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CIMETIDINE INTERACTION WITH LIVER MICROSOMES *IN VITRO* AND *IN VIVO*

INVOLVEMENT OF AN ACTIVATED COMPLEX WITH CYTOCHROME P-450*

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Abstract—The O-deethylation of 7-ethoxycoumarin was inhibited in a mixed type manner by cimetidine *in vitro* and in microsomes isolated from rats treated with cimetidine *in vivo*. It was found that the inhibition was even greater if cimetidine was preincubated with the microsomal suspension in the presence of an NADPH-generating system prior to the addition of substrate. *In vitro* the decrease in activity was accompanied by a decrease in cytochrome P-450 content. This decrease was unaffected by the addition of EDTA to the microsomal suspensions, eliminating the possibility that free radical production was responsible for the decrease in cytochrome P-450. The decrease in activity and cytochrome P-450 content following preincubation of microsomal suspensions with cimetidine could be attenuated if potassium ferricyanide was added to the suspensions. The deethylation activity and cytochrome P-450 content of liver microsomes prepared from cimetidine-treated rats was decreased compared to control animals. The activity and cytochrome P-450 content of microsomes from cimetidine-treated rats could also be restored if microsomes were washed with potassium ferricyanide prior to incubation with substrate. It is proposed that an intermediate complex of cimetidine and cytochrome P-450 could be involved in the inhibition of microsomal metabolism by cimetidine.

Cimetidine interferes with the cytochrome P-450 mediated metabolism of various drugs [1–3]. The concentration of cimetidine for inhibition *in vitro* is, however, 10–100 times higher than that needed *in vivo* [3–5]. Whereas 0.25–2.0 mM of cimetidine is used in most *in vitro* experiments, maximum plasma concentrations of only 3–10 μM are observed following a single 400 mg oral dose of cimetidine in man [4, 5], and during chronic oral dosing the plasma concentration of cimetidine does not exceed 6 μM [5]. Thus, based upon the *in vitro* data the erroneous assumption might be made that cimetidine is not present in high enough concentrations *in vivo* to affect drug metabolism.

To explain the discrepancy between the *in vitro* and *in vivo* observations it has been suggested that the inhibition of cytochrome P-450 by cimetidine could involve isozymes of P-450 [6]. Spectral binding data of cimetidine to P-450 indicate that cimetidine interacts with at least 2 binding sites, but isozyme activities were not determined [6]. An alternative possibility is the formation of an activated intermediate complex with cytochrome P-450, as has been shown for amphetamine-like compounds [7]. This work was undertaken to investigate the latter possibility and further characterize the interaction of cimetidine with cytochrome P-450.

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MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (Süddeutsche Versuchstierfarm Tuttlingen, F.R.G.) weighing between 250 and 350 g were used throughout the studies. The animals were maintained on a regular twelve hour light–dark cycle with free access to food and water. All animals were housed in polyester cages, 4–6 animals to a cage, at 20–25°. Wood shavings were used on the bottom of the cases.

Preparation of liver microsomal fraction. All subsequent procedures were carried out in a cold room at 4°. Animals were lightly anaesthetized with ether and exsanguinated by cardiac puncture. The livers were removed, rinsed with buffer, blotted dry, weighed, and then minced into fine pieces. Homogenization of the liver pieces (20% w/v in 0.02 M phosphate buffer, pH 7.4) was done using a glass–Teflon Braun Homogenizer (Melsungen, F.R.G.). The homogenate was centrifuged as follows: 600 *g*, 10,000 *g*, and 100,000 *g* for 10, 20 and 60 min, respectively. For specific experiments the microsomal fraction was washed with 50 μM ferricyanide and recentrifuged at 100,000 *g* for 30 min. All microsomal preparations were resuspended in 0.05 M phosphate buffer, pH 7.4, to a concentration of approximately 10 mg/ml.

Enzyme assays. Cytochrome P-450 was determined by the method of Omura and Sato [8]. Because complexed cytochrome P-450 cannot bind carbon monoxide (CO) this method only determines the

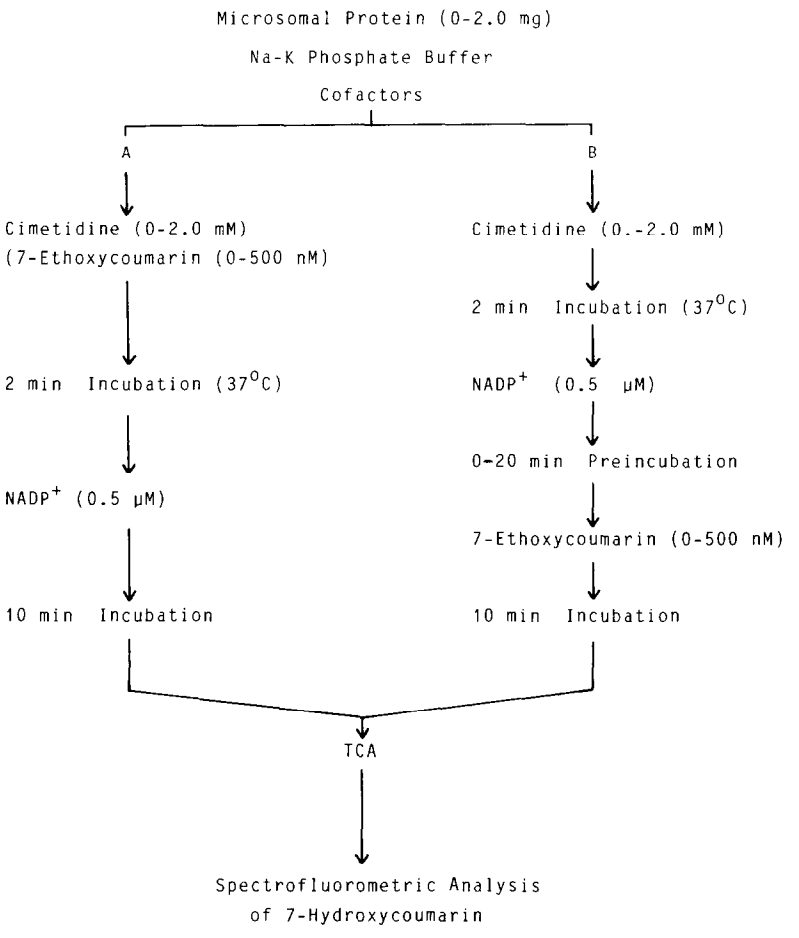

Fig. 1. Incubation scheme of microsomes with cimetidine and 7-ethoxycoumarin: (A) no preincubation; (B) preincubation of cimetidine with the NADPH-generating system before 7-EC addition.

free, uncomplexed cytochrome P-450 present. Ferricyanide was used to decomplex cytochrome P-450, with the subsequent measurement of total cytochrome P-450 [9].

Protein determinations were done using the method of Lowry *et al.* [10]. NADPH-cytochrome c reductase and NADPH-cytochrome P-450 reductase activities were assayed as described by Macleod *et al.* [11]. Microsomal deethylase activity was determined, using 7-ethoxycoumarin (7-EC) as substrate, by the sensitive fluorometric method of Greenlee and Poland [12]. All incubations were carried out at 37° under an air atmosphere in phosphate buffer (Na–K-phosphate; 50 mM, pH 7.4) to which was added: KCl 50 mM, $MgCl_2$ 2.5 μM, glucose-6-phosphate 3 μM, glucose-6-phosphate dehydrogenase 5 units, $NADP^+$ 0.5 μM, microsomal enzyme preparation 0.5–2.0 mg, and substrate (7-EC, 50–500 nM in 50% ethanol/H_2O) in a final volume of 2.4 ml. Cimetidine was added to the incubation solution in a final volume of 20 μl.

Incubations were carried out according to two procedures. In the first procedure (A; Fig. 1), The cimetidine and 7-EC were added to microsomes and cofactors followed by a 2 min incubation to bring the mixture to 37°. The reaction was then started by the addition of $NADP^+$ and further incubated for 10 min. In the second procedure (B; Fig. 1), cimetidine was added followed by a 2 min incubation to bring the mixture to 37°. $NADP^+$ was then added to bring the mixture incubated up to 20 min prior to the addition of 7-EC (preincubation). After the addition of 7-EC, the mixture was further incubated for 10 min. In both cases the enzyme reaction was stopped by the addition of trichloroacetic acid (TCA). The mixture was assayed for 7-hydroxycoumarin. Results are reported as nMoles 7-hydroxycoumarin found per minute per mg microsomal protein.

Treatment with cimetidine. Male Sprague–Dawley rats (initial weight 225–250 g) were administered cimetidine, 75 mg/kg, once daily for 4 days. The cimetidine solutions was 18.75 mg/ml in saline injected i.p. in a volume of 4 ml/kg. Blood was collected and livers were removed one hour after the final administration of cimetidine. This time point was chosen because peak plasma concentrations of cimetidine could be expected at this time [4]. The plasma concentration of cimetidine was determined as previously described [13].

Spectral study. The binding of CO in the presence of cimetidine to microsomes was determined with

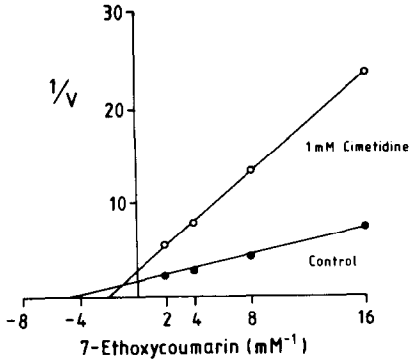

Fig. 2. Double-reciprocal plot of the 7-ethoxycoumarin deethylase activity versus 7-ethoxycoumarin concentration without cimetidine (control) and in the presence of cimetidine.

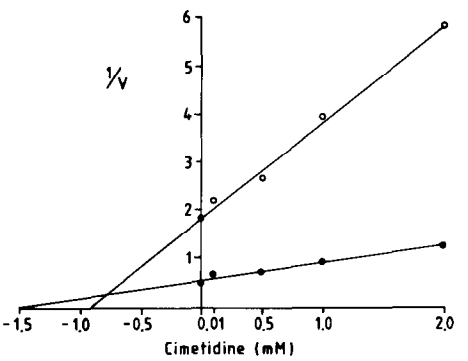

Fig. 3. Dixon plot of the inhibition of 7-ethoxycoumarin deethylase activity in the presence of increasing concentrations of cimetidine. The concentration of 7-ethoxycoumarin in the upper curve was 100 mM (○) and in the lower curve 500 mM (●).

microsomes preincubated with cimetidine, with cimetidine and the NADPH-generating system, and with microsomes preincubated with cimetidine, the NADPH generating system plus ferricyanide.

RESULTS

When cimetidine and 7-ethoxycoumarin were added simultaneously to microsomes, a concentration-dependent mixed-type inhibition of deethylase activity was observed (Fig. 2). The calculated inhibition constant (K_i) of cimetidine was 0.8 mM determined by the method of Dixon (Fig. 3) [14].

When microsomes were preincubated for various time periods with cimetidine and the NADPH-generating system prior to the addition of 7-EC, there was a further decrease in the deethylase activity (Fig. 4). It is shown in the upper portion of Fig. 4 that the decrease in deethylation activity was dependent on the preincubation time of cimetidine. It is also noted that the maximum inhibition of 7-EC deethylase activity of 75% was reached following 10 min preincubation with cimetidine with no further inhibition observed when microsomes were preincubated for 20 min (Fig. 4; lower portion).

The addition of cimetidine to oxidized liver microsomes, before the reduction by dithionite, did not change the cytochrome P-450 absorption spectrum of the microsome-CO complex compared to control microsomes (Fig. 5). However, the binding of CO to cytochrome P-450 was decreased when cimetidine

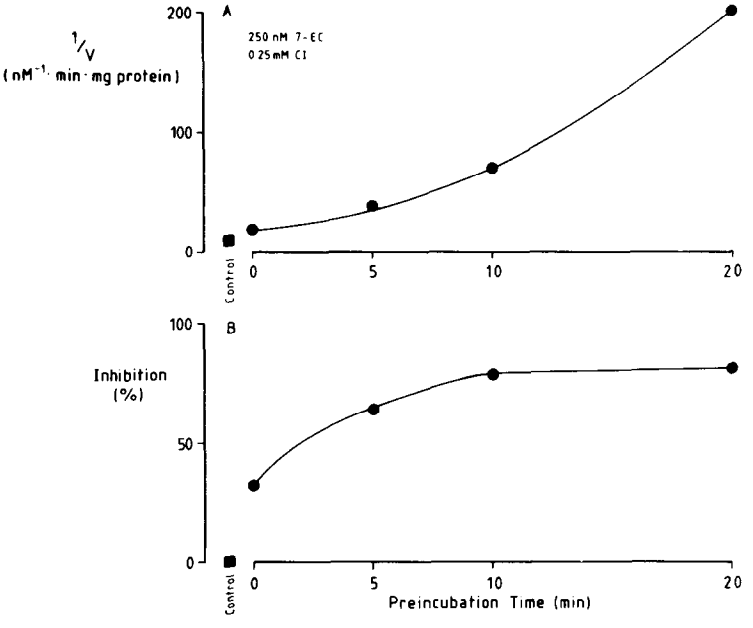

Fig. 4. The effect of preincubation time on inhibition of 7-ethoxycoumarin deethylase activity by cimetidine: (A) plot of preincubation time in presence of NADPH-generating system prior to addition of 7-ethoxycoumarin versus 1/product formation; (B) identical results, expressed in percentage of decrease of deethylase activity compared to control. All values represent the mean of three experiments.

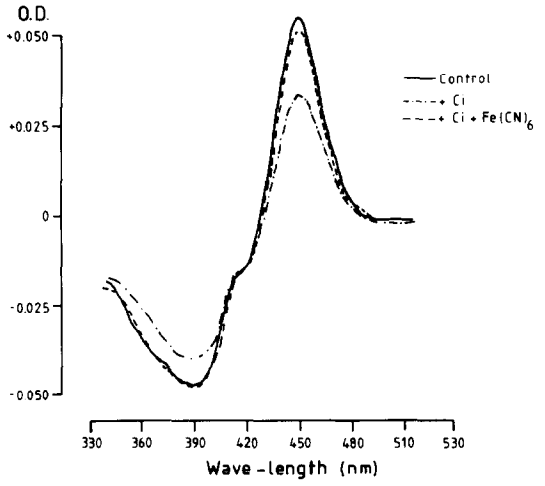

Fig. 5. Absorption spectra of dithionite-reduced cytochrome P-450–CO-complex from control microsomes (Control;—), microsomes preincubated for 10 min with cimetidine and the NADPH-generating systems (Ci;—·—) and from microsomes preincubated for 10 min with cimetidine, the NADPH-generating system, and 50 μM ferricyanide (Ci + $Fe(CN)_6$; ——). The spectrum of microsomes preincubated for 10 min with cimetidine but without the NADPH-generating system was identical to that of the control preparation.

had been preincubated with the microsomes and NADPH (Fig. 5). As the binding of CO was decreased the apparent content of cytochrome P-450 was also decreased (Fig. 6). This decrease in P-450 content was also preincubation time-dependent, as shown in the upper portion of Fig. 6. The percent decrease in cytochrome P-450 content reached a maximum of 75% after 10 min preincubation with cimetidine (Fig. 6; lower portion). When microsomes were preincubated with cimetidine and the NADPH-generating system in the presence of ferricyanide the binding spectrum of CO was not different from that of the untreated microsomes (Fig. 5). Ferricyanide alone had no effect on carbon monoxide binding to diethionite reduced microsomal cytochrome P-450.

Because it has been shown that the incubation of microsomes with a NADPH-generating system can lead to the production of free radicals due to the production of hydrogen peroxide or lipid peroxidation [15, 16], microsomes were preincubated with cimetidine in the presence of EDTA to eliminate this possibility. No change in activity was observed when microsomes treated with EDTA were compared to controls (251.3 ± 5.0 vs 241.3 ± 7.3 nM/min/mg protein; EDTA-treated vs controls respectively).

In rats treated with cimetidine for 4 days microsomal P-450 content, 1 hr after the final dose, was decreased by 25% when compared to saline-treated control rats (Table 1). Deethylase activity decreased from 237.9 ± 16.5 to 215.4 ± 10.2 nmoles/min/mg protein, control vs cimetidine-treated, respectively. In the cimetidine-treated rats, the plasma concentration of cimetidine was 1.1 ± 0.3 μg/ml. When microsomes from cimetidine-treated rats were washed with ferricyanide and recentrifuged at 100,000 *g*, the subsequently measured 7-EC deethylase activity and cytochrome P-450 content was equal to that of the control group (Table 1).

DISCUSSION

This study confirms previous reports that cimetidine is a potent inhibitor of drug metabolism *in vivo*

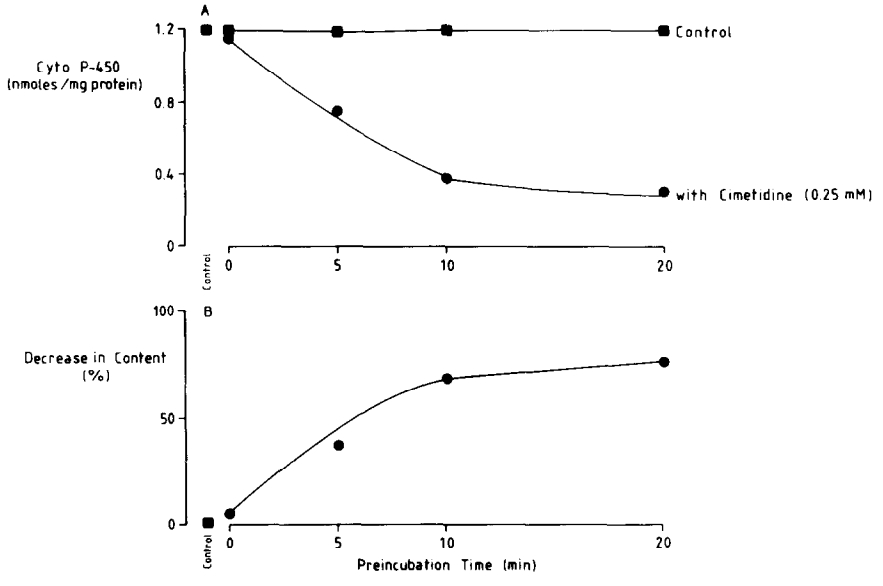

Fig. 6. Effect of preincubation of cimetidine with microsomes on cytochrome P-450 content: (A) cytochrome P-450 content in microsomes incubated without cimetidine for 0–20 min (upper line) and with 0.25 mM cimetidine for 0–20 min prior to determination of P-450 content. Values are the mean of three experiments; (B) identical results expressed in percentage of decrease of cytochrome P-450 compared to control values.

Table 1. Effect of potassium ferricyanide on the cytochrome P-450 content and deethylase activity of microsomes prepared from control and cimetidine treated rats

	Cytochrome P-450 content (nmoles/mg protein)			7-Ethoxycoumarin activity (nmoles/min / mg protein)		
	Potassium ferricyanide without	Potassium ferricyanide with		Potassium ferricyanide without	Potassium ferricyanide with	
Control rats	1.15 ± 0.04	1.15 ± 0.04	n.s.*	237.9 ± 16.5	239.3 ± 10.1	n.s.
			P < 0.05			P < 0.05
Cimetidine-treated rats	0.86 ± 0.03	1.16 ± 0.05	P < 0.05	215.4 ± 10.2	264.5 ± 20.1	n.s.

Hepatic microsomes were prepared from control rats or rats killed 1 hr after the last of four repeated doses of cimetidine (75 mg/kg, i.p.) daily for 4 days. Potassium ferricyanide (50 μM) was added to part of the microsome suspensions. The 7-ethoxycoumarin deethylase activity was measured under Materials and Methods. Results are expressed as the mean ± S.D. from three experiments.

* n.s., not significant.

and *in vitro* due to its interaction with microsomal cytochrome P-450 [17, 18]. The inhibition of cytochrome P-450 has been reported to be non-competitive, competitive, and mixed type [17, 19, 20]. Our data indicate that cimetidine is a mixed-type inhibitor of 7-EC deethylation. The differences in the type of inhibition reported may be due to either the enzyme reaction studied (hydroxylation, dealkylation, etc.) or the different concentration ranges of substrate and/or enzyme employed. The calculated K_i of 0.8 mM is similar to previously reported values [17–20].

Preincubation of cimetidine with liver microsomes and the NADPH-generating system prior to the addition of substrate led to an enhancement of cimetidine inhibition of 7-EC deethylation. The preincubation effect was time-dependent with maximum inhibition reached after 10 min preincubation. Besides reducing enzyme activity, preincubation was also associated with a decrease in measurable cytochrome P-450.

Preincubation of cimetidine with the cytochrome P-450 and the NADPH-generating system more closely parallels the *in vivo* situation than the simultaneous addition of inhibitor and substrate. The increase in inhibition after preincubation would therefore explain why the K_i of cimetidine *in vitro* is in the millimolar range, while *in vivo* inhibition occurs with blood concentrations in the micromolar range.

Enhanced inhibition of enzyme activity by preincubation with cimetidine could be due to the following mechanisms: (1) suicide inhibition of cytochrome P-450; (2) formation of a metabolite with a stronger inhibitory potential of cytochrome P-450 than cimetidine; (3) inhibition of a specific isozyme responsible for the metabolism of the substrate studied; (4) the formation of an "activated" complex of cimetidine and cytochrome P-450 leading to increased inhibition of enzyme activity.

No evidence is available which indicates that cimetidine is an irreversible suicide inhibitor. Because enzyme activity in man returns to normal shortly after termination of cimetidine administration (<24 hr) suicide inhibition of cytochrome P-450 can be excluded [3].

It cannot be totally excluded that the enhanced inhibition by cimetidine after preincubation is due to the formation of a more active metabolite. Speeg *et al.* [20] have reported that the K_i of the sulfoxide metabolite of cimetidine, the main metabolite of cimetidine found in both man and animals [2, 4, 5], is approximately 5 times higher than that of cimetidine when inhibition of aminopyrine demethylation was measured. Therefore, it is unlikely that the sulfoxide metabolite of cimetidine plays a major role in the inhibition of mixed function oxidases by cimetidine, but other minor metabolites of cimetidine have not been studied in this respect.

The possibility of selective inhibition of isoenzymes by cimetidine is based upon the assumption that those sites of high affinity cimetidine binding are identical to those which are the most important for the metabolism of the particular drug studied. Reilly *et al.* have shown that cimetidine displays high and low affinity binding to microsomal preparations [6], but did not present data on the activity of the assumed isozymes. We have shown here that cimetidine is unable to completely eliminate deethylase activity *in vitro*, indicating the presence of isozymes which are not inhibited by cimetidine. However, the properties of isolated isozymes in the presence of cimetidine remain to be investigated.

Our data best support the hypothesis that *in vivo* or during preincubation of microsomes with the NADPH generating system and cimetidine an activation of either cytochrome P-450 or cimetidine occurs resulting in a ligand interaction between cimetidine and P-450 leading to strong inhibition of enzyme activity. Preincubation of microsomes with cimetidine also produced a time dependent decrease in P-450 content, as measured by carbon monoxide binding. When the microsomes were washed with ferricyanide after preincubation the decrease in the binding of CO to cytochrome P-450 indicative of a decrease in the apparent content of cytochrome P-

450, was not observed. This is similar to the results of Pessayre *et al.* [9] and Muakassah and Yang [21] who have shown that active intermediate complexes between oleandomycin or phenelzine and cytochrome P-450 are characterized by a decrease in CO binding, and an apparent decrease in P-450 content. They also found that the decrease in enzyme activity and P-450 content could be prevented when microsomes after preincubation with the inhibitors were washed with ferricyanide. This is due to the oxidation of the heme iron in the ferric state by ferricyanide which destroyed the intermediate complexes of oleandomycin or phenelzine with cytochrome P-450, liberating free cytochrome P-450.

The administration of 75 mg/kg of cimetidine to rats over 4 days led to a decrease in deethylase activity *in vitro*. This is in contradiction to the findings by Pelkonen and Puurunen [19], who administered cimetidine over 7 days. However, animals in their study were sacrificed 24 hr after the final dose of cimetidine, whereas in the present study animals were sacrificed only 1 hr after the final cimetidine dose when concentrations of cimetidine in plasma were about 1 μg/ml. Since the half-life of cimetidine is less than 2 hr in rat and man [3, 4, 22], a 24 hr time interval after the dose represents 12 half-lives. It is therefore unlikely that in the Pelkonnen and Puurunen study appreciable amounts of cimetidine were present at this time point in plasma or tissues. The activity of ferricyanide-washed microsomes from cimetidine-treated rats was equal to that of control rat microsomes.

This study supports the assumption that either a strong ligand interaction of cimetidine with cytochrome P-450 exists or that active intermediate metabolites of cimetidine are formed *in vivo*, which can be disassociated from cytochrome P-450 by the washing with ferricyanide. In addition, one must also take into account not only the metabolite disposition of the inhibitor but also strive to reproduce conditions *in vivo* which influence outcome or extent of inhibition.

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