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Human Anti-Mitochondria Autoantibodies Appearing in Iproniazid-Induced Immunoallergic Hepatitis Recognize Human Liver Monoamine Oxidase B

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Anti-mitochondria (anti-M6) autoantibodies have been found in the serum of patients with immunoallergic iproniazid (Marsilid)-induced hepatitis, but to date the identity of the protein antigen has not been determined. Here we show, using immunoprecipitation of pargyline-labelled proteins, that among the mitochondrial proteins, liver MAO-B is specifically recognized by the sera containing anti-M6 antibodies. Moreover the enzymatic activity of MAO-B towards phenylethylamine and tyramine is also suppressed after this immunoprecipitation, contrary to the MAO-A activity towards 5-hydroxy-tryptamine. As MAO is irreversibly inhibited by iproniazid, these results suggest that the mechanism of iproniazid-induced appearance of anti-M6 antibodies could be another example of the reactive metabolite/enzyme haptenization mechanism already proposed in the case of tienilic acid for the appearance of anti-organelle antibodies in a drug-induced hepatitis.

The mechanisms of drug-induced immuno-allergic hepatitis in humans are poorly understood. In some cases, circulating anti-organelle antibodies have been detected in the sera of patients by using indirect immunofluorescence techniques on rat or mouse liver and kidney sections (1, 2). These autoantibodies appear to be good tools for understanding the detailed mechanism of some drug-induced hepatitis. Unfortunately, for most drugs leading to such effects several different anti-organelle autoantibodies have been detected in patient sera (2, 3). However, three of these anti-organelle autoantibodies appear to be specifically associated with hepatitis induced by a given drug. Anti-liver kidney microsome (LKM2) antibodies have been detected only in patients suffering from hepatitis and treated by tienilic acid (2), anti-liver microsome (LM) antibodies in patients suffering from dihydralazine induced hepatitis (4) and anti-M6 antibodies (M for mitochondria) have been only found in iproniazid-induced hepatitis (5, 6). The anti-LKM2 antibodies are specifically directed against one protein of the endoplasmic reticulum of human liver, a cytochrome P-450 of the 2C subfamily (CYP 2C9) which is mainly responsible for the metabolism and activation of tienilic acid into alkylating metabolites in human liver (7, 8). This activation of tienilic acid through covalent binding to CYP 2C9 is thought to initiate the appearance of antibodies against the cytochrome P-450, and could be related to the observed hepatotoxic effects (9). The sequence of events could be described as follows:

$$\text{Drug} + \text{Activating enzyme} \rightarrow \text{Reactivemetabolite} \rightarrow$$
$$\text{Alkylated enzyme} \rightarrow \rightarrow \text{Appearance of anti-enzyme antibodies} \rightarrow \text{Toxic effects}$$

Similarly in dihydralazine induced hepatitis, anti-LM autoantibodies have been demonstrated to be

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Abbreviations: MAO, monoamine oxidase; anti-M6, anti-mitochondria autoantibodies nb 6; anti-LKM2, anti liver kidney microsome autoantibodies nb 2; anti-LM, anti liver microsome autoantibodies; IC_{50} concentration inhibiting 50% of the activity.

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directed against CYP 1A2 which also metabolizes the drug (4, 10). It was tempting to study whether a similar mechanism would operate for the appearance of anti-M6 antibodies in iproniazid-induced hepatitis. Iproniazid (Marsilid&otrade;) is an irreversible inhibitor of monoamine oxidases (MAO) (11). Its oxidation into reactive metabolites (12) by mitochondrial MAO could lead to an irreversible modification of these enzymes and eventually to the appearance of antibodies against them. The present communication describes a series of experiments which show that anti-M6 autoantibodies appearing in iproniazid-induced hepatitis specifically recognize MAO-B from human liver mitochondria.

MATERIALS AND METHODS

Chemicals. All chemical reagents were of the highest quality available. Electrophoresis reagents and detergents were from Serva (Heidelberg), protein A-Ultrogel was from IBF (France), nitrocellulose sheets were from Biorad, peroxidase-conjugated immunoglobulins were from Dako Immunoglobulin (Copenhagen). [^{3}H]-pargyline (23 Ci/mmol), [^{14}C]-phenylethylamine (50 Ci/mol) were purchased from NEN (Boston, USA) and [^{14}C]-tyramine (1 Ci/mol) and [^{14}C]-5-hydroxy-tryptamine (40 Ci/mol) from Amersham (Bucks, UK). Deprenyl and clorgyline were from Interchim (gifts of Laboratoire Fournier, France).

Human liver mitochondria. Human liver mitochondria were prepared from several livers from renal transplantation donors after accidental death. The livers were removed, perfused and chilled on ice within 15 min after death. Small portions (about 10 g) were frozen in liquid nitrogen and stored at −80°C (7). They were thawed and homogenised for 10 s with an ultraturrax. Mitochondrial fractions were prepared by centrifugations at 800 g and 15000 g for 20 min. They were washed in phosphate buffer (0.1M, pH 7.4) and stored at −80°C. The protein concentration was measured colorimetrically (13).

Human placenta mitochondria. Mitochondria were harvested from placentas (gift of Maternité Baudelocque) within 1 to 2 h of delivery by differential centrifugation (14).

Human sera. Two sera containing anti-M6 antibodies (6) were obtained from Pr J.C. Homberg from patients suffering from iproniazid-induced hepatitis. The anti-M6 titers by immunofluorescence of patients BIM and JAC were respectively 1:2000 and 1:200. Control sera were obtained from ten human volunteers or from ten patients suffering from hepatitis of another origin (7).

Immunoblot analysis. Human liver or placenta mitochondria were subjected to electrophoresis (15) on NaDodSO4 polyacrylamide gels (9%). After completion of electrophoresis, the separated proteins were electrically transferred to nitrocellulose sheets according to Towbin et al. (16). Nitrocellulose sheets were saturated with bovine serum albumin and fetal calf serum and incubated 30 min with either anti-MAO antibodies or human sera at room temperature. Then blots were incubated with peroxidase conjugated immunoglobulins raised against mouse or human immunoglobulins (Dako) under the same conditions. Peroxidase was revealed by 3,3′-diaminobenzidine and H_2O_2 as reported (7, 8).

Fluorography. Fluorography was performed after separation of [^{3}H]-pargyline labelled mitochondrial proteins by electrophoresis (15, 17). The Coomassie blue stained gel was incubated 30 min in Enlightning (NEN) before drying and exposure to Kodak film (XOMAT) for 3 weeks.

Covalent labelling of MAO in mitochondria with [^{3}H]-pargyline. Samples (25 μl) of mitochondria (325 μg protein) were incubated with 10 μl of clorgyline or deprenyl (1.5 μM) for 60 min at 37°C in a total volume of 50 μl in order to inhibit respectively MAO A and MAO B. Then, aliquot samples (50 μl) were incubated for 60 min at 37°C with [^{3}H]-pargyline (1 μCi, 0.9 μM final) (17, 18). Five washes were done with PBS buffer. The pellets were obtained after centrifugation at 12000 g and solubilized with 1% CHAPS, 0.4% Triton X-100 for 30 min and centrifuged at 12000 g for 5 min (Eppendorf Sigma centrifuge). The supernatants were used for immunoprecipitation.

Immunoprecipitation of labelled MAO. Samples of the above supernatant in a total volume of 100 μl were incubated with 50 μl of several dilutions of human sera (control or containing anti-M6 antibodies) for 60 min at room temperature. Fifty μl of protein A-Ultrogel (30% suspension) were added and incubated 60 min. The samples were centrifuged at 12000 g. Radioactivity irreversibly bound to proteins in the supernatant fraction was quantified according to Fenselau (7, 19). Aliquots were deposited on glass fiber filter paper discs (Whatman GFB); the proteins were precipitated by dipping in 15% TCA, washed twice in methanol, once in ethyl acetate, dried and the radioactivity was counted in a Packard Tricarb 300 after adding 2 ml of toluene scintillator (Packard). The immunoprecipitated pellets were washed twice with PBS and solubilized with 1 M NaOH and the radioactivity counted after addition of Picofluor 40 (Packard).

Enzymatic assay and immunoprecipitation. Mitochondria (liver or placenta) were solubilized with 1% CHAPS, 0.4% Triton X-100 for 30 min and centrifuged at 12000 g for 5 min (Eppendorf Sigma centrifuge) and immunoprecipitation was performed as above. MAO activity remaining in solution was assayed in duplicates by adding 20 μl [^{14}C]-tyramine (45 μM final) to 50 μl of the supernatant obtained after incubation with human sera and protein A-Ultrogel in a total volume of 220 μl for 15 min at 37°C (20). The reaction was stopped by adding 200 μl 0.5 M HCl and extracted with 3 ml toluene/ethylacetate (v/v). The organic phase was counted after adding 10 ml of toluene scintillator. MAO-B and MAO-A activities were measured similarly with respectively [^{14}C]-phenylethylamine (45 μM) and [^{14}C]-5-hydroxy-tryptamine (100 μM).

RESULTS

Labelling of Human Liver and Placenta Mitochondrial MAO by [^{3}H]-pargyline

In order to recognize MAO-A and MAO-B among the human liver mitochondrial proteins, labelling with [^{3}H]-pargyline, which binds covalently and specifically to MAO, was performed according to a previously described technique (17, 18). A fluorography study of labelled human liver mitochondria, after protein separation by gel electrophoresis, confirmed the presence of two radioactive bands at 60 and 55 kD corresponding to MAO-A and MAO-B (data not shown). Moreover the lowest band was immunorevealed by monoclonal anti-MAO-B antibodies (21). Identical experiments performed on human placenta mitochondria, which contain only MAO-A, showed only one radioactive band corresponding to the heavier one.

Immunoprecipitation of Human Liver MAO by Anti-M6 Antibodies

After solubilization with detergents, labelled mitochondria were incubated with antibodies and the immune complexes were precipitated with protein A-Ultrogel. As shown in Fig 1, the two human sera containing anti-M6 antibodies (JAC, BIM) were both able to immunoprecipitate the major part of the labelled human liver MAO. Control sera precipitated very little labelled protein. The precipitation curves were almost linear and no saturation occurred showing that protein A-Ultrogel and sera were not limiting. In the absence of protein A-Ultrogel, anti-M6 antibodies were not precipitated (not shown). Attempts to detect them by Ouchterlony method were unsuccessful. From the data of Fig 1A and B, we could compute the percentage of labelled mitochondria in both the pellet and the supernatant. The total recovery of labelled mitochondria was about 80% of the initial radioactivity, 20% being probably lost during the washing steps (Fig 1C) and the precipitated radioactivity represented 57% (JAC) and 74% (BIM) of total counts recovered. A similar experiment performed using 240 μg human liver mitochondrial proteins and variable amounts of serum (0 to 1 μl) is shown in Fig 2. BIM and JAC sera precipitated pargyline labelled proteins while control sera did not. The immunoprecipitation curve reached saturation at 0.25 μl serum for BIM serum but was still linear for JAC serum at 1 μl. About 25% of the total

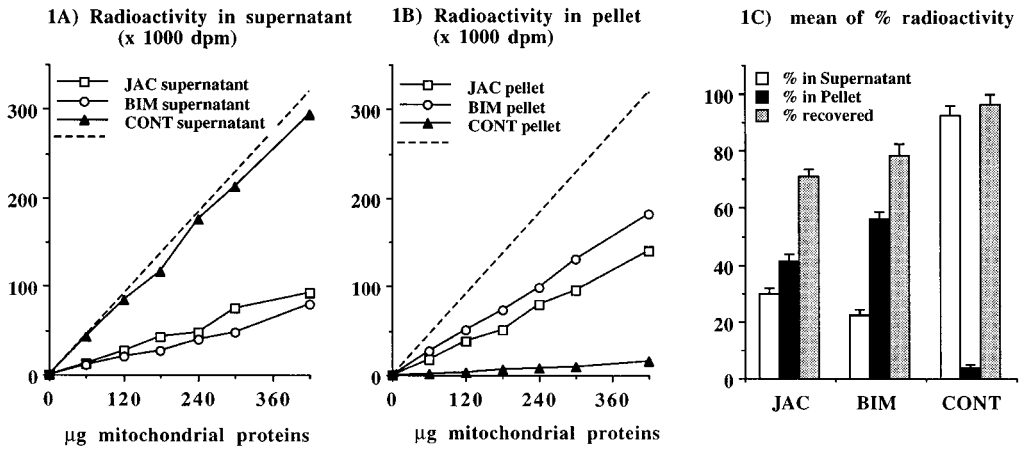

FIG. 1. Immunoprecipitation of human liver MAO by anti-M6 autoantibodies. Immunoprecipitation of [^{3}H]-pargyline-labelled mitochondria (760 dpm/μg or 15 pmol pargyline bound/mg prot) solubilized as described in Methods by 1 μl of a human control serum (CONT) or sera containing anti-M6 antibodies (BIM, JAC). The radioactivity (dpm) of the supernatant or the pellet was counted on an aliquot and is given for the total incubation. Fig 1A represents the radioactivity in the supernatant, Fig 1B in the pellet and Fig 1C represents the average slope ±SEM (calculated on 6 points) of the curves in 1A and 1B and their sum expressed as percentage of total radioactivity. For simplicity only one control serum is shown. The broken lines represent theoretical 100% added radioactivity.

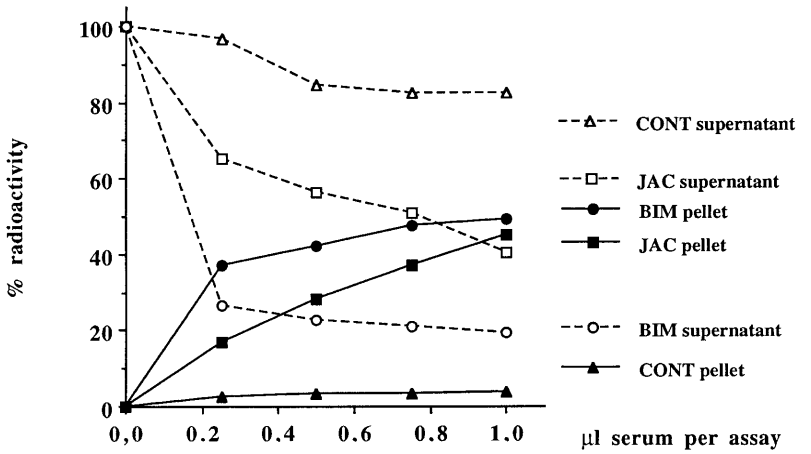

FIG. 2. Titration curves of [^{3}H]-pargyline-labelled human liver MAO by anti-M6 autoantibodies. Pargyline-labelled mitochondria (240 μg protein, 760 dpm/μg or 15 pmol pargyline bound/mg prot) were immunoprecipitated by 0.25, 0.5, 0.75 or 1 μl of human sera containing anti-M6 antibodies (BIM, JAC) or control human sera (CONT). The radioactivity was counted in the supernatant or in the pellet as described in Methods.

radioactivity could not be precipitated by both sera. All control sera tested precipitated less than 7% of the total radioactivity. Thus, the above experiments show that human sera containing anti-M6 antibodies, JAC and BIM, are able to immunoprecipitate human liver MAO. Serum BIM exhibits a higher affinity, in agreement with its higher titer by immunofluorescence.

Immunoprecipitation of Human Liver MAO-A or B by Anti-M6 Antibodies

As anti-M6 antibodies recognize human liver MAO, it was interesting to know whether MAO-A or B is specifically recognized. Liver MAO-A and B were specifically labelled by using unlabelled irreversible specific inhibitors of MAO-A or B before labelling with pargyline. Thus human liver mitochondria were preincubated for 60 min at 37° with 1.6 μM MAO-A or -B specific inhibitors (17, 18), respectively clorgyline and deprenyl, before [^{3}H]-pargyline labelling. Without inhibitor, 15 pmol pargyline are bound/mg protein; after preincubation with clorgyline only MAO-B reacted with pargyline leading to a level of covalent binding of 9 pmol/mg protein and after deprenyl pretreatment only MAO-A was labelled (0.6 pmol/mg protein). Then the immunoprecipitation experiment were performed as above. The results are represented in Fig 3. When MAO-A was inhibited by clorgyline (only MAO-B was labelled by [^{3}H]-pargyline), similar results as those of Fig 1C were obtained. These data (Fig 3 left) suggest that MAO B is immunoprecipitated by anti-M6 antibodies. When MAO-B was inhibited (only MAO-A was labelled by [^{3}H]-pargyline), the sera containing anti-M6 antibodies could not be differentiated from control sera (Fig 3 center). However, these last experiments were not conclusive because of the low levels of MAO-A labelling. In order to determine whether MAO-A is recognized by anti-M6 antibodies, human placenta mitochondria were used.

Immunoprecipitation of Human Placenta MAO (MAO A) by Anti-M6 Antibodies

Labelling of human placenta mitochondria, which contains almost only MAO-A by [^{3}H]-pargyline was more efficient than that of liver mitochondria (23 pmol pargyline bound/mg protein). The same experiment as the one described in Fig 1 was performed. The results (Fig 3 right) show that the three sera used could not be differentiated, indicating that anti-M6 antibodies do not recognize MAO-A and appear to be specifically directed against MAO-B.

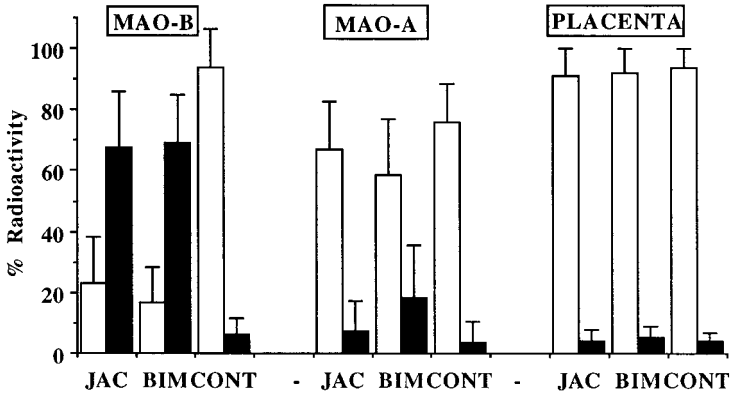

FIG. 3. Immunoprecipitation of pargyline-labelled human liver MAO-A or -B and placenta MAO-A by anti-M6 autoantibodies. Mean percentage of radioactivity present in the pellet or in the supernatant after immunoprecipitating mitochondria by human sera containing anti-M6 antibodies (BIM, JAC) or control human sera (CONT). Liver mitochondria were first preincubated with a specific inhibitor of MAO-A (clorgyline) in order to specifically label MAO-B (left) or an inhibitor of MAO-B (deprenyl) in order to specifically label MAO-A (center) then they were labelled with [^{3}H]-pargyline(respectively, 9 pmol and 0.6 pmol pargyline bound/mg protein or 460 and 30 dpm/μg protein). Similarly human placenta mitochondria were labelled as described for liver mitochondria (23 pmole pargyline bound/mg prot and about 1160 dpm/μg prot) (right). The immunoprecipitations were then done as described in Fig. 1A and 1B with 1 μl of serum and are represented as in Fig 1C.

Effect of Anti-M6 Antibodies on MAO Activity

A direct method was used in order to confirm the previous results. Using the same immunoprecipitation technique on solubilized *active* mitochondrial MAO, the MAO enzymatic activity was measured in the supernatant. Titration curves obtained with JAC, BIM and control sera are shown in Fig 4 using three typical substrates for monitoring MAO activity: tyramine, phenylethylamine and 5-hydroxy-tryptamine as A/B, B and A specific substrates. They demonstrate that only the MAO B activity is suppressed after immunoprecipitation by anti-M6

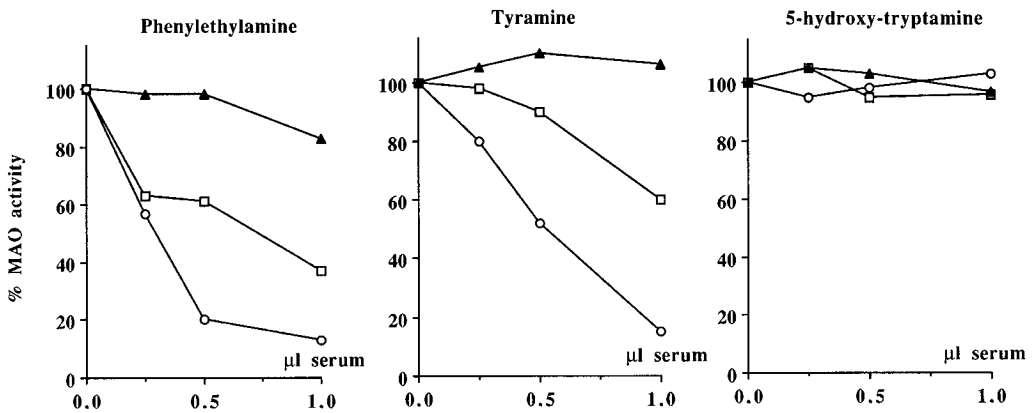

FIG. 4. Loss of MAO-dependent enzymatic activity after immunoprecipitation by anti-M6 autoantibodies. Native mitochondria were solubilized in 0.4% Triton X-100, 1% CHAPS as described in Methods and 240 μg protein was incubated with increasing amounts of serum BIM (&hb9;), JAC (□) and CONT (▲) for 60 min at 37°C; then protein A-Ultrogel was added and 60 min later the beads were separated by centrifugation. Determination of enzymatic MAO activity was performed using 50 μl of the supernatant and liver mitochondria with [^{14}C]-tyramine (45 μM) and [^{14}C]-phenylethylamine (45 μM) or placenta mitochondria with [^{14}C]-5-hydroxy-tryptamine (100 μM), respectively, for MAO-A/B, B and A activities as described (18). In the presence of the diluted detergent mixture, 100% activities were, respectively, 2.9, 4.2 and 2.1 nmole product per min per mg protein.

antibodies. Direct immunoinhibition of the MAO enzymatic activity by anti-M6 antibodies was attempted by preincubating whole mitochondrial fraction with sera before [^{3}H]-pargyline labelling or before tyramine oxidation assay. No inhibition occurred with the human sera containing anti-M6 antibodies (data not shown).

DISCUSSION

The aforementioned results clearly show that human sera containing anti-M6 antibodies are able to immunoprecipitate the MAO-B protein and to suppress MAO-B-dependent activities while they are totally unable to immunoprecipitate MAO-A. Thus these anti-M6 antibodies produced in iproniazid-induced hepatitis (an MAO irreversible inhibitor) specifically recognize MAO-B. These results were obtained with the only two sera containing antibodies of good titer that were available; however, it is noteworthy that none of the twenty control sera not containing anti-M6 antibodies (ten coming from control individuals and ten from other origins) immunoprecipitated MAO-B.

Direct recognition on immunoblots was not successful (data not shown) either because of the very low concentration of MAO-B in mitochondrial fractions or possibly because of the denaturation of the protein in SDS-PAGE. On the contrary, native MAO-B is immunoprecipitated with a good efficiency by BIM and JAC sera, BIM exhibiting a better affinity for MAO-B than JAC and having also a better titer by immunofluorescence. Although a real titer cannot be determined, it is clear from Fig 2 that 0.2 and 0.6 μl serum BIM and JAC respectively were needed to immunoprecipitate 50% ($\sim$7 pmol) MAO, corresponding to IC_{50}'s of 30 and 90 μl serum per nmol MAO. This is comparable with the range of IC_{50} found for inhibition of CYP 2C9-dependent activities by sera containing anti-LKM2 antibodies (7). However, contrary to anti-LKM2 antibodies appearing in tienilic acid-induced hepatitis which were able to inhibit more than 90% of CYP 2C9-dependent activities, anti-M6 antibodies of sera JAC and BIM were completely unable to directly inhibit MAO-B-dependent activities. It is noteworthy that none of the anti-MAO antibodies described in the literature were able to inhibit the MAO-dependent enzymatic activities (20, 21, 22, 23, 24).

The above data suggest that the proposed general mechanism (reactive metabolite/haptenisation of the activating enzyme) for the appearance of drug-induced autoantibodies (7, 26) could also apply to the case of iproniazid. In agreement with this hypothesis, iproniazid is known to act as a pro-drug and can be converted into isopropyl hydrazine which binds covalently to liver MAO (12). Moreover, it is known that iproniazid can be activated by microsomal cytochrome P-450 to isopropyl hydrazine then to an isopropyl radical which binds to microsomal proteins (25). Thus in a similar manner, isopropyl hydrazine could be activated by MAO to a radical which binds to the flavin or protein moiety of MAO leading to inactivation and haptenization. Then the modified protein could be presented to the immune system and lead to adverse reactions.

Similar mechanisms have been shown for other drugs with cytochromes P450 as targets (26): tienilic acid and CYP 2C9 (7, 8, 9), dihydralazine and CYP 1A2 (10) and more recently anticonvulsive drugs and a cytochrome P-450 of the 3A family (27, 28).

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