Biochemical Pharmacology, Vol. 38, No. 19, pp. 3241–3246, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

METABOLIC ACTIVATION OF THE ANTIDEPRESSANT TIANEPTINE

I. CYTOCHROME P-450-MEDIATED *IN VITRO* COVALENT BINDING

PHILIPPE LETTERON,* VERONIQUE DESCATOIRE,* MARINA TINEL,* PATRICK MAUREL,† GILLES LABBE,* JACQUELINE LOEPER,* DOMINIQUE LARREY,* ERIC FRENEAUX* and DOMINIQUE PESSAYRE*‡

* Unité de Recherches de Physiopathologie Hépatique (INSERM U 24), Hôpital Beaujon, 92118 Clichy, France, and † INSERM U 128, 34033 Montpellier, France

(*Received* 19 *January* 1989; *accepted* 31 *March* 1989)

Abstract—Incubation under air of [^{14}C]tianeptine (0.5 mM) with a NADPH-generating system and hamster, mouse or rat liver microsomes resulted in the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to microsomal proteins. Covalent binding to hamster liver microsomes required NADPH and oxygen; it was decreased in the presence of the cytochrome P-450 inhibitors, carbon monoxide, piperonyl butoxide (4 mM), and SKF 525-A (4 mM) or in the presence of the nucleophile, glutathione (1 or 4 mM). *In vitro* covalent binding to hamster liver microsomes was not decreased in the presence of quinidine (1 μM), and was similar with microsomes from either female Dark Agouti, or female Sprague–Dawley rats. In contrast, *in vitro* covalent binding to hamster liver microsomes was decreased in the presence of troleandomycin (0.25 mM), while covalent binding was increased with microsomes from either hamsters, mice or rats pretreated with dexamethasone. Preincubation with IgG antibodies directed against rabbit liver glucocorticoid-inducible cytochrome P-450 3*c*(P-450 IIIA4) decreased *in vitro* covalent binding by 53 and 89%, respectively, with microsomes from control hamsters and dexamethasone-pretreated hamsters, and by 60 and 81%, respectively, with microsomes from control and dexamethasone-pretreated rats. We conclude that tianeptine is activated by hamster, mouse and rat liver cytochrome P-450 into a reactive metabolite. Metabolic activation is mediated in part by glucocorticoid-inducible isoenzymes but not by the isoenzyme metabolizing debrisoquine. *In vivo* studies are reported in the accompanying paper.

The hepatotoxicity of about 30 drugs [1], including that of several tricyclic antidepressants [2–5], has been attributed to the formation of chemically reactive metabolites. Such studies, however, were performed long after the drugs had been marketed and shown to produce hepatitis. To assess the predictive value (if any) of metabolic activation studies, it would seem desirable that several of these studies be reported before the hepatotoxic potential of the drug (if any) is eventually known.

Tianeptine (Fig. 1), is a new tricyclic antidepressant which has been marketed in France by Ardix Laboratories since May 1988. Its main biochemical effect is an increased serotonin uptake [6]. As yet, toxicologic studies and clinical trials have not detected any hepatotoxic potential of tianeptine. Drug-induced hepatitis, however, is a rare phenomenon which often is recognized only after the drug has been released on the market and used on a large scale [7, 8].

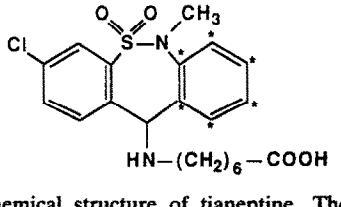

Fig. 1. Chemical structure of tianeptine. The asterisks indicate the position of ^{14}C in the labelled molecule used in this study.

We report here the *in vitro* metabolic activation of tianeptine by hamster, mouse and rat liver microsomal cytochrome P-450. *In vivo* studies are reported in the following paper [9].

MATERIALS AND METHODS

Chemicals. Tianeptine sodium, i.e. 7-[(3-chloro-6,11-dihydro-6-methyl-dibenzo [c, f] [1–2]thiazepin-11-*yl*)amino] heptanoic acid, sodium salt, was kindly provided by Institut de Recherches Internationales Servier. [^{14}C]Tianeptine sodium (30 mCi/mmol), uniformly labelled on one of the benzene rings (Fig. 1), was prepared by Commissariat à l'Energie Atomique (Gif-sur-Yvette, France); its radiochemical purity was found to be 98.8% by HPLC. Troleandomycin and dexamethasone phosphate were generous gifts from Pfizer (Sandwich, U.K.) and Merck Sharp and Dohme (Rahway, NJ), respectively.

Animals and treatments. Male Golden Syrian hamsters, weighing 90–110 g, were purchased from Fichot (Ormesson, France). Male and female Crl:CD(SD)BR Sprague–Dawley rats and male Crl:CD-1(ICR)BR Swiss mice were purchased from

‡ To whom correspondence should be addressed at: INSERM U 24, Hôpital Beaujon, 92118 Clichy, France.

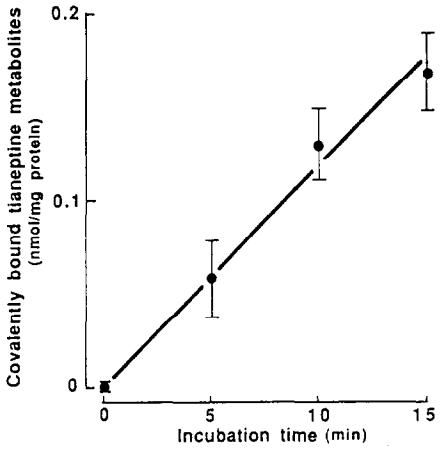

Fig. 2. Time course for the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to male hamster liver microsomal proteins. [^{14}C]Tianeptine (0.5 mM, 1 μCi/ml) was incubated under air with hepatic microsomes (5 mg protein/ml) and a NADPH-generating system for 0–15 min. Results are means ± SEM for 4 determinations.

Charles River France (Saint-Aubin-lès-Elbeuf, France). Female Dark Agouti rats were purchased from Centre de Sélection et d'Elevage d'Animaux de Laboratoire (Orléans, France). Animals were fed a normal standard diet given *ad lib.* (M25 biscuits ExtraLabo, Provins, France for hamsters and mice, and Autoclavé 113, Usine d'Alimentation Rationnelle, Villemoisson, France for rats).

Some hamsters were given phenobarbital sodium (100 mg/kg, i.e. 0.4 mmol/kg i.p. in 0.5 ml of water), 3-methylcholanthrene (20 mg/kg, i.e. 0.07 mmol/kg i.p. in 0.5 ml of corn oil), dexamethasone phosphate (50 mg/kg, i.e. 0.13 mmol/kg i.p. in 0.5 ml of water), isosafrole (100 mg/kg, i.e. 0.62 mmol/kg i.p. in 0.7 ml of corn oil) or pyrazole (150 mg/kg, i.e. 2.2 mmol/kg i.p. in 0.7 ml of corn oil), daily for 3 days and were killed 24 hr after the last dose of the inducer. Some male Sprague–Dawley rats and some male mice received the same dexamethasone-pretreatment.

Preparation of microsomes. Liver samples were homogenized in 3 vol. of ice-cold 0.154 M KCl, 0.01 M phosphate buffer (K_2HPO_4/NaH_2PO_4), pH 7.4. The homogenates were centrifuged at 10,000 *g* (average) for 10 min. The 10,000 *g* supernatants were centrifuged at 100,000 *g* (average) for 60 min. Microsomal pellets were stored at −20° until used, 1–15 days later. Microsomal protein concentration was determined by the method of Lowry *et al.* [10].

In vitro *covalent binding studies.* Unless otherwise indicated, the standard incubation was performed as follows: [^{14}C]tianeptine (0.5 mM, 1 μCi/ml) was added to an incubation mixture containing NADP (1 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (3 enzyme units/ml), $MgCl_2$ (6 mM) and hepatic microsomes (5 mg protein/ml) in a total volume of 1 ml of 0.07 M KCl, 0.13 M sodium–potassium phosphate buffer, pH 7.4. A zero-time sample (500 μl) was removed, and the remaining mixture was incubated under air, with shaking at 37° for 10 min.

In some experiments, we varied the incubation time. In other experiments, the NADPH-generating system was omitted. For experiments in anaerobiosis, an oxygen-consuming system, consisting of glucose (13 mM), glucose oxidase (8 enzyme units/ml) and catalase (1000 enzyme units/ml) was added to the incubation mixture. Nitrogen was bubbled in the ice-cold incubation mixture for 5 min, and then for the 10 min of incubation at 37°. In other flasks, CO was bubbled, first in the ice-cold incubation mixture for 5 min, and then for 10 min during the incubation at 37°. In other experiments, piperonyl butoxide (4 mM), SKF 525-A (4 mM), troleandomycin (0.25 mM), quinidine hydrochloride (1 μM), 1,1,1-trichloropropene 2,3-oxide (0.1 or 0.5 mM), cyclohexene oxide (0.1 or 0.5 mM) or glutathione (0.5–4 mM) was added to the incubation mixture.

Covalent binding to microsomal proteins was measured as described by Hoellinger *et al.* [11]. Briefly, proteins in the zero-time and the incubated samples were precipitated with 2 ml of 10% perchloric acid, and extracted four times with 2 ml of ethylacetate. Proteins where then washed with successively, 2 ml of 10% perchloric acid (twice), acetone, ethanol and 0.1 M sodium–potassium phosphate buffer, pH 7.4. Radioactivity could not be removed further by adding additional extraction steps. After drying, the pellets were dissolved overnight in 1 ml of 1 N NaOH. Aliquots (250 μl) were acidified with 50 μl of 12 N H_2SO_4, and counted for ^{14}C activity. Another aliquot (80 μl) was used to determine proteins by the method of Lowry *et al.* [10]. Non-specific binding (in the zero-time sample) was subtracted from that in the incubated sample.

Immunoinhibition experiments. Antibodies to highly purified rabbit liver cytochrome P-450 3c (P-450 IIIA4) were raised in goats and the IgG fraction was isolated, as previously described [12]. Such antibodies have been shown to cross-react with the orthologous proteins in rat (P-450 IIIA1, IIIA2), hamster, mouse and man (P-450 IIIA3) [13, 14].

Microsomes (1.25 mg protein) from untreated or dexamethasone-pretreated hamsters or rats were incubated with preimmune IgG (1.75 or 3.5 mg) or anti-P-450 3c IgG (1.75 or 3.5 mg) in a total volume of 250 μl, at 37° for 5 min. [^{14}C]Tianeptine and the various cofactors (at concentrations described above) were added, bringing the total volume to 500 μl. A 250-μl zero-time sample was taken, placed on ice, and centrifuged at 100,000 *g* for 60 min at 3°, while the remaining incubation mixture was first incubated at 37° for 10 min, and then similarly processed. Supernatants were discarded. Covalent binding to microsomal proteins was measured as described above. Since the presence of preimmune IgG itself slightly decreased covalent binding to microsomal proteins, the covalent binding observed in the presence of anti-P-450 3c IgG was expressed as a percentage of that in the presence of a similar amount of preimmune IgG.

Statistical analysis. The Student's *t*-test for independent data was used to assess the significance of differences between means.

Table 1. Cofactors requirements for, and inhibitors of, the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to hamster liver microsomal proteins

	Covalently bound [^{14}C]tianeptine metabolites (nmol/mg protein/10 min)
Complete system	0.109 ± 0.010
Without NADPH	0.023 ± 0.009*
Without oxygen	0.031 ± 0.006*
With carbon monoxide	0.033 ± 0.009*
With piperonyl butoxide (4 mM)	0.030 ± 0.006*
With SKF 525-A (4 mM)	0.047 ± 0.003*
With troleandomycin (0.25 mM)	0.035 ± 0.012*
With quinidine (1 μM)	0.101 ± 0.024

In the "complete system", [^{14}C]tianeptine (0.5 mM, 1 μCi/ml) was incubated under air with male hamster liver microsomes (5 mg protein/ml) and a NADPH-generating system for 10 min. In other flasks, the incubation mixture was modified as described in Materials and Methods. Results are means ± SEM for 43 determinations (complete system) or 4–10 determinations (other systems).

* Significantly different from the complete system, $P < 0.001$.

RESULTS

Incubation of [^{14}C]tianeptine with hamster liver microsomes and a NADPH-generating system resulted in the *in vitro* covalent binding of a ^{14}C-labelled material to microsomal proteins (Fig. 2). The amount of covalently bound material increased linearly as a function of time for at least 15 min of incubation (Fig. 2). Further incubations were ended after 10 min (Tables 1–5, Figs 3 and 4). Metabolic activation, and therefore covalent binding (Fig. 3) followed Michaelis–Menten kinetics.

In vitro covalent binding to hamster liver microsomal proteins required NADPH and O_2, and was inhibited in the presence of carbon monoxide, piperonyl butoxide and SKF 525-A (Table 1). Troleandomycin, which selectively forms an inactive cytochrome P-450-metabolite complex with glucocorticoid-inducible cytochrome P-450 isoenzymes [15], markedly decreased covalent binding (Table 1). In contrast, quinidine, a potent inhibitor of the cytochrome P-450 isoenzyme which metabolizes debrisoquine [16] and several tricyclic antidepressants [17–19], exerted no significant inhibitory effect on covalent binding (Table 1), even at a concentration (1000 nM) much higher than the K_i (60 nM) for the inhibition by quinidine of debrisoquine 4-hydroxylase activity [16]. By the same token, *in vitro* covalent binding was similar with rat

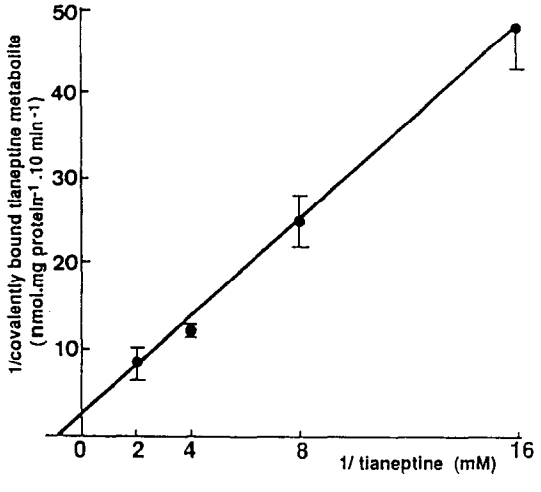

Fig. 3. Double reciprocal plot of *in vitro* covalent binding to male hamster liver microsomal proteins in the presence of various concentrations of [^{14}C]tianeptine. [^{14}C]Tianeptine (0.062–0.500 mM) was incubated under air with hepatic microsomes (5 mg protein/ml) and a NADPH-generating system for 10 min. Results are means ± SEM for 4 experiments. The V_{max} for covalent binding was 0.46 nmol bound/mg protein/10 min, the K_m was 1.3 mM.

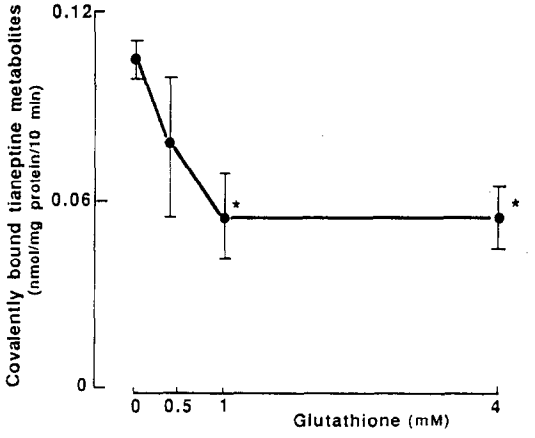

Fig. 4. Effects of glutathione on the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to male hamster liver microsomal proteins. [^{14}C]Tianeptine (0.5 mM, 1 μCi/ml) was incubated under air with hepatic microsomes (5 mg protein/ml), a NADPH-generating system, and glutathione (GSH) in various concentrations (0–4 mM) for 10 min. Results are means ± SEM for 8–16 determinations. * Significantly different from the incubation without glutathione, $P < 0.001$.

Table 2. *In vitro* covalent binding of [^{14}C]tianeptine metabolites to liver microsomal proteins from female Sprague–Dawley and female Dark Agouti rats

	Covalently bound [^{14}C]tianeptine metabolites (nmol/mg protein/10 min)
Sprague–Dawley	0.091 ± 0.016
Dark Agouti	0.107 ± 0.014

[^{14}C]Tianeptine (0.5 mM, 1 μCi/ml) was incubated under air with rat liver microsomes (5 mg protein/ml) and a NADPH-generating system for 10 min. Results are means ± SEM for 9 rats.

Table 3. Effects of various cytochrome P-450 inducers on the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to hamster, mice or rat liver microsomal proteins

	Covalently bound [^{14}C]tianeptine metabolites (nmol/mg protein/10 min)
Hamsters	
Non-treated	0.101 ± 0.010
3-Methylcholanthrene	0.060 ± 0.007*
Pyrazole	0.060 ± 0.003*
Isosafrole	0.095 ± 0.006
Phenobarbital	0.120 ± 0.008*
Dexamethasone	0.122 ± 0.006*
Mice	
Non-treated	0.251 ± 0.021
Dexamethasone	0.339 ± 0.025*
Rats	
Non-treated	0.102 ± 0.010
Dexamethasone	0.237 ± 0.014*

Male hamsters, male mice or male Sprague–Dawley rats were pretreated for 3 days with various cytochrome P-450 inducers as described in Materials and Methods. [^{14}C]Tianeptine (0.5 mM, 1 μCi/ml) was incubated under air with liver microsomes (5 mg protein/ml) and a NADPH-generating system for 10 min. Results are means ± SEM for 7–12 animals, except for microsomes from non-treated hamsters (28 animals).

* Significantly different from that in non-treated animals, $P < 0.05$.

liver microsomes from female Sprague–Dawley rats and from female Dark Agouti rats (Table 2), although the latter are genetically deficient in debrisoquine 4-hydroxylase activity [20].

Table 3 shows the effects of microsomal enzyme inducers on *in vitro* covalent binding in various species. Covalent binding to hamster liver microsomal proteins was decreased by pretreatments with 3-methylcholanthrene or pyrazole; it was unchanged by pretreatment with isosafrole, but was increased by pretreatment with phenobarbital or dexamethasone (Table 3). The enhancing effect of dexamethasone pretreatment on *in vitro* covalent binding was rather slight in hamsters (21%), moderate in mice (35%), but marked in rats (132%) (Table 3).

Immunoinhibition studies were first performed with microsomes from dexamethasone-treated hamsters. As compared to that with identical amounts of preimmune IgG, covalent binding was decreased by

Table 4. Immunoinhibition by anti-cytochrome P-450 3c IgG of the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to microsomal proteins

	Per cent inhibition
Hamster liver microsomes	
Untreated	67, 40 (53)
Dexamethasone-treated	97, 82 (89)
Rat liver microsomes	
Untreated	68, 51 (60)
Dexamethasone-treated	75, 87 (81)

Microsomes (1.25 mg protein) were preincubated with either preimmune IgG (3.5 mg) or anti-P-450 3c IgG (3.5 mg) before the addition of [^{14}C]tianeptine (0.5 mM, 0.5 μCi/0.5 ml) and an NADPH-generating system. Results give the per cent inhibition of covalent binding by anti-P-450 3c IgG, as compared to covalent binding in the presence of preimmune IgG for two different experiments. The mean value is given in parentheses.

Table 5. Effects of two inhibitors of microsomal epoxide hydrolase on the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to male hamster liver microsomal proteins

	Covalently bound [^{14}C]tianeptine metabolites (nmol/mg protein/10 min)
Complete system	0.086 ± 0.005
With TCPO (0.1 mM)	0.082 ± 0.013
With TCPO (0.5 mM)	0.085 ± 0.036
With CHO (0.1 mM)	0.097 ± 0.004
With CHO (0.5 mM)	0.085 ± 0.007

In the "complete system", [^{14}C]tianeptine (0.5 mM, 1 μCi/ml) was incubated under air with hamster liver microsomes (5 mg protein/ml) and a NADPH-generating system for 10 min. In other flasks, 1,1,1-trichloropropene 2,3-oxide (TCPO) or cyclohexene oxide (CHO) was added. Results are means ± SEM for 4–8 determinations.

76 and 89%, respectively, by preincubation with 1.75 and 3.5 mg of anti-P-450 3c IgG. The latter amount (3.5 mg) was selected for further studies (Table 4). Anti-cytochrome P-450 3c IgG decreased *in vitro* covalent binding by 53 and 89%, respectively, with microsomes from control, and dexamethasone-pretreated hamsters, and by 60 and 81%, respectively, with microsomes from control, and dexamethasone-pretreated rats (Table 4).

In vitro covalent binding to hamster liver microsomal proteins was not modified in the presence of either 1,1,1-trichloropropene 2,3-oxide or cyclohexene oxide (Table 5), two inhibitors of microsomal epoxide hydrolase [21]. *In vitro* covalent binding to hamster liver microsomal proteins was decreased by 48% in the presence of 1 or 4 mM glutathione (Fig. 4).

DISCUSSION

Our results show that tianeptine is activated by hepatic microsomal cytochrome P-450 into a chemically reactive metabolite that covalently binds to microsomal proteins *in vitro*. Indeed, covalent binding required oxygen and NADPH (Table 1); it was decreased by the cytochrome P-450 inhibitors carbon monoxide, piperonyl butoxide and SKF 525-A (Table 1), or by the nucleophile glutathione (Fig. 4), and was increased by the cytochrome P-450 inducer, dexamethasone (Table 3).

The chemical structure of the tianeptine reactive metabolite(s) remains unknown. Several neuropsychiatric agents having a tricyclic structure, including amitriptyline [22], carbamazepine [23], chlorpromazine [24], cyproheptadine [25] and imipramine [8] are oxidized by cytochrome P-450 on one or several of the double bonds of the benzene rings. It has been hypothesized that the hydroxylated derivatives may be preceded by the formation of unstable epoxides [8, 22, 23]. Preliminary results with tianeptine suggest the formation of a phenolic metabolite of tianeptine in man [26]. The formation of this metabolite is very limited, however, being less than 1% of the administered dose [26]. Furthermore, *in vitro* covalent binding to hamster liver microsomal protein was not modified by the addition of two inhibitors of microsomal epoxide hydrolase (Table 5). Further studies are required to determine the chemical structure(s) of the reactive tianeptine metabolite(s).

The oxidation by cytochrome P-450 of several other tricyclic antidepressants, including amitriptyline [17], desipramine [18], imipramine [18], and nortriptyline [19], is mediated, at least in part, by the cytochrome P-450 isoenzyme which metabolizes debrisoquine. It was thus of interest to assess whether this isoenzyme was involved in the metabolic activation of tianeptine. Our results, however, suggest that this is not the case. Indeed, quinidine, an extremely potent inhibitor of this isoenzyme [16], did not decrease covalent binding (Table 1). Furthermore, *in vitro* covalent binding (Table 2) as well as *in vivo* covalent binding [9] were similar in female Sprague–Dawley rats and in female Dark Agouti rats, although the latter are deficient in debrisoquine 4-hydroxylase activity [20].

In contrast, three sets of observations suggest that glucocorticoid-inducible isoenzymes are involved, at least in part, in the metabolic activation of tianeptine. (a) Troleandomycin has been shown to selectively inhibit glucocorticoid-responsive isoenzyme(s) by forming an inactive cytochrome P-450-metabolite complex [15]. Troleandomycin decreased the *in vitro* metabolic activation of tianeptine (Table 1). (b) Dexamethasone induces the glucocorticoid-responsive family of cytochrome P-450 isoenzymes in rats, mice and hamsters [13]; some of these isoenzymes are also induced by phenobarbital [27]. Pretreatment with dexamethasone, and also phenobarbital, increased the *in vitro* (Table 3) and *in vivo* [9] covalent binding of tianeptine metabolites to liver proteins. (c) Immunoinhibition experiments showed that covalent binding was markedly reduced by anti-cytochrome P-450 3c IgG (Table 4). Antibodies against this rabbit liver, glucocorticoid-inducible isoenzyme have been shown to cross react with the orthologous, glucocorticoid-inducible proteins of rats, mice, hamsters and humans [13, 14].

We conclude that tianeptine is transformed by hepatic microsomal cytochrome P-450 into chemically reactive metabolites. Metabolic activation is mediated, in part, by glucocorticoid-inducible isoenzymes, but not by the cytochrome P-450 isoenzyme metabolizing debrisoquine. *In vivo* studies and further comments are given in the accompanying paper [9].

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