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THE SPECIFICITY OF INHIBITION OF DEBRISOQUINE 4-HYDROXYLASE ACTIVITY BY QUINIDINE AND QUININE IN THE RAT IS THE INVERSE OF THAT IN MAN

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Abstract—The kinetics of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in rat and human liver microsomes have been compared. Quinidine is a potent inhibitor of debrisoquine 4-hydroxylase activity of human liver (IC_{50}: 3.6 μM). However, its stereoisomer, quinine, is some 60 times less potent (IC_{50}:223 μM). Both compounds are able to inhibit > 95% of 4-hydroxylase activity. In rat liver microsomes quinine is approximately 50 times more potent an inhibitor (IC_{50}: 2.4 μM) than quinidine (IC_{50}: 137 μM). Again, 4-hydroxylase activity is inhibited by > 95%. Inhibition of debrisoquine 4-hydroxylase activity by both quinine and quinidine in human and rat liver is competitive. Values of K_i for quinidine in human and rat were 0.6 μM and 50 μM, whereas with quinine the K_i values were 13 μM and 1.7 μM, respectively. The data in this paper are consistent with 4-hydroxylation of debrisoquine in both rat and human liver catalysed by a specific form of cytochrome P-450. Although both quinidine and quinine are competitive inhibitors of debrisoquine 4-hydroxylase activity in rat and man, their potency is reversed. This suggests that the nature of the active site of cytochrome P-450dbl differs between the two species, and indicates that data on the specificity of this isoenzyme in the rat should be extrapolated to man with extreme caution.

The 4-hydroxylation of debrisoquine is polymorphic in man [1], with approximately 8% of the Caucasian population being classified as poor metabolisers (PM) [2]. The frequency of PM subjects from Japan and China appears to be much lower, 0.7% or less [3, 4]. Impaired 4-hydroxylation of debrisoquine is inherited as an autosomal recessive trait [1]. In most subjects the defect appears to be a reduction in the amount of a specific isoenzyme of cytochrome P-450 (P-450dbl)§ catalysing the 4-hydroxylation of debrisoquine [5].

The specificity of cytochrome P-450dbl has been extensively studied *in vitro* using samples of human liver [7, 8]. Although, in some instances, the liver samples were from subjects previously phenotyped *in vivo* as either extensive metaboliser (EM) or PM phenotype they were often obtained from organ transplant donors of unknown phenotype. During such studies it was found that quinidine is a very potent, competitive inhibitor of cytochrome P-450dbl activity [9]. The stereoisomer, quinine, was also a competitive inhibitor, but was some 100-fold less potent [9]. This led to the suggestion that quinidine might provide a highly specific inhibitor that could be used at low dose *in vivo* to determine the contribution of cytochrome P-450dbl to the elimination of a drug. This prediction was born out in subsequent studies [10].

In an effort to find a suitable animal model in which to screen drugs likely to be metabolised polymorphically by cytochrome P-450dbl in man, Al-Dabbagh *et al.* [11] found that the female DA rat had many of the characteristics of the PM phenotype. It has been suggested that females of this strain, together with the male Fischer rat, would provide an animal model of the two phenotypes. However, the validity of this model has been questioned [5, 12]. We have now investigated the effects of quinidine and quinine on debrisoquine 4-hydroxylase activity in the rat, and compared the results with those obtained with human liver.

MATERIALS AND METHODS

Materials. Debrisoquine hemisulphate and 4-hydroxydebrisoquine hemisulphate were generously provided by Roche Products Ltd. (Welwyn Garden City, U.K.). 1,1,3,3-Tetradeutero-4-hydroxydebrisoquine was a generous gift from C. O. Meese (Stuttgart, F.R.G.). Hexafluoroacetylacetone was purchased from Fluorochem Ltd. (Glossop, U.K.). All other chemicals were of analytical grade.

Liver samples. Human liver samples, all from sub-

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§ The nomenclature adopted in this manuscript for the major isoenzymes of cytochrome P450 catalysing the 4-hydroxylation of debrisoquine is as described by Gonzalez and his colleagues, initially in the rat [5], and then extended to man [6]. Cytochrome P-450dbl, the isoenzyme responsible for the polymorphic 4-hydroxylation of debrisoquine in man, is the gene product of P-450IIDI [6].

jects of EM phenotype as judged by debrisoquine 4-hydroxylase activity *in vitro*, were obtained from renal transplant donors [13]. The use of such samples in these studies had local Research Ethics Committee permission. Male Wistar rats (200–250 g) were maintained as previously described [14]. Animals were killed by stunning and exsanguination. Microsomal fractions from human and rat liver samples were isolated by differential ultracentrifugation, suspended in 0.25 M potassium phosphate buffer (pH 7.25) containing 30% (v/v) glycerol and stored at −80° until required [15].

Assay of debrisoquine 4-hydroxylase activity. Debrisoquine-4-hydroxylase activity was assayed by a modification of our previously described method [16, 17]. The standard incubation mixture comprised 50 mM Tris–HCl buffer (pH 7.4), 6 mM $MgCl_2$, 1.2 mM NADPH and approximately 0.75–1.0 mg of microsomal protein, in 50 μl of 0.25 M potassium phosphate buffer (pH 7.25) containing 30% (v/v) glycerol, in a final volume of 1 ml. In inhibition studies, aqueous solutions of quinidine and quinine (to give final concentrations of 0.01–100 μM and 1–1000 μM, respectively) were prepared freshly on the day of use. Samples were preincubated for 2 min at 37° in the presence of quinidine or quinine before the addition of the substrate, debrisoquine. The reaction was started by the addition of an aqueous solution of debrisoquine hemisulphate to give a final concentration of 50–1000 μM. In fixed substrate concentration studies, the final concentration of debrisoquine was 1 mM. Incubations were performed at 37° in air in a shaking water bath for 12 min, the time of incubation and protein concentration having been shown previously to be in the linear range [16]. The reaction was stopped by the addition of 200 μl of ice-cold 1 M NaOH and the samples were transferred to ice. Blanks were incubated without substrate, which was added after the addition of sodium hydroxide. The internal standard, tetradeutero-4-hydroxydebrisoquine, was added to each sample and to a series of standards (1 ml volume) of 4-hydroxydebrisoquine with a range of concentrations from 0–100 ng/ml. Unmetabolized debrisoquine was removed by extraction at pH 13.5 as previously described [16] and the amidine group of the metabolite and the internal standard derivatised at pH 8.5 with hexafluoroacetylacetone. Gas chromatography–mass spectrometry was performed by a modification of our earlier method [16]. The 4-hydroxyl group was not derivatised and the mass spectrometer was operated in the negative ion chemical ionization mode with ammonia as the reagent gas at a source pressure of 0.4 torr. The mass spectrometer was tuned to monitor negative ions at m/z 363 and m/z 369 (the molecular ions of the pyrimidino derivatives of 4-hydroxydebrisoquine and its deuterated internal standard, respectively).

Protein concentration was measured by a modification of the method of Lowry *et al.* [15, 18] with crystalline bovine serum albumin, fraction V, as standard.

Analysis of data. The concentrations of quinidine and quinine causing 50% reduction in debrisoquine 4-hydroxylase activity (IC_{50} values) were determined from a semi-log plot of inhibitor concentration against the percentage of debrisoquine-4-hydroxylase activity remaining. Michaelis–Menten parameters were initially estimated by graphical analysis [14]. Inhibitory constants (K_i values) were obtained graphically by the method of Dixon [19].

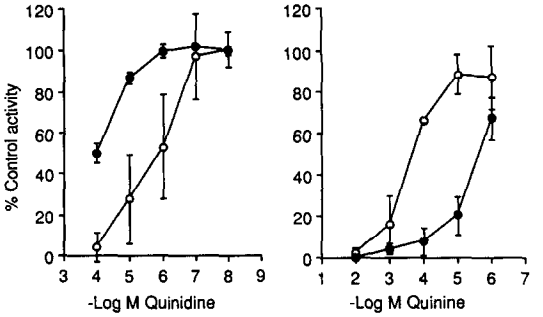

Fig. 1. Effect of quinidine and quinine on human and rat liver microsomal debrisoquine 4-hydroxylase activity. The effects of quinidine (*left*) and quinine (*right*) on human (○) and rat (●) liver microsomal debrisoquine 4-hydroxylase activity are shown. The compounds were added, in a range of concentrations, to samples containing microsomal fractions from human or rat liver which were then preincubated at 37° for 2 min. The reaction was started by the addition of substrate, debrisoquine, to give a final concentration of 1 mM. Values plotted are activities of debrisoquine 4-hydroxylase in the presence of inhibitor, expressed as a percentage of the corresponding control activity in the absence of inhibitor and are means ± SD (N = 3).

Table 1. Values of IC_{50} for the inhibition of debrisoquine 4-hydroxylase activity of rat and human liver by quinidine and quinine

Species	Quinidine	Quinine
Human	3.6 ± 2.0 μM	223 ± 54.4 μM
Rat	137 ± 41.1 μM	2.4 ± 0.6 μM

Samples were preincubated with inhibitor for 2 min at 37° prior to starting the reaction by addition of substrate, debrisoquine, to give a final concentration of 1 mM. Values of IC_{50} (concentration of inhibitor causing a 50% reduction in debrisoquine 4-hydroxylase activity) were determined from the semi-log plot inhibition curves shown in Fig. 1. Values are means ± SD (N = 3).

RESULTS

Both quinine and quinidine inhibited debrisoquine-4-hydroxylase activity of human liver by > 95% (Fig. 1). The same was true for the effects of quinine on the activity of rat liver microsomes. The maximum inhibitory effect of quinidine on activity in the rat could not be determined due to the limit of solubility of this compound.

The marked difference between quinidine and quinine in their potency of inhibition of human debri-

Table 2. Values of K_i for the inhibition of debrisoquine 4-hydroxylase activity of rat and human liver by quinidine and quinine

Species	K_m (μM)	K_i (μM) Quinidine	Quinine
Human	226	0.6	13.0
Rat	60.0	50.0	1.7

Debrisoquine 4-hydroxylase activity was determined over a range of concentrations from 50 to 500 μM, in the presence and absence of inhibitor, as described under Materials and Methods. Values of K_m for debrisoquine 4-hydroxylase activity were determined as described previously [17]. K_i values were determined graphically from Dixon plots [19].

soquine 4-hydroxylase activity [9] was confirmed (Fig. 1), with the IC_{50} for quinidine some 60-fold less than that for quinine (3.6 ± 2.0 μM cf. 223 ± 54.4 μM, Table 1). In direct contrast, quinine was a much more potent inhibitor of activity with rat liver microsomes (Fig. 1), with an IC_{50} value of 2.4 ± 0.6 μM, compared with a value of 137 ± 41.1 μM for quinidine (Table 1).

As previously reported [20], the K_m for debrisoquine 4-hydroxylase activity was lower with microsomes from rat liver than with those from human liver, by some 3–4 fold (Table 2). The kinetics of inhibition of debrisoquine 4-hydroxylase activity by quinine and quinidine were determined. Both compounds were competitive inhibitors with human liver microsomes (Fig. 2, Fig. 4) and with rat liver microsomes (Fig. 3, Fig. 5). Values of K_i for quinidine were 0.6 μM and 50 μM for human and rat liver, respectively and for quinine they were 13 μM and 1.7 μM, respectively (Table 2).

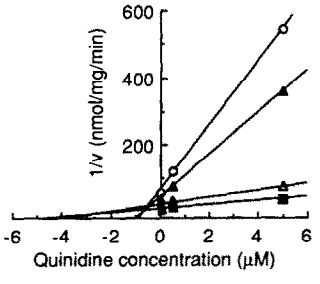

Fig. 2. Kinetics of inhibition of human liver debrisoquine 4-hydroxylase activity by quinidine. Debrisoquine 4-hydroxylase activity of microsomal fractions of human liver was determined, at concentrations of 67 (○), 100 (▲), 250 (△) and 500 (■) μM debrisoquine, in the presence of 0, 0.5 and 5 μM quinidine. Samples were incubated for 2 min at 37° prior to addition of debrisoquine. K_i values were determined graphically by the method of Dixon [19]. The *symbols* show values derived from the experimental data, whereas the *lines* were estimated by linear regression analysis.

DISCUSSION

Both quinine and quinidine are competitive inhibitors of debrisoquine 4-hydroxylase activity of rat and man. The IC_{50} value for inhibition of debrisoquine 4-hydroxylase activity by quinidine in human liver is very similar to that reported previously [10]. One interpretation of such competitive inhibition is that these compounds bind to the same active site on cytochrome P-450dbl as does debrisoquine. Indeed, cytochrome P-450dbl does metabolise quinidine, albeit at a very low turnover rate [21], indicating that binding of this compound to the active site of the isoenzyme must occur. It appears that for the human isoenzyme, this binding is of a very high affinity, explaining the potency of inhibition of debrisoquine 4-hydroxylase activity of human liver. However, Guengerich and his colleagues [21] have also shown that neither the human nor the rat isoenzyme contributes very much to the overall metabolism of quinidine.

Quinine also acts as a competitive inhibitor of cytochrome P-450dbl in man, but much less potently than quinidine. This is in agreement with previous studies [9, 10] on the effects of these two compounds on both sparteine and debrisoquine oxidation. Both compounds inhibit debrisoquine 4-hydroxylase activity by > 95%. Given the competitive and selective nature of this inhibition, this is further evidence for the predominant contribution of cytochrome P-450dbl in the 4-hydroxylation of debrisoquine [8] and supports the suggestion that such inhibition is diagnostic of such involvement [10].

Although both quinine and quinidine are competitive inhibitors of debrisoquine 4-hydroxylase activity of rat liver, the rank order of potency is the reverse of that with human liver. As with human liver, > 95% of activity in the rat could be inhibited by quinine. Thus, either several isoenzymes are all potently, and competitively inhibited by this compound, or only one isoenzyme is involved. Studies with polyclonal antibodies to P-450dbl suggest that the latter explanation is the more likely [8]. It appears that P-450dbl in the rat can metabolise quinidine, although this is not the major isoenzyme involved

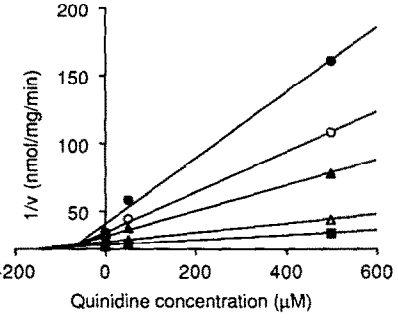

Fig. 3. Kinetics of inhibition of rat liver debrisoquine 4-hydroxylase activity by quinidine. Debrisoquine 4-hydroxylase activity of microsomal fractions of rat liver was determined at concentrations of 50 (●), 67 (○), 100 (▲), 250 (△) and 500 (■) μM debrisoquine, in the presence of 0, 50 and 500 μM quinidine. Further details are as described in the legend to Fig. 2.

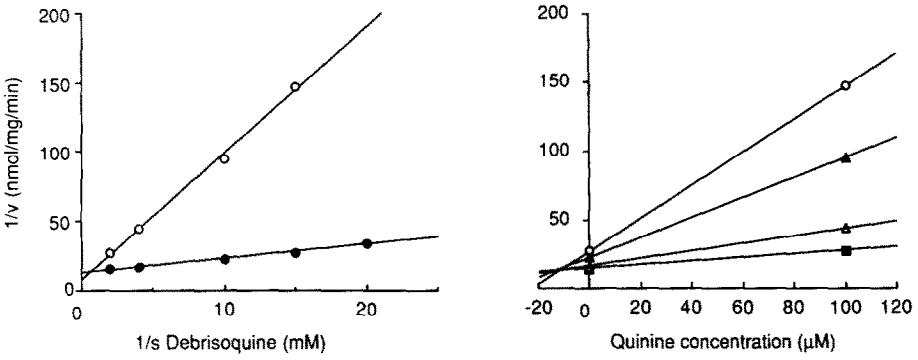

Fig. 4. Kinetics of inhibition of human liver debrisoquine 4-hydroxylase activity by quinine. Data are presented in the form of a Lineweaver–Burk plot (*left*) in which debrisoquine 4-hydroxylase activity of microsomal fractions of human liver was determined with 0 (●) and 100 (○) μM quinine and of a Dixon plot (*right*) with concentrations of 67 (○), 100 (▲), 250 (△) and 500 (■) μM debrisoquine. Further details are as described in the legend to Fig. 2.

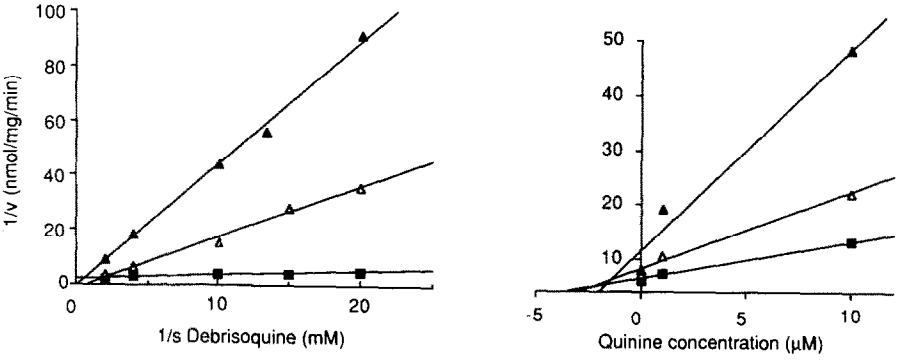

Fig. 5. Kinetics of inhibition of rat liver debrisoquine 4-hydroxylase activity by quinine. Data are presented in the form of a Lineweaver–Burk plot (*left*) in which debrisoquine 4-hydroxylase activity of microsomal fractions of rat liver was determined with 0 (■) 1 (△) and 10 (▲) μM quinine and of a Dixon plot (*right*) with concentrations of 100 (▲), 250 (△) and 500 (■) μM debrisoquine. Further details are as described in the legend to Fig. 2.

[21]. There is no information on the degree of involvement of cytochrome P-450dbl in the rat in the oxidation of quinine in this species.

The difference between rat and human debrisoquine 4-hydroxylase in response to these stereoisomers suggests a fundamental difference in the active sites of the two isoenzymes. Evidence for such differences has been obtained previously, by both substrate and antibody inhibition studies [5, 12]. With human liver, the methoxy side chain of quinidine appears necessary for potent inhibition, as cinchonine and cinchonidine, although inhibitors, have similar potency to quinine [9]. This structural feature is obviously not necessary for inhibition of the rat isoenzyme, for which quinine was the more potent inhibitor. However, the effects of cinchonine and cinchonidine on the activity of this isoenzyme are not yet known.

These studies have confirmed the potent, competitive inhibition of human debrisoquine 4-hydroxylase activity by quinidine. Although quinidine is a competitive inhibitor of activity in the rat, quinine was the more potent inhibitor in this species. This provides further weight to the suggestion that the active site of debrisoquine 4-hydroxylase in the rat is qualitatively different from that of the human isoenzyme [12]. Extrapolation from studies performed in the rat must therefore be viewed with caution.

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