune hepatitis, specifically recognize a microsomal protein of 50 kDa in rats [13], and 48 kDa in human liver [34], identified as P450 db1 and db2 in rats (P450IID1 and 2) [35] and P450 db1 in humans (P450IID6) [9]. Immunoblot analysis of primate liver microsomes showed that this auto-antibody cross-reacted with two proteins of 49 and 47 kDa. This auto-antibody recognizes denatured and native P450s. Therefore it can be assumed that, as was observed in rats, this primate expressed at least two members of the IID subfamily, found in similar amounts in adult animals. In addition, *in vitro* studies of dextromethorphan O-demethylation showed that sera from children with autoimmune hepatitis and antibody against P450 from the IID subfamily inhibited P450IID subfamily activity of primate liver microsomes. In contrast, sera containing other types of auto-antibodies and control serum had no effect on DEM metabolism.

It is now admitted that an animal model would provide advantages over human studies and the Dark Agouti rat (DA rat) has been widely investigated in this perspective [36]. However, this rat model exhibited major catalytical differences when compared to man and is not yet validated [37]. The catalytical and immunological similarities, associated with the identical substrate specificity of P450IID between humans and non-human primates indicate that this primate system may represent a useful model for studies of P450IID subfamily polymorphism. Further investigations are necessary to demonstrate the genetic origin of debrisoquine polymorphism in non-human primates. However, it seems already possible to screen the linkage of new drugs with P450IID6 in monkey microsomes and to perform *in vivo* pharmacokinetic experiments using quinidine in order to investigate the possible pharmacokinetic consequences of inhibitors of P450IID function identified *in vitro*.

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