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METOPROLOL OXIDATION BY RAT LIVER MICROSOMES

INHIBITION BY DEBRISOQUINE AND OTHER DRUGS

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Abstract—The oxidative metabolism of metoprolol has been shown to display genetic polymorphism of the debrisoquine-type. The use of *in vitro* inhibition studies has been proposed as a means of defining whether one or more forms of cytochrome P-450 are involved in the monogenically-controlled metabolism of two substrates. We have, therefore, tested the ability of debrisoquine and other substrates to inhibit the oxidation of metoprolol by rat liver microsomes. Debrisoquine and guanoxan were potent competitive inhibitors of the α-hydroxylation and *O*-desmethylation of metoprolol as well as its metabolism by all routes (measured by substrate disappearance). Cimetidine and ranitidine, drugs which are known to impair the clearance of metoprolol in man, showed an inhibitory action comparable to that of debrisoquine in rat liver microsomes. Antipyrine, a compound whose metabolism is not impaired in poor metabolisers of debrisoquine, was found to be only a weak inhibitor of the metabolism of metoprolol. These findings suggest that the oxidation of metoprolol is linked closely to that of debrisoquine, cimetidine and ranitidine but not to that of antipyrine in the rat.

The oxidative metabolism of metoprolol, a beta-adrenoceptor antagonist, exhibits genetic polymorphism in man and is under similar genetic control to that of the 4-hydroxylation of debrisoquine [1, 2]. *In vitro* studies using human liver microsomes have indicated that the debrisoquine-type polymorphism is a result of either a lack of or the functional inactivity of a single form of cytochrome P-450 [3–5]. Boobis *et al.* [4, 5] have argued that substrates for this enzyme system should be mutually competitive inhibitors and data have been reported which support this hypothesis. For example, sparteine, guanoxan, phenformin and bufuralol, drugs whose metabolism is associated with debrisoquine polymorphism *in vivo* [6–9], inhibited competitively the 4-hydroxylation of debrisoquine *in vitro* [4, 5, 10], whereas antipyrine and acetanilide, whose oxidation does not co-segregate with that of debrisoquine *in vivo* [11, 12], were only weak non-competitive inhibitors [4]. This experimental approach may serve to define whether one or more forms of cytochrome P-450 are involved in the monogenically-controlled metabolism of two substrates and to identify other drugs that may be subject to polymorphic oxidation in man.

It has been suggested that debrisoquine 4-hydroxylation is also polymorphic in rats and the female DA strain has been proposed as a model for the poor metaboliser phenotype [13]. Larrey *et al.* [14] have now isolated a cytochrome P-450 from the livers of Sprague–Dawley rats possessing high specific activity for the 4-hydroxylation of debrisoquine.

The rat may, therefore, prove valuable in the investigation of debrisoquine polymorphism in man. In the present work we have tested the ability of debrisoquine and other drugs to inhibit the oxidative metabolism of metoprolol in liver microsomes from a rat strain with a capacity to 4-hydroxylate debrisoquine.

MATERIALS AND METHODS

Metoprolol tartrate, α-hydroxymetoprolol *p*-hydroxybenzoate, *O*-desmethylmetoprolol base and pamatolol hemisulphate were gifts from AB Hassle (Molndal, Sweden), debrisoquine hemisulphate from Roche Products Ltd. (Welwyn Garden City, Herts), guanoxan hemisulphate from Pfizer Ltd. (Sandwich, Kent), cimetidine base and ranitidine hydrochloride from Dr. D. W. Holt (Poisons Unit, New Cross Hospital, London) and nadolol from Squibb & Sons Ltd. (Hounslow, Middlesex).

An outbred strain of Wistar rats from the University of Sheffield Breeding Colony was used. This strain has been demonstrated to metabolise debrisoquine extensively by 4-hydroxylation [13]. Male rats (200–250 g) were maintained on the standard laboratory diet (Clark Rat and Mouse Diet, K. K. Greeff Ltd., Croydon, U.K.) and allowed access to water *ad libitum*. They were starved for 18 hr, stunned and killed by cervical dislocation. Their livers were removed immediately and microsomes were prepared by differential centrifugation. They were resuspended in 0.2 M potassium phosphate buffer (pH 7.25) containing 30% (w/v) glycerol. Microsomal suspensions were incubated for 5 min with substrate/inhibitor at 37° and pH 7.25 in the presence of NADP (0.17 mM), glucose-6-phosphate (0.35 mM), glucose-6-phosphate dehydrogenase (2 units/ml) and magnesium chloride (42 mM). The above procedures are described in detail by Boobis *et al.* [15]. Incubations were performed in duplicate. For experiments involving the addition of substrate only (K_m and V_{max} determination) the livers from 3 rats were pooled. The determination of inhibition constants required 6 livers per experiment.

Under the experimental conditions used the rates of appearance of α-hydroxymetoprolol and *O*-desmethylmetoprolol were linear with respect to time

and protein concentration. Total protein was measured by the method of Lowry *et al.* [16] and a concentration of 1–1.5 mg/ml was chosen for the kinetic experiments. The reaction was terminated by the addition of 6% (w/v) perchloric acid (0.2 vol. to 1 vol. of incubation mixture).

Metoprolol, α-hydroxymetoprolol and *O*-desmethylmetoprolol were assayed by high performance liquid chromatography [17]. A sample, to which had been added nadolol (200 ng) as internal standard, was diluted with an equal volume of mobile phase (water:acetonitrile 88:12 containing 1% (w/v) triethylamine adjusted to pH 3 with orthophosphoric acid) and an aliquot (10–30 μl) was injected into the chromatograph. The column used was a Waters Z-Module system containing a C_{18} reversed phase packing material (Novapak). The flow rate was 3 ml/min and detection was by fluorescence (Schoeffel 970 FS fluorometer) at an excitation wavelength of 193 nm with no emission filter. In the experiments involving debrisoquine, pamatolol was used as the internal standard since nadolol and debrisoquine could not be resolved.

Apparent Michaelis–Menten constants were obtained from Hanes plots [18] and data from inhibition experiments were analysed by the method of Dixon [18]. Fitting the data using a non-linear extended least squares algorithm (ELSFIT) gave almost identical results. Inhibition of metoprolol oxidation via all routes was assessed by determining values for the concentrations at which the inhibitors impaired the disappearance of metoprolol by 50% ($IC_{50\%}$).

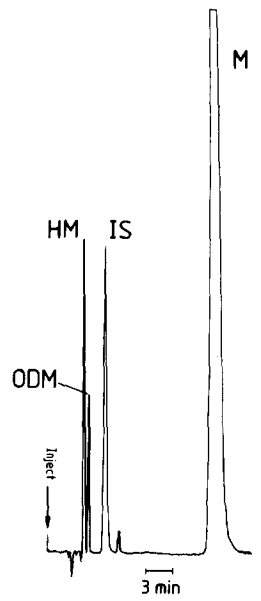

Fig. 1. High performance liquid chromatogram from the analysis of Wistar rat liver microsomes incubated for 5 min at pH 7.25 and 37° with 40 μM metoporolol. HM = α-hydroxymetoprolol, ODM = O-desmethylmetoprolol, IS = Internal Standard (Nadolol) and M = Metoprolol. The concentrations of α-hydroxymetoprolol and *O*-desmethylmetoprolol in the incubation mixture were 0.94 μM and 0.40 μM, respectively.

RESULTS

A chromatogram from a typical incubation of metoprolol with rat liver microsomes is shown in Fig. 1. At a substrate concentration of 20 μM metoprolol, α-hydroxymetoprolol and *O*-desmethylmetoprolol accounted for 17.0% (range 16.8–17.3%, N = 3 experiments from separate batches of pooled livers) and 5.3% (range 4.9–6.1%, N = 3), respectively, of the metabolised drug. Hanes plots for the appearance of α-hydroxymetoprolol and *O*-desmethylmetoprolol are shown in Fig. 2.

Debrisoquine, guanoxan, cimetidine and ranitidine were all potent inhibitors of the α-hydroxylation and *O*-demethylation of metoprolol (Fig. 3, Table 1). Plots of substrate concentration/rate vs inhibitor concentration yielded parallel lines indicating that inhibition was competitive in nature [18]. The oxidation of metoprolol by all routes was also inhibited extensively by these substrates (Table 1).

In contrast, antipyrine was only a weak inhibitor of α-hydroxymetoprolol and *O*-desmethylmetoprolol appearance. The mechanism of inhibition could not be characterised but inhibition constants for both routes were estimated to be greater than 1 mM.

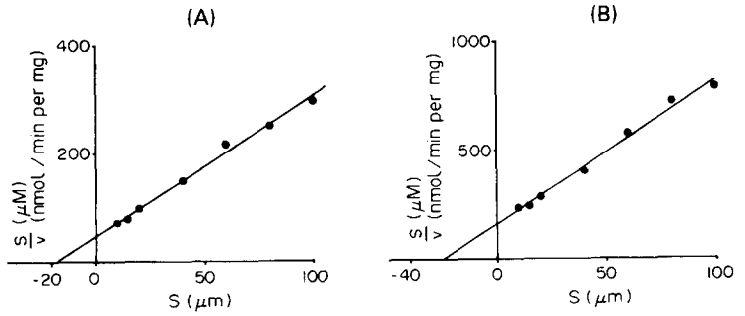

Fig. 2. Hanes plots illustrating the effect of substrate concentration (S) on the rate of appearance (V) of (A) α-hydroxymetoprolol and (B) O-desmethylmetoprolol following a 5 min incubation of metoprolol with rat liver microsomes at pH 7.25 and 37°. The points are mean values from the results of duplicate incubations, all of which differed by less than 10%. The lines represent computer best fits of the data. In 3 experiments values of apparent K_m and V_{max} were 18, 21, 17 (mean 18) μM and 0.26, 0.25, 0.39 (mean 0.29) nmoles min^{-1} mg $protein^{-1}$, respectively, for α-hydroxylation and 24, 19, 10 (mean 18) μM and 0.15, 0.22, 0.14 (mean 0.17) nmoles min^{-1} mg $protein^{-1}$ for O-desmethylation.

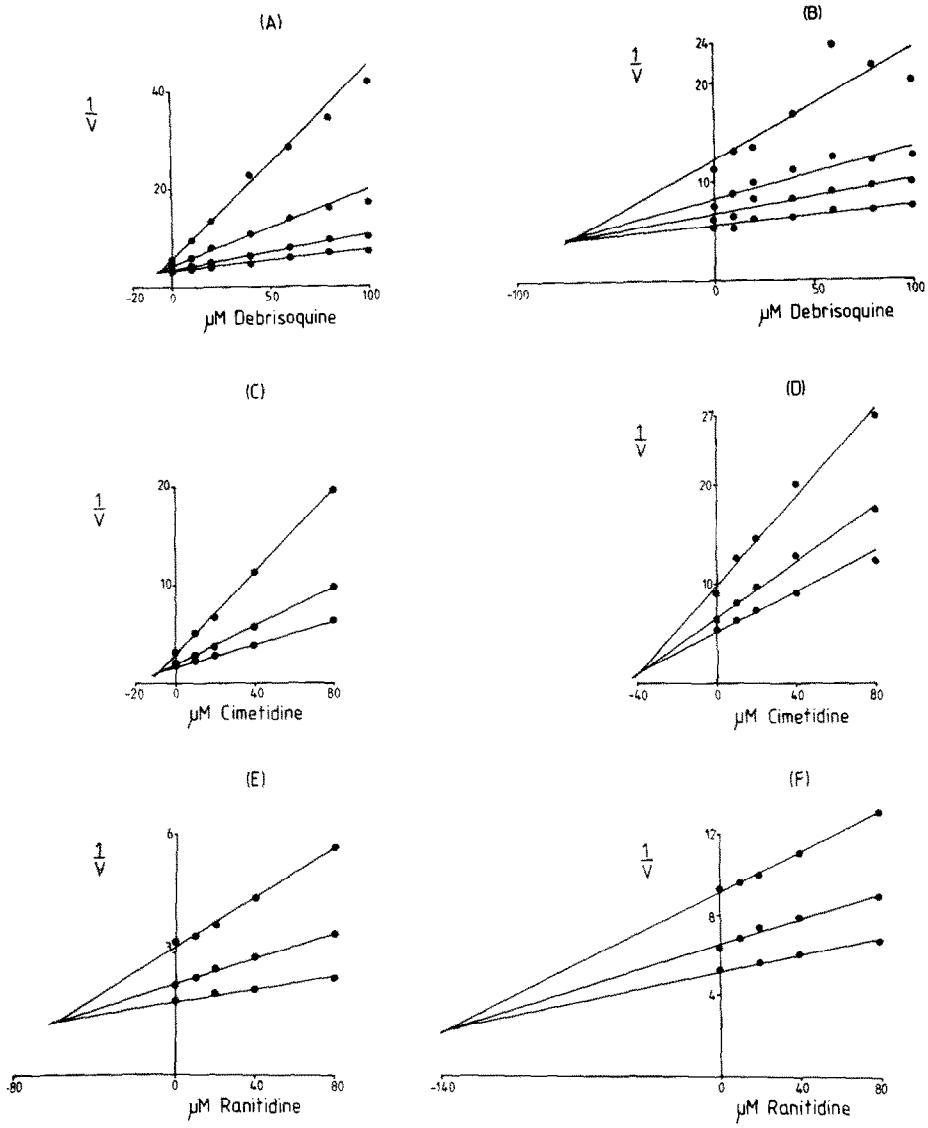

Fig. 3. Dixon plots illustrating the effect of varying concentrations of debrisoquine, cimetidine and ranitidine on the rate of appearance (V, nmoles min^{-1} mg $protein^{-1}$) of (A), (C) and (E) α-hydroxymetoprolol and (B), (D) and (F) *O*-desmethylmetoprolol following a 5 min incubation of metoprolol with rat liver microsomes at pH 7.25 and 37°. The points are mean values of results from duplicate incubations, all of which differed by less than 10%. The lines are computer best fits of the data.

Table 1. Values of (a) apparent inhibitor constants (K_i) for the α-hydroxylation and *O*-desmethylation of metoprolol and (b) concentrations of inhibitor required to impair the disappearance of substrate by 50% (IC_{50})

Drug	α-Hydroxylation K_i (μM)	mechanism	*O*-desmethylation K_i (μM)	mechanism	IC_{50} (μM)
Debrisoquine	8	Competitive	61	Competitive	93
Guanoxan	4	Competitive	48	Competitive	6
Cimetidine	9	Competitive	38	Competitive	30
Ranitidine	58	Competitive	134	Competitive	86
Antipyrine	>1000		>1000		17500

Metoprolol and inhibitor were coincubated for 5 min with liver microsomes at pH 7.25 and 37°. K_i and IC_{50} values for debrisoquine each represent the mean of 3 experiments (individual data: K_i α-hydroxylation = 9, 9 and 6 μM; K_i *O*-desmethylation = 39, 26 and 119 μM; IC_{50} = 82, 90 and 107 μM). Each of the values for the other drugs were determined from single sets of experiments.

Antipyrine also had a very small effect on the disappearance of the drug from microsomal incubates (Table 1).

DISCUSSION

The results confirm earlier findings [19] demonstrating the ability of rat liver microsomes to catalyse the aliphatic hydroxylation and *O*-desmethylation of metoprolol. The apparent linearity of Hanes plots over the substrate concentration range studied together with the low values for apparent K_m suggest the presence of single, high affinity sites of enzyme activity.

The potent competitive nature of the inhibition of metoprolol oxidation by debrisoquine and guanoxan is consistent with *in vivo* observations of defective oxidation of guanoxan [7] and metoprolol [1] in poor metabolisers of debrisoquine and provides evidence that one or more pathways of all three substrates are catalysed by a single form of cytochrome P-450.

Boobis *et al.* [4] have argued that K_i for inhibition should be the same as the K_m for metabolism of the inhibitor and the enzyme–substrate dissociation constants for binding of the inhibitor. In support of this hypothesis, previously reported type I spectral dissociation constants for the binding of debrisoquine (8.5 μM) and guanoxan (1.0 μM) to rat liver microsomes [20] are almost identical to the K_i values for inhibition of metoprolol α-hydroxylation by these substrates (Table 1). The significance of the nearly tenfold difference between the inhibition constants for α-hydroxylation and *O*-desmethylation is not clear and requires further study. No data are available on the converse interaction, that is the effect of metoprolol on debrisoquine 4-hydroxylation in rat liver microsomes. However, it has been shown that metoprolol is a potent competitive inhibitor of the human liver microsomal oxidation of debrisoquine [10] and also of sparteine [21] and desmethylimipramine [22], two other drugs whose metabolism has been linked to that of debrisoquine *in vivo* [23].

The ability of debrisoquine and other compounds to slow markedly the disappearance of unchanged metoprolol from microsomal incubates implies that these agents must exert a potent inhibitory action on metoprolol oxidation via all its major routes. The extent of this inhibition cannot be accounted for solely on the basis of an impairment of α-hydroxymetoprolol and *O*-desmethylmetoprolol formation, since about 80% of metabolised drug was converted to unidentified products. 4-(2-Hydroxy-3-isopropylaminopropoxy)-phenylacetic acid, the product of the oxidation of *O*-desmethylmetoprolol and the major urinary metabolite of metoprolol in man, was not detected to any significant extent.

Drugs whose metabolism are not linked to that of debrisoquine in man would not be expected to interact *in vitro*. Antipyrine metabolism is essentially independent of debrisoquine oxidation phenotype [11, 24] and, as anticipated, had only a weak inhibitory effect on metoprolol metabolism via all the pathways tested in rat liver microsomes.

There have been no reports to date on the relationship between debrisoquine oxidation phenotype and the metabolism of the histamine H_2-receptor blocking agents, cimetidine and ranitidine. However, an interaction between these drugs and metoprolol has been reported to occur in humans, although this is somewhat controversial [5, 6]. Nevertheless, both cimetidine and ranitidine appear to decrease the clearance of single doses of metoprolol in healthy volunteers, the former by 60% and the latter by 44% [27–29]. Metoprolol is eliminated predominantly by Phase I metabolism [30]. The potent inhibitory effect of cimetidine and ranitidine on metoprolol metabolism in rat liver microsomes (Table 1) is compatible, therefore, with impaired clearance of metoprolol in man.

Although most interactions involving these H_2-receptor antagonists are thought to be the consequence of inhibition of cytochrome P-450-catalysed oxidation, there is some dispute as to their precise mechanism. Because several *in vitro* studies have reported that inhibition occurs only at concentrations which are very much higher than seen *in vivo* [31, 32] doubt has been cast on the ability of cimetidine to act as a competitive inhibitor *in vivo*. On the other hand Reilly and colleagues demonstrated low spectral dissociation constants for the binding of cimetidine to rat liver microsomes [33] and went on to show that cimetidine is a potent competitive inhibitor of the *N*-demethylation of morphine *in vitro* (K_i = 64 μM) [34]. These findings together with ours for metoprolol indicate that cimetidine can impair the metabolism of some drugs *in vivo* by direct competition at the enzyme site. Recent evidence suggests that a metabolite–intermediate complex of cimetidine and cytochrome P-450 may also contribute to the inhibitory action of this H_2-receptor antagonist [35].

The inhibitory effects of cimetidine on metoprolol oxidation in rat liver microsomes were almost identical to those of debrisoquine. Although ranitidine had higher K_i values, it was still a potent inhibitor. These findings suggest that one or more pathways of cimetidine and ranitidine metabolism are under similar genetic control to the oxidation of metoprolol. Both H_2-receptor antagonists are metabolised oxidatively [36, 37] and they have other features in common with drugs whose metabolism is subject to debrisoquine polymorphism in that they are nitrogenous bases which possess hydrophobic centres. However, since a high proportion of the dose of both cimetidine and ranitidine is eliminated unchanged in man [36, 37], a major influence of oxidation phenotype on the clearance of these drugs is unlikely.

There are now about 20 drugs for which data are available both on the relationship between debrisoquine oxidation phenotype and their metabolism and on their ability to inhibit competitively the oxidation of "debrisoquine-type" substrates in liver microsomes [4, 5, 10, 21, 22, 38]. The almost complete concordance of these *in vivo* and *in vitro* observations suggest that *in vitro* inhibition studies may be an accurate predictor of polymorphic oxidation in man. However, further data are required to validate fully this technique as a method of screening for polymorphic oxidation in man. Thus, it is particularly important to demonstrate a lack of inhibitory effect *in vitro* for other drugs whose metabolism has been

shown not to cosegregate with that of debrisoquine *in vivo*.

Recent studies have shown that monooxygenase enzymes catalysing the oxidation of debrisoquine and other substrates are closely related in rat and man [14, 39]. When samples of human liver tissue are difficult to obtain the rat may be a useful alternative for the study of debrisoquine polymorphism in man.

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