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Adverse drug interactions with cimetidine: competitive inhibition of monooxygenase-dependent *N*-demethylation of morphine

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Inhibition by cimetidine of the monooxygenase-dependent metabolism of a number of other drugs is considered to be the source of the adverse side effects when subjects, receiving these drugs, also receive cimetidine [1]. However, studies of cimetidine as a monooxygenase inhibitor *in vitro* have shown extremely high concentrations of the drug to be required for demonstrable inhibition; and no clear mechanism of inhibition has emerged [2, 3]. In this communication we show unequivocally that cimetidine is a competitive inhibitor of the *N*-demethylation of morphine by a rat liver microsomal monooxygenase preparation. Furthermore, the demonstration of inhibitory action by low cimetidine concentrations provides tangible support for the hypothesis that the adverse drug interactions observed between this drug and morphine [4, 5], a matter of dispute [6], may be due to monooxygenase inhibition.

Materials and methods

Aliquots of a stock microsomal suspension prepared by the Netter method [7] from the livers of adult male, random outbred Wistar rats were stored at −75° prior to use. The microsomal cytochrome P-450 concentration, determined by the Estabrook procedure [8] with an Aminco DW-2a spectrophotometer in the dual wavelength mode, was 0.84 nmoles/mg protein. This monooxygenase preparation was diluted to a final pigment concentration of 1 μM for kinetic studies of the production of formaldehyde resulting from *N*-demethylation of morphine. Reaction mixtures (3 ml, pH 7.5) contained, in addition to microsomes, 0.125 M Tris–HCl, 0.025 M tetrasodium pyrophosphate, 2.5 mM glucose-6-phosphate, 1 mM NADP, 1 mM semicarbazide, D-glucose-6-phosphate dehydrogenase (1 μl, 0.7 U), morphine hydrochloride (8.0 μM to 1.64 mM), and cimetidine (0, 12, 37 or 95 μM): reaction mixtures were incubated at 26° for 25 min. Preliminary experiments had established the linear time-dependence, under these conditions, of formaldehyde production over the lower range of morphine concentrations (17–155 μM), where substrate depletion may have introduced error in the use of initial concentrations for the calculation of kinetic parameters. Preliminary experiments also showed that formaldehyde was not produced from cimetidine under these incubation conditions. Monooxygenase catalysis was initiated by the rapid addition of an aliquot of the stock microsomal suspension to the above reaction mixtures, and terminated by the addition of undiluted perchloric acid. Formaldehyde was measured by adding an equal volume of Nash reagent [9] to weighed aliquots (1.3–1.5 ml) of supernatant obtained by centrifuging the terminated reaction mixtures in an Eppendorf Zentrifuge (model 5412); and allowing colour development to proceed for 1 hr at 37° [10]. Absorbances at 410 nm were recorded on an Aminco DW-2a spectrophotometer operated in the split-beam mode; and converted to concentrations on the basis of a standard curve obtained with similarly treated formaldehyde solutions of predetermined concentration. Cimetidine was a gift from Smith Kline and French Laboratories (Australia) Limited, and morphine was a product of Macfarlan Smith (Batch 12666): NADP was a Sigma product. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer-Mannheim Corporation, and other chemicals were of reagent grade.

Results and discussion

Results of the kinetic experiments are summarized in Fig. 1, about which the following points are noted. First, *N*-demethylation of morphine by the rat liver monooxygenase preparation is adequately described by Michaelis–Menten kinetics with values of 300 ± 24 μM and 1.34 ± 0.05 min^{-1} for the Michaelis constant, K_m, and k_{cat}, respectively, the latter value being based on the pigment content of the assay mixtures. These estimates (±2 SEM) were obtained with a

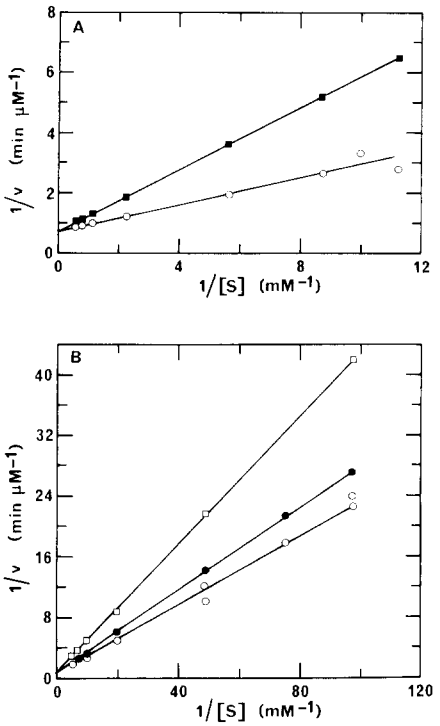

Fig. 1. Competitive inhibition of the monooxygenase-dependent *N*-demethylation of morphine by cimetidine in reaction mixtures containing rat liver microsomes (1 μM cytochrome P-450) and morphine hydrochloride, *S* (8 μM to 1.64 mM), the concentrations of cimetidine being as follows: ○, zero; ●, 12 μM; □, 37 μM; ■, 95 μM. Reaction velocities, *v*, refer to the formation of formaldehyde.

modification of the method described by Duggleby [11] for non-linear regression analysis of the untransformed (v, $[S]$) data. Secondly, Fig. 1(A) clearly shows the competitive nature of the inhibition of *N*-demethylation of morphine by cimetidine (95 μM), the results for lower concentrations of this drug (37 and 12 μM) also being consistent with this concept (Fig. 1B). Thirdly, at variance with the findings of Rendić and coworkers [2] but in keeping with the requirements for competitive inhibition, the same value (68 ± 10 μM) for K_i describes the inhibition by all three concentrations of cimetidine. Fourthly, this magnitude of K_i is sufficiently low for considerable inhibition of morphine *N*-demethylation to occur with concentrations of cimetidine pertinent to the pharmacological situation. For example, in man the concentration of cimetidine in the systemic circulation typically reaches peak values of 4–10 μM some 2 hr after each normal oral dose of the drug [12, 13]; and the present kinetic results with 12 μM cimetidine (Fig. 1B) indicate that such concentrations would suffice to impair *N*-demethylation of morphine, which could well lead to the adverse side effects observed in subjects (man and animal) receiving both drugs simultaneously [4, 5]. Finally, the value of K_i obtained in this investigation is also entirely consistent with the range of dissociation constants (3–104 μM) detected [14] by spectral measurements of the interaction between cimetidine and cytochrome P-450 of a rat liver microsomal preparation equivalent to that used here.

Previous kinetic studies of cimetidine's inhibitory effects on monooxygenase catalysis by rat liver microsomes have led to no clear conclusions. Thus, Rendić and coworkers [2] obtained high estimates (> 220 μM) of K_i for cimetidine inhibition of the dealkylation of 7-ethoxycoumarin; but also showed dependence of the magnitude of K_i upon the concentration of cimetidine used for its measurement, thereby signifying incompatibility of the kinetic data with competitive inhibition. On the other hand, Pelkonen and Puurunen [3] found cimetidine to be a non-competitive inhibitor of benzo[α]pyrene hydroxylation in studies conducted over a narrow range of substrate concentration. Awareness of the findings of Rendić and coworkers [2] led Pelkonen and Puurunen [3] to conclude that the mechanism of cimetidine's inhibitory effect on monooxygenase catalysis remained unclear. In this regard it should be noted that non-Michaelis kinetics have been observed with a number of monooxygenase substrates; and that the consequent desirability of examining monooxygenase catalysis over a very wide range of substrate concentrations has been emphasized [15]. Furthermore, competitive inhibitors of monooxygenase catalysis have also been shown seemingly to act in a non-competitive manner when tested with such substrates over low, narrow concentration ranges [15]. No such complications were observed in the monooxygenase-catalyzed *N*-demethylation of morphine (Fig. 1), which clearly obeyed Michaelis–Menten kinetics over the wide range of substrate concentration (8 μM to 1.64 mM) employed. With this substrate no ambiguity exists in interpreting the nature of monooxygenase inhibition by cimetidine: competitive inhibition is clearly indicated (Fig. 1).

Other kinetic studies of the inhibition by cimetidine of monooxygenase activity [3, 16, 17] have only involved determination of the cimetidine concentration required to decrease the reaction rate by 50%, a procedure that is commonly employed in studies of drug metabolism, but which is in fact of little value if performed at only a single substrate concentration. Considerations of the competitive inhibition of an enzyme exhibiting Michaelis–Menten kinetics show that $[I]_{50}$, the concentration of inhibitor required for a 50% decrease in reaction velocity for a concentration $[S]$ of substrate, is given by $[I]_{50} = K_i\{1 + ([S]/K_m)\}$. Clearly, $[I]_{50}$ is governed to a great extent by the magnitude of K_m in relation to the substrate concentration used for its determination. Since $[I]_{50}$ estimates are usually based on assay conditions designed for measurement of enzyme concentration, $[S] \gg K_m$ and accordingly $[I]_{50} \gg K_i$. Furthermore, choice of a different value of $[S]$ for the same substrate alters the magnitude of $[I]_{50}$, a factor that presumably contributes to the discrepancy between the values (10 and 1.5 mM) reported by Pelkonen and Puurunen [3] and Serlin and coworkers [17] for cimetidine on the basis of kinetic studies of the *N*-demethylation of aminopyrine. It is also important to realize that those values are not necessarily at variance with the present estimate of 68 μM for K_i.

In summary, a kinetic study has been made of the inhibition by cimetidine of monooxygenase-dependent *N*-demethylation of morphine in 0.125 M Tris–HCl–0.025 M tetrasodium pyrophosphate buffer, pH 7.5. These studies with a microsomal preparation from rat liver as the source of monooxygenase activity have established the adequacy of the Michaelis–Menten scheme to describe the production of formaldehyde from morphine, and also the competitive nature of the inhibition by cimetidine, which is characterized by an inhibition constant of 68 μM. The demonstration of inhibitory action at low, pharmacologically relevant drug concentrations provides the first acceptable enzyme kinetic evidence for the concept [1] that adverse drug interactions encountered with cimetidine could be the result of this inhibitory effect on the monooxygenase-dependent metabolism of some drugs with which it is administered.

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Effects of benfluorex and fenofibrate treatment on mitochondrial and peroxisomal marker enzymes in rat liver

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Clofibrate (ethyl-*p*-chlorophenoxyisobutyrate) and related compounds are commonly used in the treatment of hyperlipidemia. Besides their beneficial lowering effect on plasma cholesterol and triacylglycerol levels, these drugs may produce some potentially harmful side-effects. Indeed, studies in rodents have demonstrated that clofibrate-like drugs, as well as chemically unrelated hypolipidemic compounds, induce hepatomegaly, hepatic peroxisome proliferation and hepatocarcinoma. These compounds have been labelled peroxisome proliferators and have been proposed as a new class of carcinogens (for a recent review, see ref. [1]). Following the discovery of a β-oxidation pathway for long-chain fatty acids in peroxisomes and of its dramatic stimulation after clofibrate treatment, a causal link has been proposed, but not demonstrated, between the hypolipidemic effect of peroxisome proliferators and the induction of peroxisomal β-oxidation [2]. However, it should be mentioned that these compounds also induce the proliferation of hepatic mitochondria in rats [3], and that peroxisome proliferation in the liver of treated patients is absent or low [4, 5].

Benfluorex (1-(3-trifluoromethylphenyl)-2-[*N*-(2-benoyloxyethyl)amino]propane) is a hypolipidemic agent, chemically unrelated to clofibrate, which does not induce hepatomegaly in experimental animals [6]. It is important to establish whether benfluorex belongs to the family of hypolipidemic peroxisome proliferators or not. In the study presented here this question was approached biochemically, i.e. through measurements of marker enzyme activities specific for peroxisomes and also for mitochondria. This approach rests on the assumption that organelle proliferation should be paralleled by increases in the activity of one or more specific marker enzymes. Benfluorex treatment was compared to treatment with fenofibrate (isopropyl-[4-(*p*-chlorobenzoyl)2-phenoxy-2-methyl]propionate), a powerful clofibrate-like hypolipidemic drug.

Materials and methods

Treatment of animals. Benfluorex (25 or 50 mg/kg) or fenofibrate (25 mg/kg) was given by tube feeding for ten consecutive days to male Wistar rats (starting weight, 100 g). The drugs were suspended (0.25%, w/v) in arabic gum (20%, w/v in H_2O). Control animals received the vehicle only. All animals had free access to food and water.

At 25–50 mg/kg, benfluorex lowers serum triacylglycerols by 40–90% in rats maintained on a high-fat or high-carbohydrate diet, whereas serum cholesterol does not decrease under these conditions [6]. At 25 mg/kg, fenofibrate slightly lowers total serum lipids and cholesterol in the normal rat, but is not, or is less, effective in the hyperlipidemic rat [7].

Liver homogenates and assays. Animals were killed by cervical dislocation. The liver was removed and weighed. A portion was homogenized in 3 vols. of 0.25 M sucrose–3 mM imidazole (pH 7.4)–1 mM EDTA (pH 7.4)–0.1% ethanol. Another portion was freeze-clamped and subsequently homogenized in 0.06 N HCl for glycogen determination [8]. The following parameters were measured on sucrose homogenates: protein [9], total carnitine [10], catalase [11], urate oxidase [12], carnitine acetyl- and palmitoyltransferase [13], glutamate dehydrogenase [14], acyl-CoA oxidase [15, 16], mitochondrial and peroxisomal [17] β-oxidation of long-chain fatty acids. Enzyme activities are reported in the units used by the authors mentioned. Minor changes introduced by the authors have been reported in detail elsewhere [14, 17].