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BUFURALOL 1′-HYDROXYLASE ACTIVITY OF THE RAT

STRAIN DIFFERENCES AND THE EFFECTS OF INHIBITORS

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Abstract—The kinetics of bufuralol 1′-hydroxylase activity of hepatic microsomal fractions have been determined in female DA and Fischer 344 rats, strains between which there is a large difference in debrisoquine 4-hydroxylase activity. Two components of bufuralol 1′-hydroxylase activity could be observed in both strains. Although there were differences between the strains in V_{max} and K_m of both components of activity, these were much less marked than the differences previously reported for debrisoquine 4-hydroxylase (Kahn *et al., Drug Metab. Dispos.* **13**, 510 (1985)). The kinetics of bufuralol 1′-hydroxylase activity were such that the difference in activity between the strains varied with the concentration of bufuralol, 4–5-fold at 2.5 μM, no difference at 100 μM. Competitive inhibitors of debrisoquine 4-hydroxylase activity in man were competitive inhibitors of bufuralol 1′-hydroxylase activity in the Fischer 344 rat, but not in the DA rat. The K_i for inhibition of bufuralol 1′-hydroxylase activity by debrisoquine in the Fischer 344 rat was 184 μM, compared with a K_m for the 4-hydroxylation of this compound of 10.5 μM. It is concluded that the major isozyme of cytochrome P-450 catalysing the 1′-hydroxylation of bufuralol in the rat is different from that catalysing debrisoquine 4-hydroxylation ($P\text{-}450_{UT\text{-}H}$).

Since it was first reported that the 4-hydroxylation of debrisoquine exhibits a genetic polymorphism in man [1], numerous other drugs have been shown to exhibit impaired oxidation in the poor metaboliser (PM)† phenotype [2, 3]. Amongst the most extensively studied of these are bufuralol, for which the 1′-hydroxylation is impaired [4] and sparteine [5]. In an early report [6], it was suggested that the defect was due to the absence or deficiency of a specific isozyme of cytochrome P-450. This has since been confirmed by others [7], and work by Guengerich and co-workers [8] and by Meyer and co-workers [9], using techniques of protein purification and immunoinhibition has demonstrated beyond doubt that the PM phenotype has a functional deficiency in a specific form of cytochrome P-450 (which has been designated $P\text{-}450_{DB}$ [10]). However, it is still not clear whether the defect is structural or regulatory.

Many workers are now using the female DA rat as a model for the human PM phenotype for debrisoquine 4-hydroxylation, following a report by Al-Dabbagh *et al.* [11], that the female of this strain is deficient in debrisoquine 4-hydroxylase activity, relative to other strains. This has been confirmed in studies with microsomal fractions [12] and the purified enzyme [13]. It was suggested that the defect in the DA rat is due simply to a reduction in the amount of a specific isozyme produced ($P\text{-}450_{UT\text{-}H}$) [13]. However, studies on the kinetics of debrisoquine 4-hydroxylation suggest that the situation might be somewhat more complex [14]. The female DA rat also shows impaired oxidation of propranolol and encainide [13], but not of phenacetin [13, 15], all substrates with impaired oxidation in the PM phenotype [3, 16]. However, there is now good evidence that the *O*-deethylation of phenacetin is catalysed by a different isozyme to that catalysing the 4-hydroxylation of debrisoquine in both rat [13] and man [10, 17]. Thus, the question remains as to how faithful a model is the female DA rat for the human PM phenotype.

Studies involving a variety of techniques have shown that the 1′-hydroxylation of bufuralol and the 4-hydroxylation of debrisoquine are catalysed by the same isozyme of cytochrome P-450 in man [9, 10, 18, 19]. There is very little information on the metabolism of bufuralol in the rat, although Guengerich and co-workers have shown that cytochrome $P\text{-}450_{UT\text{-}H}$ is active in catalysing its 1′-hydroxylation [20]. We have performed a detailed comparison *in vitro* of the kinetics and inhibition of the hepatic oxidation of bufuralol in female Fischer 344 and DA rats to compare with the results of a similar study performed in man [19], in an attempt to validate the female DA rat model and to determine the explanation for the functional deficiency in oxidising activity in this strain.

MATERIALS AND METHODS

(±)Bufuralol hydrochloride and 1′-hydroxybufuralol were generous gifts of Roche Products plc (Welwyn Garden City, U.K.). NADPH (tetrasodium salt, type I) and bovine serum albumin (fraction V) were purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Methyl-*t*-butyl

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† Abbreviations used: HPLC, high pressure liquid chromatography; IC_{50}, the concentration of an inhibitor producing 50% inhibition of activity; PM phenotype, poor metaboliser phenotype as defined in [1].

ether and acetonitrile, both of HPLC grade, were purchased from Rathburn Chemicals (Walkerburn, Scotland). All other reagents were of Analar grade.

Female DA and Fischer 344 rats, 150–200 g body wt, were obtained from Bantin and Kingman Limited (Hull, Humberside, U.K.) and maintained in a controlled environment (constant heating and lighting cycles) for at least one week prior to use. The animals were kept in wire-bottom cages, 6 per cage, and permitted free access to food (PRD diet, Labsure Animal Diets, Poole, U.K.) and water until 16 hr before sacrifice, when the food was withdrawn.

Hepatic microsomal fractions were isolated from the rats as previously described [21] and stored as aliquots at −80° in suspension in 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol. Bufuralol 1′-hydroxylase activity was determined essentially as described previously [19]. The incubation mixture, in a total volume of 0.25 ml, comprised 50 mM Tris–hydrochloride buffer, pH 7.4, 5 mM magnesium chloride, 2.4 mM NADPH and 0.1 mg microsomal protein. Both the substrate and, when used, the inhibitors were added as aqueous solutions. In the inhibition studies, the samples were preincubated for 5 min at 37° and the reaction started by the addition of NADPH. The reaction was performed under conditions where velocity was linear with respect to both protein concentration and time of incubation. The samples were extracted and analysed by normal phase HPLC, using Spherisorb 5 μm silica (Shandon Southern Products Limited, Runcorn, Cheshire, U.K.) as stationary phase, as previously reported [19].

Microsomal protein content, with bovine serum albumin, fraction V, as standard, was determined as previously described [21].

Analysis of results. The kinetics of the 1′-hydroxylation of bufuralol by microsomal fractions from rat liver were biphasic. The data have therefore been analysed, assuming a two component system, by initially estimating the Michaelis–Menten parameters by graphical analysis followed by computerised, non-linear iteration using the graphically determined values as first estimates [14]. IC_{50} and K_i values were calculated as before [22]. Where shown, variation was determined with microsomal fractions from different animals.

RESULTS

The kinetics of the 1′-hydroxylation of bufuralol by microsmal fractions from liver were biphasic for both the DA and Fischer 344 rat (Fig. 1). All four of the Michaelis–Menten constants characterising the two components of 1′-hydroxylation differed between the strains (Table 1). The DA rat showed reduced activity (relative to the Fischer 344 strain) of the high affinity component. This was reflected in both a 2.5-fold decrease in V_{max} 1 and a 2.5-fold increase in K_m1. As a consequence, intrinsic clearance* by this component of activity was approxi-

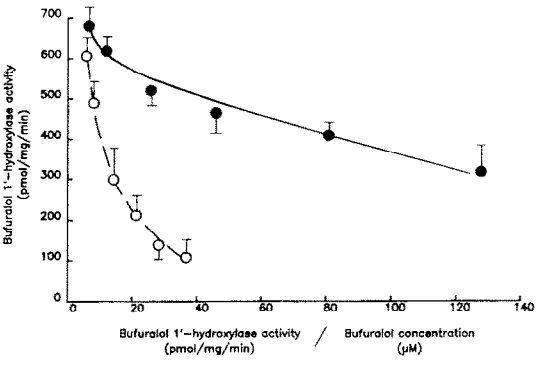

Fig. 1. Strain differences in the Eadie–Hofstee plots for bufuralol 1′-hydroxylase activity of rat liver. The *points* show the mean data ± SE (N = 3) for female Fischer 344 (●) and DA (○) rats. The *lines* were fitted by computer as described in Materials and Methods.

Table 1. Strain differences in the kinetics of hepatic bufuralol 1′-hydroxylase activity of female Fischer 344 and DA rats

Parameter	Strain	
	Fischer 344	DA
$V_{max}1$* (pmole/mg/min)	600 ± 54†	251 ± 21
K_m1 (μM)	2.0 ± 0.1	4.7 ± 0.8
Cl_i1 (μl/min/mg) ($V_{max}1/K_m1$)	296 ± 34	54.6 ± 11.1
$V_{max}2$ (pmole/mg/min)	500 ± 86	1134 ± 110
K_m2 (μM)	299 ± 92	140 ± 46
Cl_i2 (μl/min/mg) ($V_{max}2/K_m2$)	1.7 ± 0.6	8.6 ± 2.4

* $V_{max}1$, K_m1, $V_{max}2$, K_m2, the Michaelis–Menten constants characterising the high affinity (1) and low affinity (2) components of bufuralol 1′-hydroxylase activity respectively. Cl_i1 and Cl_i2, the intrinsic clearance (ratio of V_{max} to K_m) of the two components of bufuralol 1′-hydroxylase activity.

† Values are mean ± SD (N = 3).

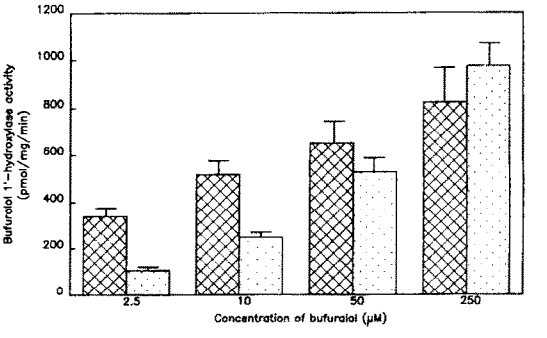

Fig. 2. Effect of substrate concentration on the difference between female Fischer 344 and DA rats in bufuralol 1′-hydroxylase activity. Values are mean ± SE (N = 3) for Fischer 344 (▩) and DA (▨) rats.

* Intrinsic clearance (Cl_i) was calculated from the ratio of V_{max} to K_m. It is a measure of the activity of the enzyme at non-saturating concentrations of substrate, such as usually occur *in vivo*.

Table 2. IC_{50} values of inhibitors of bufuralol 1'-hydroxylase activity* of female Fischer 344 and DA rats

Inhibitor	IC_{50} (mM)† Fischer 344	DA
Phenformin	0.34	>1.0‡
Guanoxan	0.30	>0.5
Sparteine	0.27	>0.5
Debrisoquine	>1.0	>1.0
Acetanilide	>5.0	2.3
Amylobarbitone	>10.0 (stimulation)	0.38

* Activity was determined at a substrate concentration of 10 μM.

† Values are the mean of duplicate determinations that varied by less than 10% from each other.

‡ Highest concentration of compound tested.

mately 6-fold lower in the DA strain. In contrast, activity of the low affinity component of activity was elevated in the DA strain, reflected in both an increase in $V_{max}2$ and a decrease in K_m2. These changes resulted in a 5-fold difference in intrinsic clearance for this component of activity between the strains. However, the intrinsic clearance due to the high affinity component of activity was still some 7-fold higher than that of the low affinity component in the DA rat, and was 180-fold higher in the Fischer 344 strain. Thus, there was a major difference in the ratio of intrinsic clearances due to the high and low affinity components of activity, respectively, in the two strains. This had a marked effect on bufuralol 1'-hydroxylase activity determined at different substrate concentrations in the Fischer 344 and DA rat (Fig. 2). At low concentrations, the Fischer 344 rat was 4–5 times more active than the DA rat but as the concentration of bufuralol increased, this difference reduced so that at high substrate concentrations, the DA rat was more active than the Fischer 344 strain.

The effects of a variety of compounds, of known specificity for cytochrome $P\text{-}450_{DB}$ in man, on bufuralol 1'-hydroxylase activity of the rat were determined (Table 2). Over the concentration range tested, all of the compounds known to show impaired oxidation in the PM phenotype, with the exception of debrisoquine, were more potent inhibitors of bufuralol 1'-hydroxylase activity in the Fischer 344 rat than in the DA rat. Acetanilide and amylobarbitone, two compounds whose oxidation is not impaired in the PM phenotype, were more potent inhibitors in the DA strain than in the Fischer 344 strain. A major difference in effect was obtained with amylobarbitone. At 5 mM this compound inhibited bufuralol 1'-hydroxylase activity in the DA rat by 85% whereas in the Fischer 344 strain it stimulated activity to 156% of control values.

The kinetics of inhibition by the compounds shown in Table 2 were determined in both DA and Fischer 344 rats. The results are shown in Table 3. In the Fischer 344 rat, the four compounds with impaired oxidation in the PM phenotype: debrisoquine, sparteine, guanoxan and phenformin, were all competitive inhibitors of the high affinity component of activity and, with the exception of guanoxan, were without effect on the low affinity component of activity. K_i values for the high affinity component were 40 to 180 μM. Guanoxan was a weak, non-competitive inhibitor, K_i 0.35 mM, of the low affinity component of activity.

Kinetic studies in the DA rat showed that debrisoquine and sparteine were without effect on either component of bufuralol 1'-hydroxylase activity in this strain. Amylobarbitone was a non-competitive inhibitor of both components of activity (K_i 0.46 and 0.87 mM, respectively for the high and low affinity components). Acetanilide was a competitive inhibitor of the high affinity component of activity (K_i 0.7 mM) and a non-competitive inhibitor of the low affinity component (K_i 0.8 mM).

DISCUSSION

The kinetics of the 1'-hydroxylation of bufuralol by hepatic microsomal fractions from both female DA and Fischer 344 rats are biphasic, like those of 4-hydroxylation of debrisoquine [14]. When bufuralol 1'-hydroxylase activity is determined in micro-

Table 3. Strain differences in the kinetics of inhibition of hepatic bufuralol 1'-hydroxylase activity of female Fischer 344 and DA rats

Inhibitor	Component of activity*	Fischer 344 Type of inhibition	Fischer 344 K_i (μM)	DA Type of inhibition	DA K_i (μM)
Phenformin	V 1	C†	41.3	N.D.‡	
	V 2	None	—	N.D.	
Guanoxan	V 1	C	42.0	N.D.	
	V 2	NC	350	N.D.	
Sparteine	V 1	C	98.3	None	—
	V 2	None	—	None	—
Debrisoquine	V 1	C	184	C	904
	V 2	None	—	None	—
Acetanilide	V 1	N.D.		C	705
	V 2	N.D.		NC	810
Amylobarbitone	V 1	N.D.		NC	461
	V 2	N.D.		NC	872

* V 1, V 2 indicate the high affinity and low affinity components of activity respectively.

† C, NC indicate competitive and non-competitive inhibition, respectively.

‡ N.D. Not determined.

somal fractions at low substrate concentrations, less than 10 μM, the DA rat has substantially lower activity than the Fischer 344 rat. However, at higher concentrations, >50 μM, there is little or no difference in activity between the strains. Debrisoquine 4-hydroxylase activity shows a similar pattern, the most marked difference in activity being apparent at the lowest substrate concentrations. However, with this substrate, the DA rat always has lower activity than the Fischer 344 rat, even at concentrations of debrisoquine as high as 2.5 mM [14].

When the kinetics of bufuralol 1'-hydroxylase activity were determined in the 2 strains of rat, it was apparent that V_{max} for the high affinity component is not dramatically reduced in the DA rat, unlike debrisoquine 4-hydroxylase activity [14]. However, the K_m of this component is increased in the DA strain, so that there is a 5-fold difference in intrinsic clearance by the high affinity component of activity between the 2 strains. Of some interest was the observation that intrinsic clearance by the low affinity component is some 5-fold greater in the DA rat than in the Fischer 344 rat. Intrinsic clearance by this component of activity is less than by the high affinity component, in both strains. However, in the Fischer 344 rat the ratio of intrinsic clearances is over 150-fold, whereas in the DA rat the ratio is only 7-fold. Thus, the differences one will observe between the strains in the metabolism of the drug *in vivo* will depend very substantially on the dose administered. Indeed, this must be a very powerful argument for performing full kinetic analysis *in vitro*.

It has been suggested that the substrate specificity of the isozyme of cytochrome P-450 impaired in the DA rat (UT-H) is similar to that impaired in the debrisoquine oxidation polymorphism in man [8]. Two questions that arise from this are:

(1) Does such specificity, indeed, exist?
(2) Are the causes of impaired oxidation the same in rat and man?

The results of the present study suggest that relative substrate specificity differs between rat and man. Although purified cytochrome P-450$_{UT-H}$ has been shown to catalyse the 1'-hydroxylation of bufuralol [20], this does not appear to be the major enzyme catalysing this reaction in the rat. Whereas there is a 15-fold difference in V_{max} of the high affinity component of debrisoquine 4-hydroxylase between the strains, with no difference in K_m [14], for bufuralol 1'-hydroxylase there is only a 2.5-fold difference in V_{max} of this component, with a 2.5- to 3-fold difference in K_m. In addition, although the K_m of the high affinity component of debrisoquine 4-hydroxylation in the Fischer 344 rat is 10.4 μM [14], its K_i for inhibition of bufuralol 1'-hydroxylation is 184 μM. Thus, presumably the isozyme contributing most to the high affinity component of bufuralol 1'-hydroxylase activity is not P-450$_{UT-H}$. However, these conclusions notwithstanding, there does appear to be some similarity in the relative specificity of the enzyme catalysing bufuralol 1'-hydroxylation in the Fischer 344 rat with the analogous human enzyme. Both activities are competitively inhibited by debrisoquine, sparteine, guanoxan and phenformin, whereas neither activity is competitively inhibited by acetanilide or amylobarbitone [22, this study]. Indeed, activity in both species is stimulated by the latter compound.

It has been shown by Guengerich and his colleagues [13] that the hepatic microsomal fraction from the female DA rat contains only 5% as much cytochrome P-450$_{UT-H}$ as that from other strains of rat. This suggests that the defect in the DA rat is regulatory. However, if the difference in the strains was only in the amount of isozyme present, then the inhibition profile of bufuralol 1'-hydroxylase activity should be the same in the two strains. This is obviously not so. None of the compounds inhibiting activity in the Fischer 344 rat had any effect on activity in the DA rat. In addition, acetanilide and amylobarbitone inhibit activity in the DA rat, whereas they do not inhibit activity in the Fischer 344 strain. If the difference between the strains is due to an isozyme other than P-450$_{UT-H}$ then this difference appears to be structural rather than regulatory. But, in the Fischer 344 rat, the affected enzyme also appears to have a similar, relative specificity to that of cytochrome P-450$_{UT-H}$ and indeed the same as human cytochrome P-450$_{DB}$, based on inhibition studies.

There seems little doubt that the female DA rat cannot be used as a model for determining the molecular basis for the polymorphism in the oxidation of debrisoquine in man. The use of this strain to predict which substrates might show impaired oxidation in man has yet to be validated. However, if more than one isozyme is affected in the DA rat then it seems likely that their specificity will differ and some substrates affected in the rat will not be affected in man and vice versa.

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