Biochemical Pharmacology, Vol. 41, No. 12, pp. 1949–1956, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

STRUCTURE–ACTIVITY RELATIONSHIPS IN THE INDUCTION OF HEPATIC MICROSOMAL CYTOCHROME P450 BY CLOTRIMAZOLE AND ITS STRUCTURALLY RELATED COMPOUNDS IN RATS

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(*Received* 14 *July* 1990; *accepted* 25 *January* 1991)

Abstract—We investigated the structure–activity relationship in the induction of hepatic microsomal cytochrome P450 by clotrimazole and its structurally related compounds. For this purpose, we synthesized various compounds structurally analogous to clotrimazole and injected them into rats at a fixed dose of 0.2 mmol/kg. We found that the chlorine atom in clotrimazole was not necessary for the induction of cytochrome P450. The imidazole moiety of clotrimazole, however, was a very important structural component for the induction of cytochrome P450; triazole, but not pyrrole, could be substituted for this moiety. 1-Tritylimidazole, 1-diphenylmethylimidazole and 1-benzylimidazole were also found to be inducers of cytochrome P450, but, to a somewhat lesser extent, with a decreasing number of substituted phenyl groups. Thus, 1-benzylimidazole was a minimum structurally active unit for inducing cytochrome P450. In addition, 4-diphenylmethylpyridine and 4-benzylpyridine also induced cytochrome P450 to extents similar to those induced by the corresponding imidazole derivatives, but 4-benzylpiperidine lacked this effect. When the methylene unit of clotrimazole-related compounds was introduced by a hydroxy or amino group instead of imidazole, there was a less extensive increase in cytochrome P450 content. This inducing effect was lost completely by the lack of an imidazole moiety and imidazole itself. 1-Phenylethylimidazole and 1-benzylimidazole induced cytochrome P450 to a similar extent. All of these findings suggest that 1-substituted heteroaromatic compounds having two or more nitrogen atoms are likely to be required for inducing cytochrome P450. Immunoblot analysis revealed that clotrimazole and other various inducers found in this study increased cytochrome P450b/e content. These results could provide information on the effects of drugs and chemicals on cytochrome P450 induction.

It has been shown that many antimycotic agents containing an imidazole group, such as clotrimazole, miconazole and ketoconazole, are able to inhibit and/or induce hepatic microsomal cytochrome P450 and its associated drug-metabolizing enzyme activities in animals [1–5]. The inhibitory mechanism on drug-metabolizing enzymes is believed to be the result of high-affinity binding of these agents to the heme moiety of the cytochrome P450 molecule and subsequent interference with oxygen binding [4, 5]. More recently, Hostetler *et al.* [6] and Rodrigues *et al.* [7] demonstrated that clotrimazole and miconazole induce cytochrome P450b/e and P450p, while ketoconazole induces a different type of the cytochrome. Because these antimycotic agents have biphasic effects on drug-metabolizing enzymes and have structurally common properties, they are likely to be a new class of inducer of cytochrome P450.

In relation to chemical structure, imidazole hydrochloride has been shown to induce slightly hepatic microsomal cytochrome P450 when administered to rats in a large dose [8]. It has also been shown that 1-phenylimidazole [9] and 1-benzylimidazole [10] induce cytochrome P450, but 1-phenylimidazole to a lesser extent than antimycotics having an imidazole moiety. However, the structure–activity relationship in the inducing effect of this series of antimycotics still remains to be elucidated.

Of the antimycotics containing an imidazole moiety, clotrimazole has been shown to be the most potent inducer of cytochrome P450 [5, 6]. Because of the relatively simple chemical structure of clotrimazole, imidazole and the trityl moiety, we synthesized various structurally related compounds and examined their effects on cytochrome P450 content in rats.

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MATERIALS AND METHODS

Chemicals. Clotrimazole and imidazole hydrochloride were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diphenyl-4-pyridylmethane, tritylamine, 1-benzylimidazole, 4-benzylpyridine, 1-phenylpyrrole and 4-benzylpiperidine were purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Triphenylmethanol, diphenylmethanol and diphenylmethane were purchased from the Wako Pure Chemical Co. (Tokyo, Japan). 1-Phenylimidazole was purchased from Transworld Chemicals (Rockville, MD, U.S.A.). All other chemicals used were of the highest grade commercially available.

Chemical synthesis. (2-Chlorophenyl)-diphenylmethanol was obtained by the acid solvolysis of

clotrimazole. 1-Tritylimidazole and 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-1,2,4-triazole were prepared by the method of Helmut and coworkers [11]. 1-Tritylpyrrole was prepared by the method of VanderWerf and coworkers [12]. 1-Diphenylmethylimidazole and 1-phenylethylimidazole were obtained by heating a mixture of imidazole and diphenylmethylchloride or phenylethylchloride according to the method of Wilkinson *et al.* [13]. Chemical structures of these synthesized analogues were identified by determining their NMR and IR spectra.

Animals and treatment. Male Wistar rats, weighing 190–220 g, were used in these experiments. They were fed a commercial solid diet *ad lib*. Clotrimazole and its analogous compounds were suspended or dissolved in corn oil by sonication and administered intraperitoneally to rats at a dose of 0.2 mmol/kg. Imidazole hydrochloride was dissolved in 0.9% NaCl solution and administered intraperitoneally. These injection solutions were prepared immediately before use. Control rats received the appropriate vehicle only. Rats were starved for 24 hr before being killed.

Tissue preparation. The rats were killed by decapitation and their livers were perfused *in situ* with 0.9% of NaCl solution. The liver was homogenized with 9 vol. of 1.15% KCl solution using a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 9000 *g* for 15 min and the resulting supernatant was centrifuged at 105,000 *g* for 1 hr. The pellet, microsomal fraction, was suspended in 0.1 M phosphate buffer (pH 7.4) and used for determining cytochrome P450 content.

Enzyme assays. Cytochrome P450 content was determined by the CO difference spectrum of dithionite-treated microsomes as described by Omura and Sato [14], but microsomes had been bubbled for 5 or 10 min with CO gas as described by Kahl *et al.* [1]. Aminopyrine demethylase activity was measured by determining formaldehyde formation according to the method of Nash [15]. To determine the inhibitory effects of clotrimazole and its analogues on aminopyrine demethylase activity *in vitro*, these compounds were dissolved in a minimum volume of dimethyl sulfoxide and added to the assay medium at various concentrations. The final concentration of dimethyl sulfoxide in the assay medium did not exceed 0.1%.

Electrophoresis and immunoblot analysis. Microsomes obtained from control and drug-treated rats were solubilized in sodium dodecyl sulfate (SDS) and resolved by polyacrylamide gel electrophoresis according to the method of Laemmli [16] and then transferred to a nitro-cellulose sheet [17]. Antigenic components reactive with APF3 monoclonal antibody raised against cytochrome P450b, which is also reactive to P450e, were visualized with 4-chloro-1-naphthol in 0.006% hydrogen peroxide solution.

RESULTS

To examine the structural requirement for the inductive effect of clotrimazole on hepatic microsomal cytochrome P450, we considered its structure from two moieties, the imidazole and the (2-chlorophenyl)diphenylmethyl moiety. From this viewpoint, we synthesized and employed various analogues of clotrimazole. First, we examined whether the chlorine atom in clotrimazole is necessary for the induction of cytochrome P450. Figure 1 shows that treatment of rats with clotrimazole resulted in a marked increase in cytochrome P450 content to about 2.8 times that of the controls when examined 24 and 48 hr later. Compound I (C-I), which has a dechlorinated structure of clotrimazole, induced cytochrome P450 to an extent similar to that induced by the parent compound, as shown in Fig. 1. Accordingly, we disregarded the chlorine atom when choosing and synthesizing compounds for further study. The effects of displacement of the imidazole moiety of clotrimazole by another heteroaromatic moiety on the induction of cytochrome P450 are also shown in Fig. 1. The triazole substituent (C-II) induced cytochrome P450 to an extent similar to clotrimazole, whereas the pyrrole substituent (C-III) did not induce the cytochrome. C-III failed to induce an increase in cytochrome P450 content even at a dose of 2 mmol/kg (data not shown).

Next, the possible contribution of the trityl moiety of clotrimazole to cytochrome P450 induction was investigated by changing the number of phenyl groups. Figure 2 shows that both diphenylmethylimidazole (C-IV) and benzylimidazole (C-V) were able to induce cytochrome P450 to levels similar to those induced by the parent compound when examined 24 and 48 hr after the treatment.

Subsequently, we examined the imidazole and other residual moiety of clorimazole independently. The structures of compounds used in these experiments and their effects on cytochrome P450 content are shown in Fig. 3. C-VI and imidazole (C-XII) correspond to cleavage products of clotrimazole. As shown in Fig. 3, although the extent of induction of cytochrome P450 was small, all compounds having a hydroxy group (C-VI, C-VII and C-VIII) or an amino group (C-IX) were inducers of the cytochrome. On the other hand, the compounds without a hydroxy or amino group, C-X and C-XI, or imidazole hydrochloride lacked the ability to induce cytochrome P450.

It is well known that the chemical properties of the imidazole ring are similar to those of the pyridine ring. These facts prompted us to examine the effects of related pyridine derivatives on the induction of cytochrome P450. As depicted in Fig. 4, both 4-diphenylmethyl pyridine (C-XIII) and 4-benzylpyridine (C-XIV) induced cytochrome P450 to an extent similar to that induced by the corresponding imidazole derivatives. However, 4-benzylpiperidine (C-XV) failed to induce cytochrome P450. C-XV did not induce an increase in cytochrome P450 content even at a dose of 2 mmol/kg (data not shown).

Murray *et al.* [9] have revealed that phenylimidazole induces cytochrome P450 slightly. Therefore, we compared the inducing effects of 1-benzylimidazole and 1-phenylimidazole on cytochrome P450. As shown in Table 1, the effect of induction of cytochrome P450 by 1-benzylimidazole (2.4 times that of the control at 24 hr after the

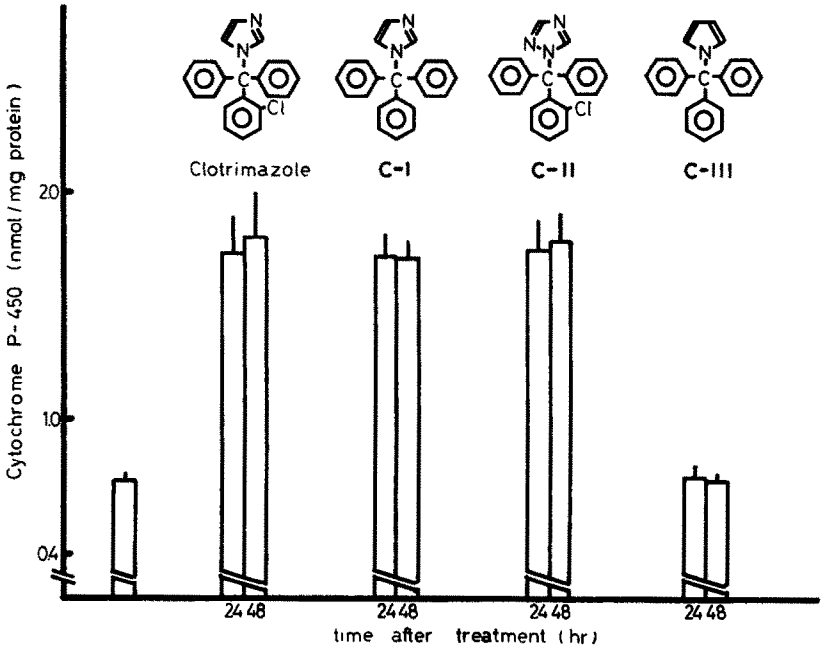

Fig. 1. Hepatic microsomal cytochrome P450 content in rats treated with clotrimazole, C-I, C-II and C-III. Rats were treated intraperitoneally with clotrimazole, C-I, C-II and C-III suspended in corn oil at a dose of 0.2 mmol/kg and were killed at 24 or 48 hr after the treatment. Control rats were treated with an appropriate volume of vehicle. All rats were fasted for 24 hr before being killed. The amount of CO-binding cytochrome P450 was determined by the method as described in Materials and Methods. Each value is the mean ± SD of four or eight rats.

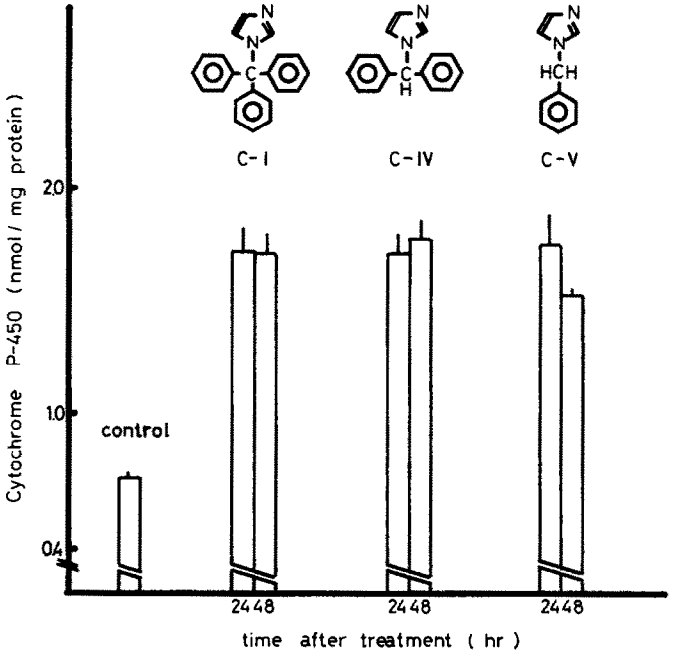

Fig. 2. Hepatic microsomal cytochrome P450 content in rats treated with C-I, C-IV and C-V. Rats were treated intraperitoneally with C-I, C-IV and C-V suspended in corn oil at a dose of 0.2 mmol/kg and were killed at 24 or 48 hr after the treatment. Other experimental conditions and methods were identical to those described in the legend of Fig. 1.

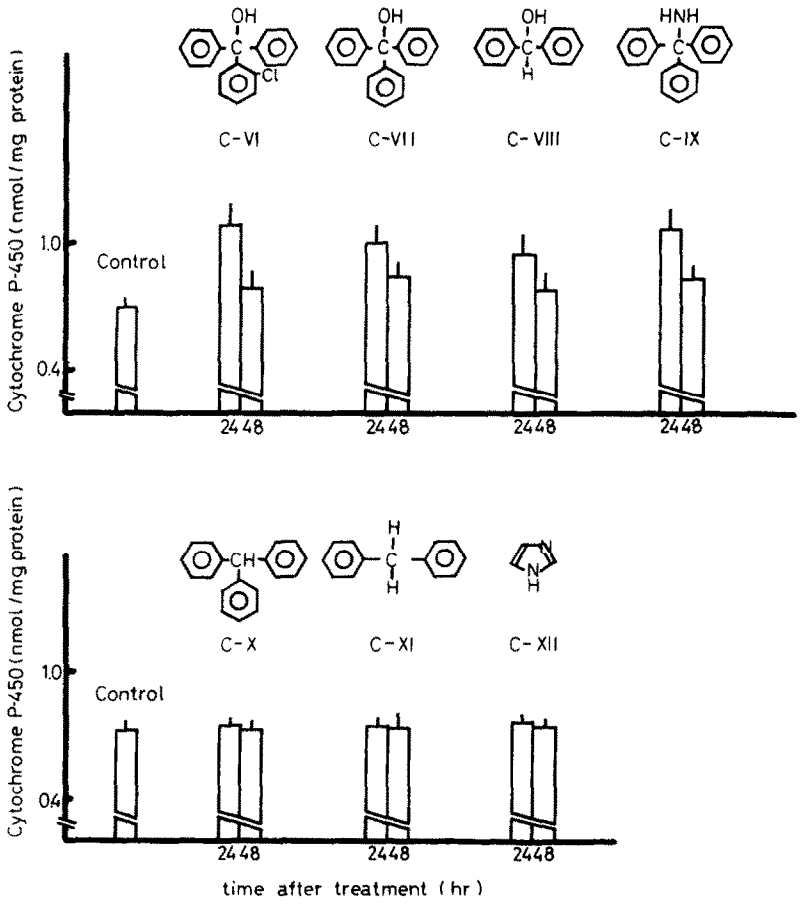

Fig. 3. Hepatic microsomal cytochrome P450 content in rats treated with C-VI, C-VII, C-VIII, C-IX, C-X, C-XI and C-XII. Rats were treated intraperitoneally with the compound suspended in corn oil at a dose of 0.2 mmol/kg and were killed at 24 or 48 hr after the treatment. Other experimental conditions and methods were identical to those described in the legend of Fig. 1.

treatment) was greater than that of 1-phenylimidazole (1.4 times the control). Further, 1-phenylethylimidazole and 1-benzylimidazole induced cytochrome P450 to almost similar extents. These results suggest that the partition between the imidazole and phenyl moieties of one or two methylene units could be ascribed to the intensity of cytochrome P450 induction. Thus, 1-benzylimidazole (C-V) would be an active minimum structural unit of clotrimazole and related compounds for the induction of cytochrome P450.

It is also well known that clotrimazole is not only an inducer of cytochrome P450 but also an inhibitor of the cytochrome-dependent drug-metabolizing enzyme activities. We therefore examined whether the compounds employed in this study could cause such biphasic effects on drug-metabolizing enzyme activities. The experiments were carried out by addition of the compounds at various concentrations to an assay medium for aminopyrine demethylase. As shown in Fig. 5, the inducers of cytochrome P450, such as C-I, C-II, C-V, C-VII and C-XIV, also inhibited aminopyrine demethylase activity in proportion to the concentration of the compound added *in vitro* to the reaction mixture. C-III, C-X and C-XV showed neither an inductive nor an inhibitory effect on aminopyrine demethylase activity.

Clotrimazole has been shown to induce phenobarbital-type cytochrome P450b/e [6, 7] and their mRNAs [6]. We therefore examined the effects of cytochrome P450 inducers found in this study on the induction of the cytochrome P450b/e species by using APF3 monoclonal antibody raised against P450b. Figure 6 shows that cytochrome P450b/e was increased in the microsomes from clotrimazole-treated rats as previously reported by Hostetler *et al.* [6] and Rodrigues *et al.* [7]. Likewise, immunoblottable cytochrome P450b/e was increased in the microsomes obtained from C-I-, C-II-, C-IV-, C-V-, C-XIII- and C-XIV-treated rats. The non-inducer C-III did not increase this cytochrome P450 species.

DISCUSSION

The present investigation has dealt with the structure–activity relationship of clotrimazole and

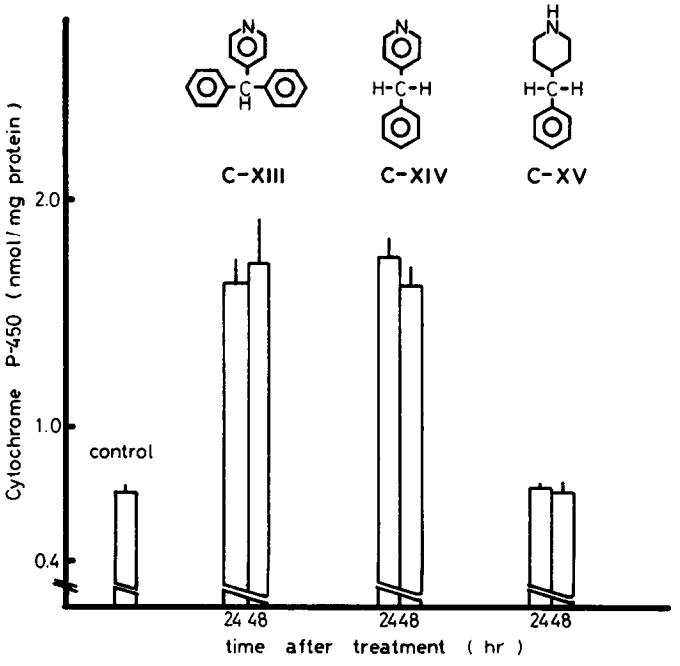

Fig. 4. Hepatic microsomal cytochrome P450 content in rats treated with C-XIII, C-XIV and C-XV. Rats were treated intraperitoneally with C-XIII, C-XIV and C-XV suspended in corn oil at a dose of 0.2 mmol/kg and were killed at 24 or 48 hr after the treatment. Other experimental conditions and methods were identical to those described in the legend of Fig. 1.

Table 1. Hepatic microsomal cytochrome P450 content in rats treated with 1-phenylimidazole, 1-benzylimidazole and 1-phenylethylimidazole

Treatment	Cytochrome P450 (nmol/mg protein)
None	0.703 ± 0.027
1-Phenylimidazole	1.018 ± 0.319
1-Benzylimidazole	1.740 ± 0.123
1-Phenylethylimidazole	1.648 ± 0.097

Rats were treated with 1-phenylimidazole, 1-benzylimidazole and 1-phenylethylimidazole, dissolved in corn oil, at a dose of 0.2 mmol/kg. Control rats were treated with vehicle alone. Rats were killed 24 hr after the treatment, and the cytochrome P450 content was determined by the method as described in Materials and Methods. Each value is the mean ± SD of four rats.

related compounds with respect to the induction of hepatic microsomal cytochrome P450. Our results have revealed that the imidazole moiety of clotrimazole could be substituted by a triazole moiety (C-II) without affecting the ability to induce cytochrome P450, but not by a pyrrole moiety (C-III) (Fig. 1). These results clearly suggest that 1-substituted heteroaromatic compounds having two or more nitrogen atoms, such as imidazole and triazole, would be an indispensable structural component for the induction of cytochrome P450. Interestingly, the 4-substituted pyridine derivatives (C-XIII and C-XIV) also exhibited cytochrome P450 induction to extents similar to those of the corresponding imidazole derivatives, but not the piperidine derivative (Fig. 4). The introduction of a hydroxy and amino group instead of imidazole, or the absence of imidazole from clotrimazole, reduced or completely negated the ability of the compound to induce cytochrome P450. All of these findings would be supportive evidence for the importance of 1-substituted heteroaromatic compounds having two or more nitrogen atoms and 4-substituted pyridine.

In turn, 1-diphenylmethylimidazole (C-IV) and 1-benzylimidazole (C-V) were inducers of cytochrome P450; however, the intensities of induction were dependent on the number of phenyl groups (Fig. 2). These results, together with the lack of effect of imidazole alone on cytochrome P450 induction, suggest that lipophilicity of these compounds, namely the number of phenyl groups, is likely to be important for the intensity and duration of induction of cytochrome P450.

Interestingly, 1-benzylimidazole (C-V) was more potent in inducing cytochrome P450 than 1-phenylimidazole (Table 1), suggesting that the number of methylene units between imidazole and the phenyl moiety has an important role in the intensity of cytochrome P450 induction. Therefore, it is reasonable to assume that the induction of cytochrome P450 by many antimycotics containing imidazole, such as miconazole, isoconazole, tioconazole, ketoconazole, sulconazole and itraconazole, is ascribed mainly to the phenylethylimidazole moiety in their structures. Likewise, our findings clearly indicate that 1-benzylimidazole would be an active

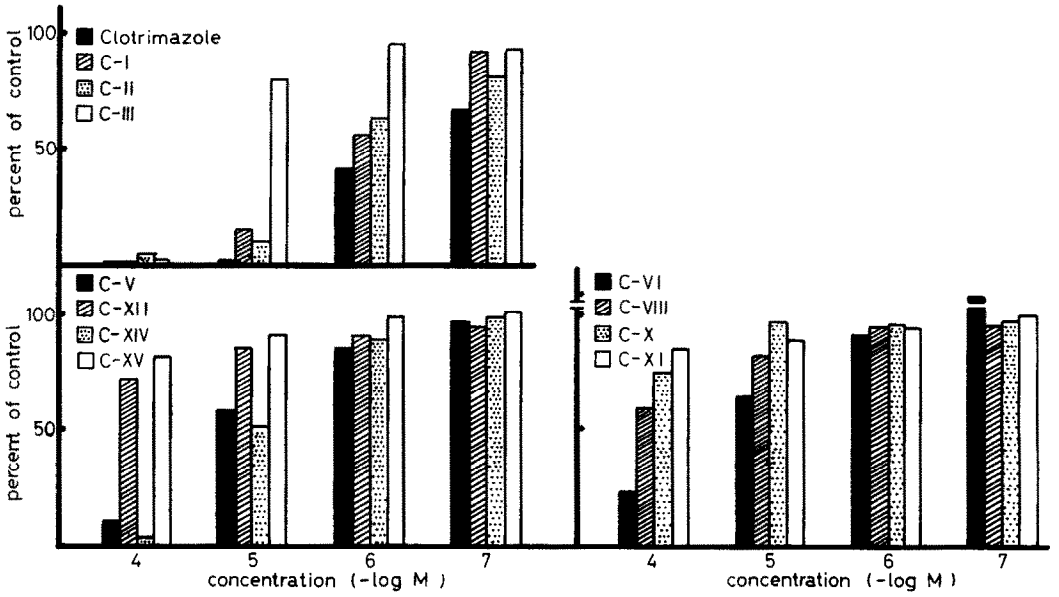

Fig. 5. Effects of clotrimazole and its structural analogues on aminopyrine demethylase activity *in vitro*. The compounds were added at various concentrations to the assay mixture for aminopyrine demethylase. The enzyme activity was measured by determining formaldehyde formation according to the method as described in Materials and Methods. The control value of three determinations was: 4.827 ± 0.082 nmol/mg protein/min.

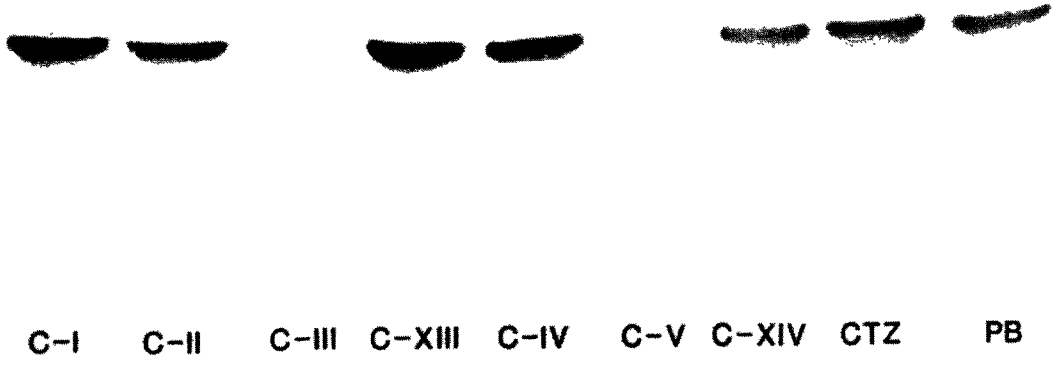

Fig. 6. Immunoblot analysis for cytochrome P450b/e in liver microsomes from rats treated with clotrimazole (CTZ) and its analogues. Microsomal samples were obtained from rats treated with clotrimazole and its analogues at a dose of 0.2 mmol/kg; the rats were killed 24 hr after the treatment. Microsomes were also obtained from rats treated with sodium phenobarbital (PB) at a dose of 80 mg/kg for two consecutive days and then killed 24 hr after the final treatment.

minimum structural unit in a series of clotrimazole-related compounds for inducing cytochrome P450.

On the contrary, many compounds having an imidazole moiety, including antimycotics, have been shown to inhibit various drug-metabolizing enzyme activities *in vitro* [1–3, 5]. The inhibition mechanism is believed to be the result of high-affinity binding of these compounds to the heme moiety of cytochrome P450 and subsequent interference with oxygen binding [2–4]. Similar inhibitory effects of various pyridine derivatives have also been reported by Jonen *et al.* [18]. In the present study, we also found that many inducers of cytochrome P450 (C-I, C-II, C-IV, C-V, C-XIII and C-XV) also inhibited aminopyrine demethylase activity in a concentration-dependent manner when added *in vitro* to the reaction mixture. These results suggest that such opposite effects of these compounds on cytochrome P450 and its related enzymatic activities are likely to be intimately related.

Clotrimazole has been shown to induce cytochrome P450b/e and P450p [6, 7]. Likewise, our compounds, which induced cytochrome P450, were able to induce cytochrome P450b/e. Because of a lack in our laboratory of antibody against cytochrome P450p, it is not clear at present whether our compounds can also induce this cytochrome species. In this respect, further studies will be required. With regard to the molecular species of cytochrome P450 induced by antimycotics having an imidazole moiety, ketoconazole has been shown to induce cytochrome P450j [6]. Similarly, imidazole hydrochloride has been shown to induce different cytochrome P450 species from clotrimazole [19]. A reason for such divergencies of the induced cytochrome P450 species by various compounds having an imidazole moiety is unclear at this time.

Although a mechanism for the induction of cytochrome P450 by our compounds is unclear at present, our findings with imidazole, triazole and pyridine derivatives suggest that cytochrome P450 induction by these compounds could be partially taking place in a similar manner. In this respect, the 1-substituted heteroaromatic moiety having two or more nitrogen atoms and the 4-substituted pyridine moiety may have an important role in the induction of cytochrome P450b/e. Montellano and Costa [20] suggested that inactivation of cytochrome P450b/e could trigger the inductive response of this type of cytochrome P450. However, a detailed mechanism for the induction of this type of cytochrome P450 species still remains to be elucidated. Interestingly, Poland *et al.* [21] have reported that 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is a powerful inducer of the phenobarbital-responsible species of cytochrome P450. This compound induced cytochrome P450 at the lowest dose of 3 mg/kg for long-term periods (about 3 months). The findings suggest the existence of a specific receptor responsible for the induction of cytochrome P450 by this type of inducer. In this respect, however, further detailed studies will be required. In addition to nicotinamide [22], isoniazide [23], 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene [21] and many antimycotics [1–5, 24] having imidazole or triazole rings, clotrimazole and its structurally-related compounds employed in this study could be classified as a new group of inducers of cytochrome P450 with respect to their structural similarities in having heteroaromatic moieties.

In conclusion, we have revealed the structure–activity relationship in the induction of cytochrome P450 by clotrimazole and its structurally related compounds, suggesting that the compounds having a 1-substituted heteroaromatic moiety containing two or more nitrogen atoms together with a somewhat bulky lipophilic moiety could be able to induce the cytochrome. The findings could provide information on the ability of a compound to induce cytochrome P450.

Acknowledgements—We thank Drs. T. Masuko and Hashimoto, Pharmaceutical Institute, Tohoku University, Sendai, Miyagi, Japan, for donating APF3 monoclonal antibody.

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