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THE INTERACTION OF CIMETIDINE WITH RAT LIVER MICROSOMES

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Abstract—The binding of cimetidine to rat liver microsomes in M/15 phosphate buffer, pH 7.9, has been investigated by difference spectroscopy and also by equilibrium partition studies, the latter method providing the more definitive characterization of the interaction in the pharmacologically relevant, low micromolar range of drug concn. In addition, the effect of cimetidine on the rate of dilution-induced displacement of [^{3}H]cimetidine from rat liver microsomes has been used to justify consideration of the binding results in terms of two distinct and independent classes of microsomal site, governed by dissociation constants of 8.3 and 104 μM under the above conditions. By demonstrating unequivocally the existence of the stronger interaction, this investigation has provided an acceptable experimental basis for considering the undesired side effect of cimetidine in concomitant use with a number of other drugs to be the consequence of its inhibition of their monooxygenase-dependent metabolism.

Cimetidine is an antagonist of histamine H_2-receptors which has found a number of therapeutic and prophylactic applications in human medicine [1]. The plasma half-life of the drug is about 100 min [2, 3] and its concn in the systemic circulation typically reaches peak values in the 4–10 μM range some 2 hr after each oral dose [4, 5]. Although it is a relatively safe drug, cimetidine does have toxic side effects, one being to cause prolongation of the half-lives of a number of other concomitantly administered drugs, apparently due to its inhibition of their monooxygenase-dependent metabolism [6–14]. Since pharmacological assessments of the efficacy of the drug as an inhibitor of the cytochrome P-450 dependent monooxygenase system have often been based on *in vitro* studies [15–20] with cimetidine concns that are 10–100 times greater than the *in vivo* levels quoted earlier, it seems logical to question whether the results obtained in those studies bear any relevance to the particular interactions of the drug that are responsible for the toxic effect. This question assumes even greater importance when note is taken of reports, based on spectral evidence [19, 21], that the P-450 system of rat liver microsomes possesses two classes of cimetidine-binding site. If the reported interpretation of those spectral data is correct, the requirement of high concns of cimetidine for inhibition to be observed in the *in vitro* studies [15–20] could well indicate that the interaction being studied is between the drug and the weaker, clinically irrelevant class of binding site. However, although not so interpreted, the spectral data [19, 21] are also consistent with the concept that the microsomal P-450 complex possesses equivalent but negatively cooperative sites [22], a suggestion recently put forward [23] on the basis of kinetic studies using *p*-nitroanisole as the monooxygenase substrate. In that event, the results of the *in vitro* studies using high concns of the drug could still retain some relevance to the therapeutic situation.

To resolve this problem, more definitive evidence of cimetidine binding to rat liver microsomes at low concns (0.04–14 μM) of the drug was obtained by direct binding studies employing [^{3}H]cimetidine. Advantage was then taken of the fact that the two models considered earlier may be distinguished by comparing the rates of displacement of labelled ligand from microsomes upon dilution with buffer alone and with buffer containing an excess of unlabelled cimetidine [24–28]. The purpose of this communication is to present those results which signify receptor-site heterogeneity as the more plausible explanation of the complex spectral binding curves referred to earlier. Furthermore, the equilibrium binding study employing radiolabelled cimetidine yielded a dissociation constant of 8.3 μM for the high-affinity class of binding site, which would thus bind the drug in the concn range associated with its therapeutic use. The significance of these findings is discussed in relation to the *in vitro* studies of monooxygenase inhibition by cimetidine; and to the occurrence of the various adverse drug interactions reported to be associated with its clinical use.

MATERIALS AND METHODS

Microsomal suspensions were prepared by the Netter [29] method from the livers of adult male, random outbred Wistar rats that were obtained from the University of Queensland Central Animal Breeding Facility. Suspensions were stored at −75° in the manner described previously [23]. Based on measurements of pigment concn by the Estabrook procedure [30] and Lowry estimations [31] of protein concn, the P-450 content of the liver microsomal suspensions was 0.7 nmoles/mg protein. Cimetidine was generously donated by Smith Kline and French (Australia), and [*N*-methyl-^{3}H]cimetidine, 22 Ci/mmole, obtained from Amersham Australia Pty Ltd. Other chemicals were of reagent grade.

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Spectral studies of cimetidine binding. In order to verify that the interaction of cimetidine with rat liver microsomes is characterized [19, 21] by a 'type II' spectral change [32], difference spectra were recorded on an Aminco DW-2a spectrophotometer, the cell compartment of which was fitted with a magnetic cuvette-stirring attachment and thermostatically maintained at 25°. Matched 1-cm cuvettes contained 3 ml of microsomal suspension (1 mg/ml in terms of protein content) in M/15 phosphate buffer, pH 7.9, 30 μl of cimetidine solution (100 mM) in buffer being added to one cell, and an equal vol. of buffer to the reference cell. For routine measurement of cimetidine binding in such mixtures with final drug concns in the range 6 μM–1 mM, the spectrophotometer was operated in the dual-wavelength mode to obtain directly the difference in absorbance at 432 nm, the peak of the difference spectrum, and 412 nm, the isosbestic point. The dependence of this absorbance difference, ΔA, upon cimetidine concn, [S], was analyzed in terms of the relationship:

$$\Delta A = \frac{\Delta A_1[S]}{K_1^s + [S]} + \frac{\Delta A_2[S]}{K_2^s + [S]} \qquad (1)$$

to characterize the interaction of cimetidine with microsomes in terms of apparent spectral dissociation constants (K_1^s, K_2^s) and the corresponding absorbance changes (ΔA_1, ΔA_2) associated with stoichiometric complex formation. This analysis of the untransformed (ΔA, [S]) data was effected by means of a nonlinear regression program [23] run on a PDP-11/03-L computer.

Partition equilibrium studies of cimetidine binding. Mixtures (175 μl) containing microsomal suspension (1 mg/ml protein) and cimetidine (0.04–14 μM, suitably supplemented with trace quantities of [^{3}H]cimetidine) in M/15 phosphate, pH 7.9, were prepared at room temp (23–25°) in polyallomer tubes for a Beckman Airfuge, and allowed to equilibrate for 15 min before being centrifuged at 178,000 *g* for 5 min to achieve separation of the solid phase. Accurately weighed aliquots (~ 30 μl) of the supernatant and original mixture were then mixed with Triton–toluene scintillation fluid (10 ml) prior to counting in a Packard model 2425 liquid scintillation spectrometer system. Conversion of these results to concns of cimetidine was based on reference cimetidine/[^{3}H]cimetidine solutions with known sp. acts. The Klotz [33] binding function, r, was then determined as the difference between the total and supernatant cimetidine concns divided by the total concn of P-450 (0.7 μM) in the mixtures. The resultant (r, [S]) data were then tested for conformity with a rectangular hyperbolic relationship by means of the nonlinear regression analysis mentioned in relation to the spectral studies.

Kinetics of cimetidine dissociation from microsomes. The effect of an excess of unlabelled cimetidine on the dissociation kinetics of bound [^{3}H]cimetidine by 'infinite' dilution has been used to determine whether the ligand-binding sites on rat liver microsomes are independent or negatively cooperative [24–28]. In the present experiments, conducted in a cold room with an ambient temp of 2°, a mixture (1.4 ml) containing microsomal suspension (1 mg/ml protein) and [^{3}H]cimetidine (0.33 μM) in M/15 phosphate buffer, pH 7.9, was allowed to equilibrate for 15 min to ensure equilibrium between free and bound ligand. Portions (175 μl) were then subjected to centrifugation in a Beckman Airfuge in order to define the magnitude of the binding function for this mixture. Aliquots (60 μl) of the equilibrium mixture were then diluted either with buffer or with buffer made 1 mM with respect to unlabelled cimetidine (5.94 ml in each instance), and the time-course of the release of labelled ligand followed for 5 min. At appropriate time intervals a sample (150 μl) of the diluted mixture was placed in an MF-1 microfilter (Bioanalytical Systems Inc., West Lafayette, IN) fitted with a nitrocellulose membrane (0.2 μm pore size), and air pressure (rather than centrifugal force) employed to achieve fast separation (~ 20 sec) of the supernatant phase. Aliquots (~ 30 μl) of each filtrate were then accurately weighed at room temp and mixed with scintillation fluid as described earlier to determine the concn of [^{3}H]cimetidine dissociated from the microsomes during the designated time period. Results were then tested for conformity with first-order dissociation kinetics by plotting log $[r_t^*/r_o^*]$ vs t, where r_t^* signifies the binding function for [^{3}H]cimetidine at time t.

RESULTS

Difference spectra obtained with mixtures of cimetidine and rat liver microsomes have confirmed the earlier finding [19, 21] that the interaction is characterized by a type II spectral change [32], which is considered to result from the binding of a ligand N atom to the sixth coordinating position of the haeme iron in cytochrome P-450 [34]. Fig. 1 summarizes the results of the present spectral binding study in a Scatchard [35] format, the pronounced curvilinearity of the plot indicating either heterogeneity or negative cooperativity of sites within the microsomal P-450 fraction. Nonlinear regression analysis of the untransformed results in terms of binding to two classes of independent binding sites (the former possibility) yielded values of 2.6 (± 4.3) μM and 104

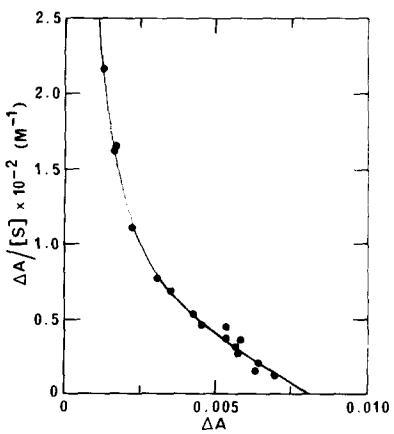

Fig. 1. Scatchard plot of spectral binding data on the interaction of cimetidine with rat liver microsomes in M/15 phosphate buffer, pH 7.9, at 25°.

(± 18) μM for K_1^s and K_2^s in equation (1); and 0.0013 (± 0.0006) and 0.0070 (± 0.0005) for the respective absorbance differences. The theoretical plot for such a system is shown as the solid line in Fig. 1. We note that the dissociation constants so deduced are very similar to the values of 3.7 and 130 μM reported by Rendić *et al.* [19] on the basis of graphical analysis of an unpublished double-reciprocal plot of spectral binding data. However, a point not evident from their study is the inability of the spectral method to provide a reliable quantitative description of the stronger binding phenomenon, the uncertainty in the value of the dissociation constant from the present results being far greater than its absolute magnitude. More definitive evidence of binding in the micromolar range of cimetidine concns was therefore sought by direct partition equilibrium studies.

Results of partition equilibrium experiments with free cimetidine concns in the range 0.04–14 μM are presented in semilogarithmic format (r vs log [S]) in Fig. 2, which also shows the theoretical relationship that provides the best-fit description of the untransformed (r, [S]) data in terms of a rectangular hyperbola. The value of 8.3 (± 1.3) μM for the dissociation constant (K_1) provides a far more definitive description of the interaction in this concn range than does K_1^s derived from Fig. 1; and the limiting value of r, viz. 0.60 (± 0.05), signifies that 60% of the microsomal binding sites participate in this interaction. No improvement in the theoretical description of the experimental results could be obtained by considering the results to reflect the sum of two rectangular hyperbolae, an observation which lends support to the concept that cimetidine is interacting with a single class of P-450 sites in this range of drug concn. The inference that this class of sites comprises 60% of the microsomal P-450 fraction may appear to be inconsistent with the earlier observation (Fig. 1) that ΔA_1 comprises only 16% of the total absorbance change associated with the interaction of cimetidine with the microsomal P-450; but the seeming discrepancy could, of course, merely signify that the less prevalent, weaker interaction gives rise to most of the spectral change. In this connection it should be stressed that such interpretation of the results implies the existence of independent classes of sites within the microsomal P-450 fraction, a situation that still remains to be proven, since the form of Fig. 1 could equally well reflect negative cooperativity of P-450 sites. A comparative kinetic study of the dissociation of [^{3}H]cimetidine by dilution in the presence and absence of unlabelled cimetidine has therefore been used to identify the relevant interpretation.

Results of experiments in which bound [^{3}H]cimetidine has been displaced by a 100-fold dilution with buffer are shown as open symbols in Fig. 3, about which the following points are noted. First, these experiments were conducted in a cold room to decrease the rate of dissociation, which was too rapid to be followed by the present techniques at the higher temp (25°) appropriate to Figs 1 and 2. Even at the lower temp the time-scale of the [^{3}H]cimetidine displacement posed considerable experimental difficulties, and accordingly greater emphasis has been placed on evaluation of the fraction of bound label, r_t^*/r_o^*, in the larger time-periods. Error bars in Fig. 3 are restricted to r_t^*/r_o^* values that are means from experiments conducted in hextuplicate. Secondly, the concentration of [^{3}H]cimetidine with which the microsomes were equilibrated was selected to ensure only a small extent of saturation of P-450 sites. From analysis of the equilibrium mixture $r_o^* = 0.03$, and hence it is reasonable to consider [24–28] that ligand attachment was confined either to high-affinity sites, or, if sites were negatively cooperative, to sites in a high-affinity state. Thirdly, the displacement of [^{3}H]cimetidine by dilution with buffer alone is adequately described by first-order kinetics, as required by either model under consideration. Finally, we note that inclusion of 1 mM cimetidine in the dilution medium had no discernible effect on the kinetics of displacement of the [^{3}H]cimetidine from the microsomes, the value of r_t^*/r_o^* obtained (●) being experimentally indistinguishable from its counterpart determined in the absence of unlabelled ligand. The fact that dissociation of labelled cimetidine is unaffected by saturation of effectively all

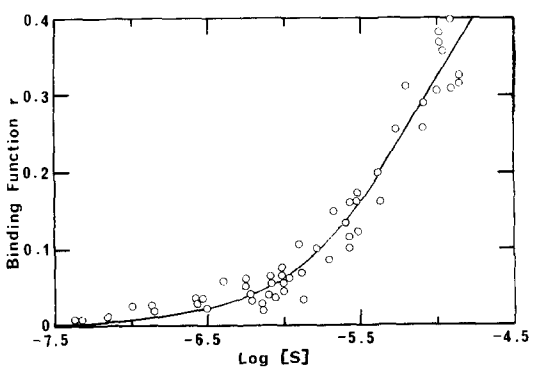

Fig. 2. Semilogarithmic plot of partition binding data on the interaction of cimetidine with rat liver microsomes in M/15 phosphate buffer, pH 7.9, at 23–25°.

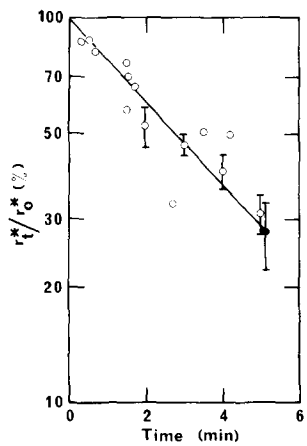

Fig. 3. Time-course of the dissociation at 2° of bound [^{3}H]cimetidine upon 100-fold dilution of an equilibrium mixture of rat liver microsomes and [^{3}H]cimetidine in M/15 phosphate buffer, pH 7.9, with buffer (○) and with buffer containing 1 mM cimetidine (●).

other P-450 sites with cimetidine signifies independence of binding sites [24]. Consequently, site heterogeneity is indeed the source of the curvilinearity of the Scatchard plot for the microsomal P-450 system (Fig. 1).

DISCUSSION

Of the various functions that this investigation has served, the provision of conclusive evidence that rat liver microsomes possess two separate and independent classes of cytochrome P-450 site capable of binding cimetidine is possibly the most important from the biochemical and pharmacological viewpoints. In this regard it is to be noted that the plasma concn of cimetidine resulting from its clinical use is in the low micromolar range [4, 5], and, accordingly, that its interaction with the weak-affinity P-450 binding sites ($K_2 > 100\ \mu M$) would only occur *in vivo* if the drug were to be concentrated some 10–100-fold in particular tissues. However, reports that the apparent steady-state vol. of distribution of cimetidine in human patients is only slightly in excess of 1 l/kg body wt [5, 36] suggests a rather uniform distribution of the drug throughout the body's extracellular and intracellular fluid compartments. Furthermore, post mortem studies of the drug's tissue distribution in patients that had been receiving cimetidine showed the kidney and liver to be the only tissues in which its concn was more than 3 times higher than the plasma value [36, 37]. When account is taken of the fact that the drug is concentrated, for purposes of excretion, in urine and bile [2, 36, 38], the high tissue to plasma concn ratios for the kidney and liver [36, 38] are not inconsistent with the concept that the concn of cimetidine in the intracellular compartments of these two tissues is also only marginally higher than in plasma. Accordingly, any adverse side effect of cimetidine due to monooxygenase inhibition may only be interpreted as the consequence of its higher-affinity interaction with cytochrome P-450.

Following the demonstration that concomitant administration of cimetidine and warfarin prolongs the half-life of the latter drug [39, 40], there was speculation [6] that monooxygenase inhibition could be the mechanism, partly on the grounds that substituted imidazoles may be potent monooxygenase inhibitors [41]. Subsequent studies of the effects of cimetidine on the biokinetics of several other drugs and model xenobiotics in human subjects [6–14, 42] and rats [15–17, 43] were also interpreted in terms of cimetidine inhibiting monooxygenase-dependent drug metabolism. Parallel investigations conducted *in vitro* using a variety of xenobiotics as substrates for monooxygenase preparations obtained from human [18] and rat [15–17, 19, 20] livers have certainly shown cimetidine to be a monooxygenase inhibitor, but the inhibition constants were very high (0.5–10 mM). Demonstrations of such weak inhibition of monooxygenase catalysis by cimetidine *in vitro* do not explain its potency in decreasing the monooxygenase-dependent clearance of a variety of drugs *in vivo*. The present evidence of a strong interaction between cimetidine and microsomal P-450 does provide the basis for such an explanation.

Since only the stronger interaction between cimetidine and microsomal P-450 is of pharmacological significance, it becomes important to have available a methodology for quantitative characterization of the phenomenon. In this regard the spectral method is particularly unsuited, since the difference spectrum is dominated by the weaker, pharmacologically irrelevant interaction between cimetidine and microsomes. A second major contribution from this investigation has therefore been the development of a simple partition equilibrium procedure that provides a reasonable quantitative description of the interaction between microsomal P-450 and cimetidine in the concn range that is likely to be encountered in drug therapy.

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