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ACTIVATION OF MIANSERIN AND ITS METABOLITES BY HUMAN LIVER MICROSOMES

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Abstract—Human liver microsomes metabolise mianserin to the stable 8-hydroxymianserin, desmethylmianserin and mianserin-2-oxide and in addition to one or more chemically reactive metabolites which bind, irreversibly, to microsomal protein. The stable metabolites were isolated by HPLC and characterized by mass spectrometry. The generation of each of these metabolites showed substantial inter-individual variation between eight sets of human liver microsomes studied. Inhibition of irreversible binding was observed with SKF-525A together with concomitant decrease in the formation of 8-hydroxymianserin and desmethylmianserin but not mianserin-2-oxide. Methimazole inhibited binding and the formation of each of the metabolites at a low concentration. Quinidine did not significantly inhibit irreversible binding but did inhibit the formation of 8-hydroxymianserin. Sulphaphenazole had no effect on irreversible binding or metabolism. The irreversible binding of mianserin was inhibited by ascorbic acid, glutathione and *N*-acetyl cysteine, whereas *N*-acetyl lysine and trichloropropane oxide had no effect. The irreversible binding of mianserin, 8-hydroxymianserin and desmethylmianserin was of the same order of magnitude however significantly greater binding was observed with the desmethyl metabolite. Incubations with [10-^{3}H/^{14}C]mianserin showed no change in the ^{3}H/^{14}C ratio when irreversible binding occurred. Inhibition of irreversible binding was demonstrated with sodium cyanide at concentrations which did not inhibit total metabolism, which suggest that metabolic activation by the cytochrome P-450 enzyme system may lead to the formation of a reactive iminium intermediate that can bind to nucleophilic groups on proteins.

Mianserin is a tetracyclic antidepressant developed during the early 1970s and has advantages over the related tricyclic antidepressants in terms of fewer cardiotoxic side effects [1]. However, it has become recognized that mianserin is associated with a number of adverse reactions including skin rashes [2], hepatotoxicity [3] and blood dyscrasias [4]. The most common of these adverse reactions is the development of blood dyscrasias with an estimated incidence of ≤ 1 in 10,000 [5, 6], although the estimate varies from country to country [7].

The mechanism(s) involved in mianserin toxicity in man has not been determined [8]. It has been suggested that the development of agranulocytosis may involve a direct suppressive effect on the bone marrow, and that this may be exacerbated by the saturation of mianserin metabolism [9]. Other workers [10] however have shown specific mianserin-dependent antibodies against platelets in the serum of a patient with thrombocytopenia and have suggested that an immunologically mediated mechanism is involved in the development of this type of blood disorder.

The role of metabolism in the toxicity of mianserin has not been defined. Several other antidepressant drugs, such as imipramine [11] and amineptine [12, 13] are transformed by cytochome P-450 into reactive metabolites that bind covalently to macromolecules. In theory, such metabolites may produce cytotoxicity directly or may function as an immunogen to initiate an immune reaction.

Studies *in vivo* indicate that mianserin undergoes extensive oxidation and 8-hydroxymianserin, desmethylmianserin and mianserin-2-oxide have been identified as major urinary metabolites [14, 15]. In this communication we describe the metabolism of mianserin by human liver microsomes *in vitro* and report that the drug undergoes variable activation to an electrophilic metabolite that reacts covalently with thiol groups on proteins.

MATERIALS AND METHODS

Materials. [13-^{3}H]Mianserin (sp. act. 3.5 Ci/mol), [10-^{3}H/^{14}C]mianserin (sp. act. 9 mCi/mol for ^{3}H and 0.4 mCi/mol for ^{14}C), [13-^{3}H]desmethylmianserin (sp. act. 0.4 Ci/mmol), [13-^{3}H]-8-hydroxymianserin (sp. act 0.2 Ci/mmol), and authentic standards were generously donated by Organon International BV (Oss, The Netherlands). All radiolabelled drugs were found to be radiochemically pure when analysed by HPLC†. SKF-525A was a gift from Smith Kline & French (Welwyn Garden City, U.K.). Sulphaphenazole was donated by Ciba-Geigy (Switzerland). NADPH (tetrasodium salt) was obtained from Boehringer Mannheim GmbH, Biochemica (Mannheim, F.R.G.). Scintillation fluid (Aqua Luma Plus) was from Lumac/3M B.V. (Schaesberg, The Netherlands). Solvents (HPLC grade) were purchased from Fison PLC (Loughborough, U.K.). All other chemicals were obtained from Sigma Chemical Co. (Poole, U.K.).

Microsomal incubations. Human livers were obtained from kidney transplant donors with ethical

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† Abbreviations used: HPLC, high performance liquid chromatography; RT, retention time on HPLC; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

approval granted. The livers were stored at −80° until required. Washed microsomes were prepared as described previously [16] and the protein content was measured by the method of Lowry *et al.* [17]. The cytochrome P-450 content was determined by the method of Omura and Sato [18].

Microsomes (8 mg of protein) from eight human livers were incubated with [13-^{3}H]mianserin (0.5 μCi; 10 μM) in 0.1 M sodium phosphate buffer (pH 7.5; 4 ml). Reactions were started by the addition of NADPH (1 mM, a concentration that has previously been shown not to be rate limiting). Control incubations containing no NADPH were performed simultaneously. Incubations were conducted at 37° for 30 min with continuous agitation.

To investigate the route of formation of the reactive intermediate human liver microsomes (8 mg of protein) were incubated with [13-^{3}H]mianserin (0.5 μCi; 10 μM) and NADPH (1 mM) in 0.1 M sodium phosphate buffer (pH 7.5; 4 ml) in the presence and absence of the cytochrome P-450 mixed function oxidase inhibitor, SKF-525A, (50 μM) and the enzyme inhibitors, methimazole (50 μM), quinidine (50 μM) and sulphaphenazole (50 μM) at 37° for 30 min.

In a separate experiment [13-^{3}H]mianserin (1 μCi; 1 μM) and the stable metabolites [13-^{3}H]-8-hydroxymianserin (0.87 μCi; 1 μM) and [13-^{3}H]desmethylmianserin (1.61 μCi; 1 μM) were co-incubated with human liver microsomes (8 mg of protein) and NADPH (1 mM) in 0.1 M sodium phosphate buffer (pH 7.5; 4 ml) at 37° for 30 min.

The chemical nature of the reactive intermediate was investigated by the incubation at 37° for 30 min of human liver microsomes (8 mg of protein), [13-^{3}H]mianserin (0.5 μCi; 10 μM) and NADPH (1 mM) with the reductant, ascorbic acid (10 mM), the epoxide hydrolase inhibitor, trichloropropane oxide (0.5 mM), the amino nucleophile, *N*-acetyl lysine (0.5 mM) and the sulphydryl nucleophiles glutathione (0.5 mM) and *N*-acetyl cysteine (0.5 mM).

To attempt the identification of the reactive species produced two further *in vitro* experiments were performed. Firstly [10-^{3}H/^{14}C]mianserin (2.3 μCi/0.1 μCi; 63 μM and 9 μCi/0.4 μCi; 250 μM) was incubated with human liver microsomes (8 mg of protein) and NADPH (1 mM) at 37° for 30 min allowing the investigation of the involvement of the 10 position in irreversible binding. Secondly [13-^{3}H]mianserin (1 μCi; 10 μM) was incubated with human liver microsomes (8 mg of protein) and NADPH (1 mM) in the presence and absence of sodium cyanide (0.1–5 mM) at 37° for 30 min.

All reactions were terminated by cooling with the addition of methanol (6 ml) to precipitate the protein.

Covalent binding to proteins. Protein pellets from the incubations were subjected to exhaustive solvent extraction [19] and the supernatant was used for HPLC analysis. The exhaustive solvent extraction consisted of washing with methanol (2 × 5 ml) and 70% methanol (2 × 5 ml). No further radioactivity could be removed by subsequent washing. Following extraction the protein pellet was resolubilized in 0.5 M sodium hydroxide (3 ml) at 60° for 1 hr. An aliquot was neutralized by glacial acetic acid and

Table 1. The metabolism and irreversible binding of mianserin by human liver microsomes

Liver	Total cytochrome P-450 content (nmol/mg protein)	% Bound without NADPH	% Bound with NADPH	NADPH-dependent % Bound	% Hydroxymianserin	% Desmethylmianserin	% Mianserin-2-oxide
1	0.60	0.85 ± 0.37	5.93 ± 0.45	5.08	12.0	11.4	1.5
2	0.23	0.50 ± 0.14	2.76 ± 0.78	2.26	0.4	2.4	0.5
3	0.20	0.42 ± 0.03	2.49 ± 0.47	2.07	0.0	4.8	1.4
4	0.67	1.35 ± 0.15	6.47 ± 1.58	5.12	5.8	3.1	0.0
5	0.29	0.63 ± 0.07	2.83 ± 0.15	2.20	0.4	0.0	1.1
6	0.26	0.43 ± 0.08	0.77 ± 0.05	0.34	0.0	0.0	0.0
7	0.37	1.07 ± 0.23	5.80 ± 0.23	4.73	1.2	0.0	0.0
8	0.23	9.98 ± 0.07	2.83 ± 0.22	1.85	3.6	16.7	2.0
Mean ± SD	0.36 ± 0.18	0.78 ± 0.34	3.74 ± 2.05	2.96 ± 1.78	2.9 ± 4.2	5.5 ± 6.3	0.8 ± 0.8

[13-^{3}H]mianserin (0.5 μCi; 10 μM) was incubated with human liver microsomes (8 mg) and NADPH (1 mM) at 37° for 30 min.
Each value is the mean ± SD of quadruplet determinations for irreversible binding and the mean of triplicate determinations for other values.

Table 2. The effect of four enzyme inhibitors (50 μM) on the metabolism of mianserin

Inhibitor	% Irreversibly bound	% Metabolized to 8-hydroxymianserin	% Metabolized to desmethylmianserin	% Metabolized to mianserin-*N*-oxide
None	4.7 ± 0.8	14.2 ± 0.7	32.9 ± 0.7	6.2 ± 0.3
SKF 525-A	1.2 ± 0.1**	8.3 ± 0.6***	14.2 ± 0.4***	5.6 ± 0.4
Methimazole	1.8 ± 0.1**	10.5 ± 1.9*	27.7 ± 0.5***	4.0 ± 0.2***
Quinidine	4.1 ± 0.5	12.0 ± 0.5*	31.6 ± 1.0	5.8 ± 0.2
Sulphaphenazole	6.6 ± 1.6	11.0 ± 2.3	32.6 ± 2.8	6.0 ± 0.3

Each value is the mean of triplicate determinations. Significantly different from control. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (Student's *t*-test).

the amount of covalently bound radioactivity was determined by liquid scintillation spectrometry. A further aliquot was taken for protein determination [17]. Results are expressed as the percentage of incubated radioactivity irreversibly bound to microsomal protein.

HPLC analysis of metabolites. Supernatants from *in vitro* incubations were analysed by radiometric reversed phase HPLC. Metabolites were identified by co-chromatography with authentic standards. A Gilson 302/111B HPLC system was employed for the analysis. Separations were achieved with a 25 cm × 4.6 i.d. octadecylsilyl 10 μM particle size column (Waters ODS 10 μM). The mobile phase consisted of ammonium phosphate buffer (pH 4.6; 0.05 M) containing heptane sulphonic acid (5 mM) and acetonitrile (62:38). The flow rate was 1.5 ml/min. Fractions were collected at 0.5 min intervals for 20 min. The order of elution of mianserin and its known metabolites was 8-hydroxymianserin (RT 3.5 min), desmethylmianserin (RT 6 min), mianserin (RT 8 min) and mianserin-2-oxide (RT 16 min). Metabolite formation was expressed as percentage of incubated radioactivity. No attempt was made to determine enzyme kinetics as this was precluded by the 30 min incubation period required to obtain measurable amounts of covalently bound radioactivity.

Mass spectrometry of metabolites. Electron impact mass spectra of unchanged mianserin and its metabolites isolated from microsomal incubations by HPLC were obtained using a VG Tritech TS-250 mass spectrometer linked to a VG 11/250J data system. Samples were introduced by direct insertion. Spectra were acquired by full scanning acquisition between *m/z* 50–*m/z* 500 (electron energy, 70 eV; emission current, 880 μV; source temperature, 180°; resolution, 500; accelerating voltage, 4120 V).

RESULTS

Mianserin was metabolized, in incubations with human liver microsomes, to three stable metabolites, 8-hydroxymianserin, desmethylmianserin and mianserin-2-oxide, which were characterized by mass spectrometry: molecular ions were observed at *m/z* 264 (mianserin), *m/z* 280 (8-hydroxymianserin) and *m/z* 250 (desmethylmianserin) while the $[M-16]^{+\cdot}$ ion was found for the mianserin-2-oxide, this corresponds to the characteristic loss of oxygen from an *N*-oxide. Diagnostic fragment ions corresponding to peaks seen in the spectrum of the relevant authentic standard were found in each case [14]. With each of the eight human livers studied incubation of [13-^{3}H]mianserin resulted in the irreversible binding of radioactive material to protein (Table 1). In addition a marked inter-individual difference in the formation of the stable metabolites was recorded, such that in two livers (4 and 6) there was apparently no hydroxylation and three samples (5, 6 and 7) produced no desmethylmianserin. Analysis of the data by Spearman's rank correlation test indicated that significant concordance occurred between total cytochome P-450 content and irreversibly bound drug ($P \leq 0.05$) and, in addition, between desmethylmianserin and mianserin-2-oxide formation ($P \leq 0.05$). However, no significant concordance was obtained between irreversible binding and the formation of any single stable metabolite. Addition of SKF-525-A resulted in significant ($P \leq 0.01$) inhibition of irreversible binding of radioactive material to microsomal protein (Table 2), and reduced formation of 8-hydroxymianserin ($P \leq 0.001$) and desmethylmianserin ($P \leq 0.001$) but not mianserin-2-oxide formation. Methimazole inhibited irreversible binding ($P \leq 0.01$) and had a significant ($P \leq 0.05$) effect on the formation of each of the metabolites (Table 2). Quinidine did not inhibit irreversible binding but produced a significant ($P \leq 0.05$) reduction in 8-hydroxymianserin formation, whereas sulphaphenazole had no significant ($P \geq 0.05$) effect on irreversible binding nor on metabolism to stable products.

Incubations of [13-^{3}H]mianserin, [13-^{3}H]desmethylmianserin and [13-^{3}H]8-hydroxymianserin with human liver microsomes led to irreversible binding of tritiated material to microsomal protein. The degree of binding with desmethylmianserin (5.97%) was significantly greater ($P \leq 0.05$) than that of mianserin (3.96%) and 8-hydroxymianserin (4.17%).

The NADPH-dependent binding was inhibited by a number of compounds (Fig. 1). A significant inhibition ($P \leq 0.05$) of irreversible binding was detected following co-incubation with the antioxidant ascorbic acid (36%) and the sulphydryl nucleophiles glutathione (70%) and *N*-acetyl cysteine (44%). No significant effects on binding were observed with the amino nucleophile *N*-acetyl lysine and the epoxide hydrolase inhibitor, trichloropropane oxide (TCPO).

The possibility that hydroxylation at the 10 posi-

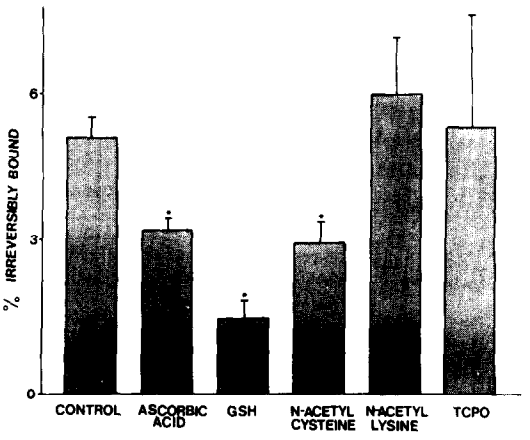

Fig. 1. The effect of several inhibitors on the irreversible binding of mianserin to human liver microsomal protein. Incubations contained mianserin (0.5 μCi; 10 μCi), human liver microsomes (8 mg of protein) and NADPH (1 mM) in 4 ml of phosphate buffer (0.1 M; pH 7.4) with 0.5 mM inhibitor or in the case of ascorbic acid 10 mM inhibitor. Each value is the mean ± SD of four determinations. * $P \leq 0.05$.

tion was involved in the irreversible binding was investigated by comparing the ratio of ^3H to ^{14}C of [10-^3H/^{14}C]mianserin when free and when bound to protein. The ratio of $^3H/^{14}C$ for unbound [10-^3H/^{14}C]mianserin was 26.0. When [10-$^3H/^{14}C$]mianserin was incubated with human liver microsomes at concentrations of 62 and 250 μM the ratios of $^3H/^{14}C$ irreversibly bound to protein were 25.0 and 23.9, respectively, which were not significantly different ($P \geq 0.05$) from the ratio for the free, unbound dual labelled drug.

Incubations of [13-^{3}H]mianserin with sodium cyanide (0.1–1.0 mM) led to significant reduction of irreversible binding (55–63% inhibition) without altering total metabolism. At a concentration of 5 mM, sodium cyanide significantly reduced (20% inhibition; $P < 0.05$) total mianserin metabolism, which was reflected in a further reduction of irreversible binding (to 83% inhibition).

DISCUSSION

Mianserin is metabolised by human liver microsomes to three stable phase I metabolites (Fig. 2). Two of these metabolites, 8-hydroxymianserin and mianserin-2-oxide, have been identified in urine from women given radiolabelled mianserin [16]. We have also demonstrated that mianserin undergoes metabolic activation to a chemically reactive metabolite(s) which binds irreversibly to microsomal protein. Mianserin shares this property with reference antidepressants like imipramine [11] and amineptine [12, 13]. The formation of the reactive metabolite and the three stable metabolites showed interindividual variation between the eight human livers studied. Inspection of Table 1 shows that *N*-demethylation may proceed independently of hydroxylation (liver 3) and that hydroxylation may be observed without concomitant demethylation or *N*-oxidation (liver 7) however, there appears to be a link between *N*-oxide formation and demethylation.

The overall lack of correlation between metabolite formation indicates that more than one enzyme is involved in the oxidation of the drug. Reactive metabolite formation was observed, albeit to a vari-

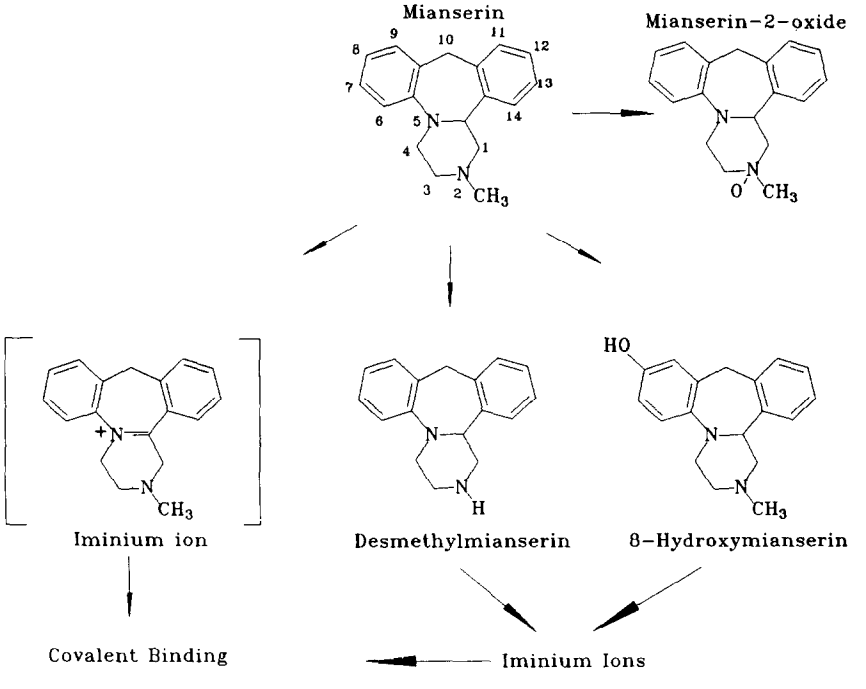

Fig. 2. Proposed metabolic pathway of mianserin producing multiple reactive iminium ions.

able extent, with all livers. In order to investigate the mechanism of activation of mianserin to a reactive metabolite we studied several selective inhibitors of drug oxidation. Generation of the chemically reactive metabolite was inhibited by SKF 525-A suggesting that its formation is mediated by a cytochrome P-450-dependent mixed function oxidase. The lack of inhibition of *N*-oxidation by SKF 525-A indicates that this metabolite is formed by other systems, and is probably not involved in the irreversible binding of mianserin. The flavin containing monooxygenases are responsible for *N*-oxidation of a number of drugs [20] and are inhibited by methimazole. At low concentrations (50 μM) methimazole was found to inhibit both the *N*-oxidation of mianserin and the irreversible binding of mianserin to protein, however the latter effect may have been due to chemical interaction of the sulphydryl group in methimazole with the reactive intermediate together with non-specific enzyme inhibition. Quinidine, a selective inhibitor of debrisoquine hydroxylase [21], produced partial inhibition of aromatic hydroxylation. Sulphaphenazole, a potent inhibitor of tolbutamide hydroxylation *in vitro* [22] had no effect on the metabolism of mianserin. Thus, it was not possible to obtain complete inhibition of reactive metabolite via enzyme inhibition. The reason for this observation became apparent when we investigated the metabolism of the major phase I metabolites 8-hydroxymianserin and desmethylmianserin. It was found that both desmethylmianserin and 8-hydroxymianserin, become irreversibly bound to a similar extent to mianserin. Thus the irreversible binding measured, after incubation of mianserin, may reflect the binding of at least three electrophilic metabolites.

The irreversible binding of mianserin to protein was blocked by thiols and by ascorbic acid, but not by *N*-acetyl lysine, indicating that the species involved are reducible, soft electrophiles. Consideration of the structure of mianserin suggested three possible sites of oxygenation which might yield such a metabolite: (1) the arene rings, forming an epoxide; (2) hydroxylation at C-10 and subsequent elimination to give a carbonium ion; and (3) hydroxylation at C-14 and subsequent elimination to give an iminium intermediate.

The epoxide hydrolase inhibitor trichloropropane oxide had no effect on irreversible binding which suggests, that oxidation of the arene ring is not involved in the activation. As indicated above, aromatic hydroxylation of mianserin does not correlate with irreversible binding. To investigate the possibility of carbonium ion formation in the 10-position, incubations were performed with mianserin labelled with both ^{3}H and ^{14}C in the 10-position. There was no change in the isotope ratio on binding to protein, or in formation of metabolites, indicating that oxidation at the 10-position does not occur to a significant extent *in vitro*.

The third possible reactive intermediate is the iminium ion formed following 14-hydroxylation of mianserin. Formation of this species would be favoured by the conjugation of two aromatic rings. A similar species has previously been identified as being responsible for the covalent binding to proteins of phencyclidine [23, 24]. Such iminium ions readily react with cyanide. We therefore investigated the effect of various concentrations of cyanide on the metabolism of mianserin. At low concentrations of cyanide it was possible to block the irreversible binding without inhibition of aromatic hydroxylation, demethylation or *N*-oxide formation. However, we have so far been unable to isolate adducts from incubations which contained radiolabelled ^{14}C-cyanide. This may be because several iminium ions may be formed from mianserin and its phase I metabolites. Moreover, it is known that adducts formed from iminium ions are relatively labile, as has recently been reported for nicotine iminium ion [25].

In conclusion, it has been found that mianserin and its major phase I metabolites undergo extensive activation, in the presence of human liver microsomes, to an electrophilic species, probably an iminium ion, which binds irreversibly to thiol nucleophiles. In theory, the formation of such species *in vivo* may be involved in the incidence of agranulocytosis, either by direct cytotoxicity, as we have demonstrated elsewhere [26], or indirectly, via haptenation of specific cells, resulting in an immunologically-mediated cytotoxic response. However, the fact that generation of potentially toxic electrophilic species was found in all eight human liver preparations, whereas the incidence of agranulocytosis is only ≤ 1 in 10000 makes a direct causal relationship unlikely. The relevance of these *in vitro* findings to the *in vivo* toxicity requires further investigation.

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