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METABOLIC ACTIVATION OF THE NEW TRICYCLIC ANTIDEPRESSANT TIANEPTINE BY HUMAN LIVER CYTOCHROME P450

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Abstract—Incubation of [^{14}C]tianeptine (0.5 mM) with human liver microsomes and a NADPH-generating system resulted in the *in vitro* covalent binding of a tianeptine metabolite to microsomal proteins. This covalent binding required oxygen and NADPH. It was decreased by piperonyl butoxide (4 mM) by 81%, and SKF 525-A (4 mM) by 87%, two relatively non-specific inhibitors of cytochrome P450, and by glutathione (4 mM) by 70%, a nucleophile. Covalent binding was decreased by 54% in the presence of troleandomycin (0.1 mM), a specific inhibitor of the glucocorticoid-inducible cytochrome P450 IIIA3, but remained unchanged in the presence of quinidine (0.1 mM) or dextromethorphan (0.1 mM), two inhibitors of cytochrome P450 IID6. Preincubation with IgG antibodies directed against cytochrome P450 IIIA3 decreased covalent binding by 65% whereas either preimmune IgG or IgG antibodies directed against P450 IA1, an isoenzyme inducible by polycyclic aromatic compounds, exhibited no significant inhibitory effect. We conclude that tianeptine is activated by human liver cytochrome P450 into a reactive metabolite. This activation is mediated in part by glucocorticoid-inducible isoenzymes but not by P450 IID6 (the isoenzyme which oxidizes debrisoquine) nor by P450 IA1 (an isoenzyme inducible by polycyclic aromatic compounds). The predictive value of this study regarding possible idiosyncratic and immunoallergic reactions in humans remains unknown.

Several tricyclic antidepressants have been shown to be potentially hepatotoxic in humans [1, 2]. For some of them, the mechanism of toxicity is believed to be related, at least in part, to metabolic activation by hepatic cytochrome P450 into chemically reactive metabolites [3–6].

Tianeptine is a new tricyclic antidepressant which has been marketed in France since May 1988. It has been shown that tianeptine is transformed by liver cytochrome P450 into a chemically reactive metabolite that covalently binds to hepatic proteins both *in vitro* and *in vivo*, in hamsters, rats and mice [7, 8]. This metabolic activation was shown to be partially mediated by glucocorticoid-inducible isoenzyme(s) of cytochrome P450 but not by P450 IID6, the isoenzyme mainly involved in debrisoquine oxidation [7, 8].

The cytochrome P450 isoenzymes present in human liver are not exactly the same as those present in animals [9]. The purpose of this study was to determine whether a similar metabolic activation may occur in human liver.

MATERIAL 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 [7], 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 dextromethorphan hydrobromide were generous gifts from Pfizer (Sandwich, U.K.), and Hoffmann-La Roche (Basel, Switzerland). Quinidine hydrochloride and glutathione were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.), 1,1,1-trichloropropene 2,3-oxide from Aldrich (Beerse, Belgium) and SKF 525-A from Smith Kline and French Laboratories (Herts, U.K.).

Human liver microsomes. The protocol to obtain human liver samples and to prepare hepatic microsomes was similar to those used in several previous studies [10–12]. A liver sample was taken by surgical biopsy in nine patients undergoing elective abdominal surgery for various reasons (digestive carcinomas, gallbladder stones, liver tumours), and in whom a histologic examination of the liver was medically required. We excluded alcoholic patients or those who had taken drugs known to induce microsomal enzymes [13] during the 2 weeks preceding surgery; patients taking other drugs were not excluded since drugs had to be given for premedication and anesthesia, anyway. Patients were premedicated with alimemazine, atropine and hydroxyzine. They were anesthetized with droperidol, enflurane, fentanyl, thiopental sodium and pancurionum bromide. This study was approved by the local Ethics Committee and informed consent was obtained from patients.

A portion of the liver specimen was used for histologic examination. The remainder of the liver samples was frozen and stored at −20° until the

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conclusion of the pathologist was available, a few days later. Only those liver specimens with a normal histology were used in this study.

The liver fragment was then thawed, blotted dry, weighed and homogenized in 3 volumes of ice-cold, 0.15 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 60 min. Microsomal pellets were stored at −20° until analysed, a few days later.

Animal liver microsomes. Some studies were performed with liver microsomes from hamsters, rats and mice. Male Sprague–Dawley rats [Crl: CD® (SD) BR] weighing 180–220 g and male Swiss mice [Crl: CD®-1 (ICR) BR] weighing 25–35 g were purchased from Charles River France (Saint-Aubin-lès-Elbeuf, France). Male golden Syrian hamsters, weighing 90–110 g, were purchased from Fichot (Ormesson, France). Animals were fed *ad lib.* with a normal diet: Autoclavé 113, UAR, France (for rats), and M 25 biscuits, Extra Labo, Provins, France (for mice and hamsters). Hepatic microsomes were prepared as described above.

*Proteins and cytochrome P*450. Microsomal protein concentration was determined by the method of Lowry *et al.* [14]. Cytochrome P450 was determined as described by Omura and Sato [15].

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, 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 experiments, piperonyl butoxide (4 mM), SKF 525-A (0.1 or 4 mM), dextromethorphan hydrobromide (0.1 mM), quinidine hydrochloride (0.1 mM), troleandomycin (0.1–4 mM), 1,1,1-trichloropropene 2,3-oxide (0.1 mM) or glutathione (4 mM) was added to the incubation mixture.

Covalent binding to microsomal proteins was measured as previously described [7, 8, 16]. Briefly, proteins in the zero-time and the incubated samples were preincubated with 2 mL of 10% perchloric acid, and extracted four times with 2 mL of ethylacetate. Proteins were 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 [7, 8]. The pellets were then 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 protein concentration. 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 P450 3c (P450 IIIA6) and cytochrome P450 6 (P450 IA1) were raised in goats and the IgG fractions were isolated as previously described [17]. Anti-P450 IIIA6 antibodies have been shown to cross-react with the orthologous proteins in rat (P450 IIIA1, IIIA2), hamster, mouse and man (P450 IIIA3) [18, 19]. The presence of P450 IA1 has been demonstrated not only in rabbit but also in rat, mouse and human [9]. Goat preimmune IgG, purchased from Sigma was used as control.

Microsomes (1.25 mg protein), corresponding to about 0.4 nmol of cytochrome P450, were preincubated with 1.25 mg of preimmune IgG or with a similar amount of anti-P450 IIIA6 or anti-P450 IA1 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. Covalent binding in the presence of anti-P450 IIIA6 or of anti-P450 IA1 antibodies was compared to that in the presence of a similar amount of preimmune IgG or in the absence of any preincubation with antibodies.

RESULTS

Incubation of [^{14}C]tianeptine with human liver microsomes and a NADPH-generating system resulted in the *in vitro* covalent binding of a ^{14}C-labelled material to microsomal proteins (Fig. 1).

In vitro covalent binding was inhibited by high concentrations (4 mM) of piperonyl butoxide and SKF 525-A, two non-specific inhibitors of cytochrome P450 (Fig. 1) as well as by glutathione (4 mM) (Fig. 2).

Covalent binding was not significantly modified by the addition of 1,1,1-trichloroproprene 2,3-oxide, an inhibitor of microsomal epoxide hydrolase [20] to the incubation mixture (Fig. 2).

The level of *in vitro* covalent binding of tianeptine metabolites to liver microsomal protein was compared in humans and in several animal species. When expressed per mg of microsomal proteins, covalent binding was not significantly different in humans, hamsters and mice, but was significantly lower in rats (Fig. 3A). When expressed per nmol of cytochrome P450, covalent binding was about twice as high in humans than in these different animal species (Fig. 3B).

The effects of several selective inhibitors of cytochrome P450 on *in vitro* covalent binding are shown in Figs 4 and 5. Quinidine, a potent inhibitor of cytochrome P450 IID6, the cytochrome P450 isoenzyme which metabolizes debrisoquine [21], and several tricyclic antidepressants [22–24], exhibited no significant inhibitory effects on covalent binding,

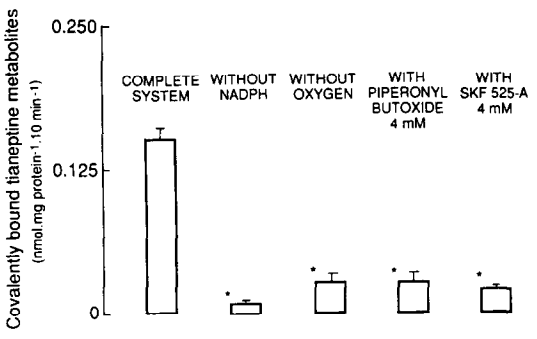

Fig. 1. Cofactors requirements and effects of non-specific inhibitors of cytochrome P450 on *in vitro* covalent binding of [^{14}C]tianeptine metabolites to human liver microsomal proteins. In the "complete system", [^{14}C]tianeptine (0.5 mM, 1 μCi/mL) was incubated under air with human liver microsomes (5 mg protein/mL) and a NADPH-generating system for 10 min at 37°. In some flasks, the NADPH-generating system was omitted. Other flasks were incubated under nitrogen, in the presence of an oxygen-depleting system (see Materials and Methods). In others, piperonyl butoxide (4 mM) or SKF 525-A (4 mM) were added to the incubation mixture. Results are means ± SE for 5–9 experiments. The asterisk indicates a significant difference from the value in the complete system (one-way analysis of variance with Dunnett's *t*-test, $P < 0.01$).

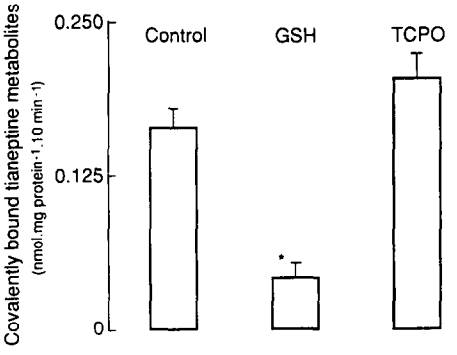

Fig. 2. Effects of glutathione or of an inhibitor of microsomal epoxide hydrolase on *in vitro* covalent binding of [^{14}C]tianeptine metabolites to human liver microsomal proteins. Incubation was made as in Fig. 1. In some flasks, 4 mM glutathione (GSH) or 0.1 mM 1,1,1-trichloropropene 2,3-oxide (TCPO) were added to the incubation mixture. Results are means ± SE for six experiments. The asterisk indicates a significant difference from the value in the control incubation (one-way analysis of variance with Dunnett's *t*-test, $P < 0.01$).

even at a concentration (0.1 mM) much higher than the K_i (60 nM) for the inhibition by quinidine of debrisoquine 4-hydroxylation (Fig. 4) [21]. Similarly, *in vitro* covalent binding was not inhibited by dextromethorphan hydrobromide (0.1 mM), a compound exhibiting a high affinity for cytochrome P450 IID6 [25] (Fig. 4). In contrast, troleandomycin, which selectively forms an inactive cytochrome P450-metabolite complex with the glucocorticoid-inducible cytochrome P450p isoenzyme [26], decreased by 54% covalent binding at a concentration (0.1 mM) at which SKF 525 A exhibited no effect (Fig. 4). This inhibitory effect was only slightly enhanced for higher doses of troleandomycin (0.25 and 4 mM) (Fig. 5).

Immunoinhibition studies were performed using various IgG antibodies. A preincubation with 1.25 mg of anti-P450 IIIA6 IgG for 5 min decreased the *in vitro* covalent binding of tianeptine metabolites by 65% (Fig. 6). In contrast, the preincubation with a similar amount of preimmune IgG or of anti-P450 IA1 antibodies exerted no significant inhibitory effect (Fig. 6).

DISCUSSION

The present study shows that tianeptine is activated by human liver microsomal cytochrome P450 into a chemically reactive metabolite that covalently binds to microsomal proteins *in vitro*, as previously demonstrated *in vivo* and *in vitro* in several animal species [7, 8]. Indeed, covalent binding to human liver microsomes required oxygen and NADPH (Fig. 1). It was decreased by cytochrome P450 inhibitors such as piperonyl butoxide and SKF 525-A (Fig. 1) and by glutathione, a nucleophile (Fig. 2).

The chemical structure of the tianeptine reactive metabolite remains unknown. It has been hypothesized that the oxidation of several potentially hepatotoxic, neuropsychiatric compounds with a tricyclic structure, in particular imipramine, amitriptyline and carbamazepine, may lead to the formation of reactive epoxides [3, 27, 28]. We observed that 1,1,1-trichloropropene 2,3-oxide, an inhibitor of microsomal epoxide hydrolase, had no significant effect on *in vitro* covalent binding of tianeptine metabolites both in hamsters [7] and in humans (Fig. 2). This finding does not support the hypothesis of an epoxide structure for tianeptine reactive metabolite but is not sufficient to rule it out. The characterization of the chemical structure of this metabolite requires further investigation.

When expressed per nmol of cytochrome P450, i.e. the enzyme responsible for metabolic activation, covalent binding of tianeptine metabolites was markedly higher with human liver microsomes than with liver microsomes from hamsters, rats and mice (Fig. 3B). This observation suggests that human liver microsomes may contain some cytochrome P450 isoenzyme(s) particularly able to form the tianeptine reactive metabolite.

We tried to assess which cytochrome P450 isoenzyme(s) may be involved in tianeptine metabolic activation, using several selective inhibitors and antibodies raised against specific cytochrome P450 isoenzymes. The absence of inhibitory effect of quinidine and dextromethorphan on covalent binding strongly suggests that cytochrome P450 IID6, the isoenzyme responsible for the oxidation of debrisoquine and several tricyclic antidepressants [29], does not contribute significantly to the metabolic activation of tianeptine in human liver (Fig. 4). These results in humans are consistent with previous results showing that this isoenzyme was not involved in tianeptine metabolic activation in hamsters, rats and mice [7, 8]. Similarly, the absence of significant

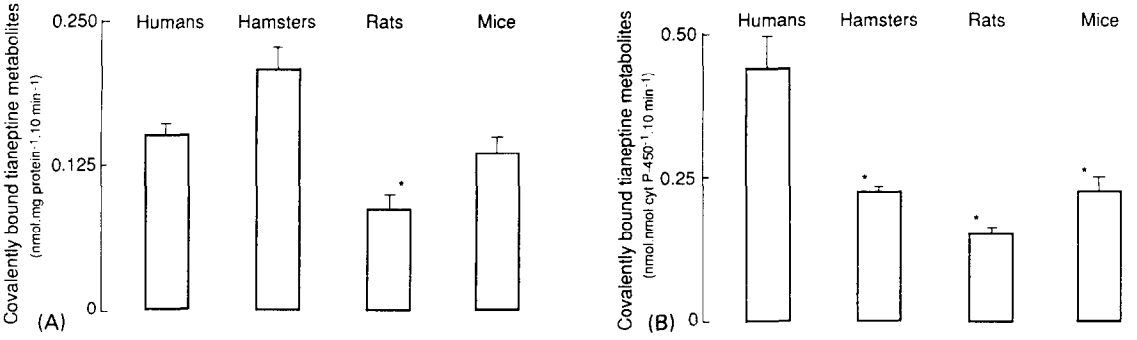

Fig. 3. Comparison of *in vitro* covalent binding with liver microsomes from humans, hamsters, rats, and mice. [^{14}C]Tianeptine was incubated with liver microsomes and a NADPH-generating system for 10 min at 37°. Microsomal protein concentration (mean ± SE) was 35 ± 2 mg/g liver in humans, 33 ± 2 in hamsters, 35 ± 3 in rats, and 38 ± 3 in mice. Microsomal cytochrome P450 content (mean ± SE) was 0.35 ± 0.06 nmol/mg microsomal protein in humans, 1.05 ± 0.09 in hamsters, 0.75 ± 0.06 in rats, and 0.71 ± 0.04 in mice. (A) shows *in vitro* covalent binding of [^{14}C]tianeptine metabolites expressed per mg of microsomal proteins. (B) shows covalent binding expressed per nmol of cytochrome P450, i.e. the enzyme responsible for metabolic activation. Results are means ± SE for six to eleven experiments. The asterisk indicates a significant difference between values found in humans and in animals (one-way analysis of variance with Dunnett's *t*-test, $P < 0.01$).

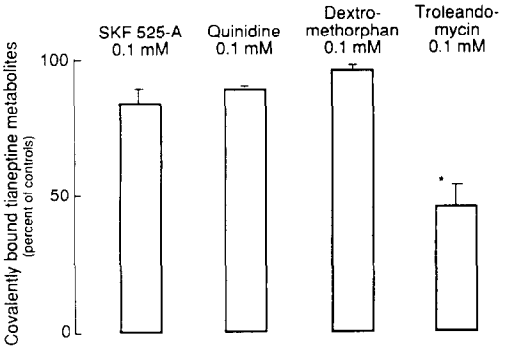

Fig. 4. Comparative effects of various selective inhibitors of cytochrome P450 isoenzymes on the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to human liver microsomal proteins. Incubation was made as in Fig. 1. In some flasks, inhibitors (0.1 mM) were added to the incubation mixture. Covalent binding in the presence of the inhibitor was expressed as the percentage of that found in the absence of inhibitor with the same human microsomes. Mean covalently bound tianeptine metabolites in the control groups varied from 0.15 to 0.26 nmol/mg protein/10 min, according to the sources of microsomal preparations. Results are means ± SE for two to six experiments. The asterisk indicates a significant difference from the value in corresponding controls (100%) (Wilcoxon signed rank test, $P < 0.01$).

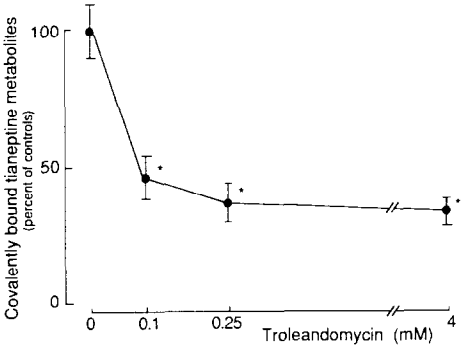

Fig. 5. Effects of various concentrations of troleandomycin on *in vitro* covalent binding of [^{14}C]tianeptine metabolites to human liver microsomal proteins. Incubation was made as in Fig. 1. In some flasks, 0.1, 0.25 or 4 mM of troleandomycin were added to the incubation mixture. Covalent binding was expressed as the percentage of that found in the same human microsomes. Control covalent binding varied from 0.25 to 0.28 nmol/mg microsomal protein/10 min between different experiments. Results are means ± SE for four or five experiments. The asterisk indicates a significant difference from the value in corresponding controls (100%) (one-way analysis of variance with Dunnett's *t*-test, $P < 0.01$).

inhibitory effect of anti-cytochrome P450 IA1 on covalent binding suggests that cytochrome P450 IA1, an isoenzyme inducible by polycyclic aromatic compounds and present in the liver of rats, mice, rabbits and humans [9], does not contribute significantly to tianeptine metabolic activation (Fig. 6).

In contrast, results of the present study strongly suggest that tianeptine metabolic activation is mediated partially by cytochrome P450 isoenzyme(s) inducible by glucocorticoids in humans as previously observed in animals [7, 8]. Indeed, *in vitro* covalent binding of tianeptine metabolites was markedly decreased in the presence of troleandomycin (Fig. 4), an antibiotic known to selectively inhibit glucocorticoid-inducible isoenzyme(s) [26]. In addition, immunoinhibition experiments showed that covalent binding was also markedly decreased by preincubation with antibodies raised against rabbit cytochrome P450 IIIA6 and cross-reacting with human cytochrome P450 IIIA3 (Fig. 6). Albeit mainly mediated by glucocorticoid-inducible isoenzyme(s), metabolic activation of tianeptine in the human liver

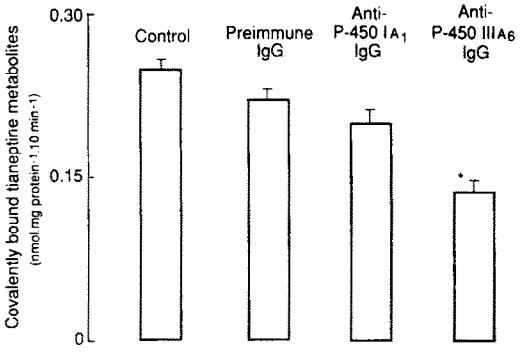

Fig. 6. Immunoinhibition of the *in vitro* covalent binding of [^{14}C]tianeptine metabolites to human liver microsomal proteins. Microsomes (1.25 mg) were preincubated with either preimmune IgG (1.25 mg) or anti-P450 IA1 IgG (1.25 mg) or anti-P450 IIIA6 IgG (1.25 mg), before the addition of [^{14}C]tianeptine (0.5 mM) and an NADPH-generating system. Results are means ± SE in three experiments. The asterisk indicates a significant difference from values found in absence of antibodies or Student's *t*-test for paired data, $P < 0.01$).

may be mediated also by other cytochrome P450 isoenzymes. This view is supported by two sets of observations. Firstly, the inhibition of covalent binding by troleandomycin (0.1–4 mM) reached a limit of about 60–70% as shown in Fig. 5. In contrast, for the same range of concentrations (0.1–4 mM), inhibition by the non-specific inhibitor of cytochrome P450, SKF 525-A increased from 20% (at 0.1 mM) to 92% (at 4 mM) (Figs 1 and 4). Secondly, the extent of inhibition of covalent binding by anti-P450 IIIA6 was also in the same range (65%) despite the use of a large amount of antibodies (2.5 mg IgG per nmol P450). From these data, it is tempting to speculate that about two-thirds of the metabolic activation of tianeptine in human liver might be mediated by glucocorticoid-inducible isoenzyme(s), and that about one-third of this metabolite activation may be related to other isoenzyme(s) of cytochrome P450, which remain(s) to be identified.

It remains unknown whether the observation of metabolic activation has a predictive value for human therapeutics. Indeed, for other drugs, the formation of reactive metabolites has been reported only after these drugs had been shown to produce hepatitis. It is generally admitted that reactive metabolites may lead to three types of hepatitis in humans: predictable toxic hepatitis, idiosyncratic toxic hepatitis and immunoallergic hepatitis. Toxicity studies performed in hamsters [8], with rat liver slices (M. Roberfroid, personal communication) or with isolated human hepatocytes [30] have suggested that the therapeutic doses of tianeptine are far too low to lead to predictable hepatotoxicity in humans. Indeed, the concentration of tianeptine required to produce some toxicity in isolated human hepatocytes was 200 μg/mL, i.e. 500-fold the peak plasma tianeptine concentration in human [30]. Consistently, no case of liver damage caused by tianeptine has been detected during clinical trials [31]. It cannot be excluded, however, that hepatitis may develop in the future, in an exceptional subject, as a result of a metabolic idiosyncrasy or, more likely, of an immunoallergic response.

We conclude that tianeptine is transformed by human liver cytochrome P450 into a reactive metabolite which covalently binds to hepatic microsomal proteins. Metabolic activation involves, at least partially, cytochrome P450 isoenzyme(s) inducible by glucocorticoids (P450 III family) but not cytochrome P450 IID6, the isoenzyme metabolizing debrisoquine nor cytochrome P450 IA1, an isoenzyme inducible by polycyclic aromatic compounds.

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