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THE EFFECTS OF PROPICONAZOLE ON HEPATIC XENOBIOTIC BIOTRANSFORMATION IN THE RAT

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Abstract—Propiconazole, a foliar fungicide used for agricultural purposes was studied for its effects on the hepatic xenobiotic biotransformation in the rat. Rats were given an intraperitoneal injection of 0.1, 1, 10 or 100 mg/kg in corn oil for seven consecutive days. Induction was seen for cytochrome P-450, ethoxyresorufin-*O*-deethylase, ethoxycoumarin-*O*-deethylase, aldrin epoxidase, aminopyrine *N*-demethylase and microsomal expoxide hydrolase activities. Aniline *p*-hydroxylase and cytosolic glutathione *S*-transferase activities were unchanged. All responses occurred at only 100 mg/kg, except for that of aminopyrine *N*-demethylase which also occurred at the 10 mg/kg dose. SDS polyacrylamide gel electrophoresis showed increased staining of a protein band of molecular weight 54,000 corresponding to cytochrome P-450b and/or P-450d. Collectively these results suggest that cytochromes P-450b and P-450d have been induced after exposure of rats to propiconazole.

Propiconazole, 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1H-1,2,4-triazole (Fig. 1) is a systemic foliar fungicide [1]. The mode of action of propiconazole as an antifungal agent is believed to be via the inhibition of cytochrome P-450-dependent synthesis of ergosterol in the fungal cell wall [2].

A literature survey has revealed no previous publications on the effects of propiconazole on xenobiotic biotransformation. However, studies on structurally related compounds, including ketoconazole, miconazole and econazole have shown this class of compounds, the N-substituted imidazoles, to be relatively nonspecific inhibitors of a range of P-450 monooxygenase activities in the rat [3, 4].

Previous studies have also shown that some antifungistatic imidazoles, including clotrimazole [5–7], miconazole and tioconazole [7] are capable of inducing, *in vivo*, hepatic cytochrome P-450 content and monooxygenase activities. Clotrimazole was found by Ritter and Franklin [5] to be a more potent inducer of cytochrome P-450 than phenobarbitone (PB).†

Isozymes of cytochrome P-450 have been shown to be partially distinguishable by substrate specificity [8, 9]. Alteration of their activities is of interest because of the possible changes in the ability of the body to metabolize other endogenous and foreign compounds.

It was the purpose of this study to investigate the effect of propiconazole on several hepatic cytochrome P-450 dependent and independent enzyme activities in the rat. Some identification of the form(s) of cytochrome P-450 induced by this fungicide was also sought.

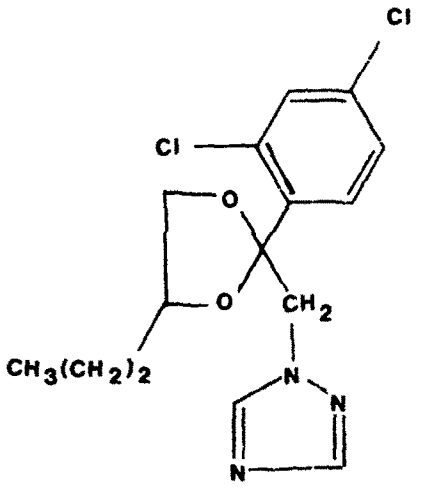

Fig. 1. Structure of propiconazole.

MATERIALS AND METHODS

Chemicals. Technical grade propiconazole was obtained from Ciba Geigy, Australia. Ethoxycoumarin, 7-hydroxycoumarin, resorufin and 1,2-dichloro-4-nitrobenzene (DCNB) were purchased from Aldrich Chemical Company (Milwaukee, WI); PB from BDH (Poole, U.K.); 3-methylcholanthrene (3-MC) from Sigma Chemical Company (St Louis, MO); isosafrole (ISF) was purchased from Eastman Kodak Company (Rochester, NY) and redistilled prior to use; aldrin and dieldrin from Shell (Australia) Ltd; ethoxyresorufin from Pierce Chemical Company (Rockford, IL); and ^{14}C-styrene oxide from

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† Abbreviations: AE, aldrin epoxidase; AD, aminopyrine *N*-demethylase; AH, aniline-*p*-hydroxylase; DCNB, 1,2-dichloro-4-nitrobenzene; ECOD, ethoxycoumarin-*O*-deethylase; EH, epoxide hydrolase; EROD, ethoxyresorufin-*O*-deethylase; GST, glutathione *S*-transferase; 3-MC, 3-methylcholanthrene; PB, phenobarbitone; ISF, isosafrole.

Amersham (Sydney). Chemicals for electrophoresis were obtained from Bio Rad, Sydney, NSW. All other chemicals used were of the highest quality commercially available.

Animals. Male Sprague–Dawley rats (240–400 g) were allowed food and water *ad libitum* until sacrifice. Rats received propiconazole (0.1, 1, 10 or 100 mg/kg) dissolved in corn oil via an intraperitoneal (i.p.) injection for seven consecutive days. For the electrophoresis experiments rats received 3-MC (20 mg/kg) or ISF (150 mg/kg) in corn oil i.p. once daily for three consecutive days. Controls received 1 ml/kg of the vehicle while all other animals received 1 ml/kg of the specified chemical. The animals were sacrificed 24 hr after the final dose.

Preparation of microsomes. Livers were perfused with ice-cold buffer (0.02 M Tris, 1.15% KCl, 3 mM EDTA, pH 7.6 at 0–4°) and then homogenized in 3 vol. of buffer w/v. The homogenate was centrifuged at 10,000 g for 15 min and the resultant supernatant centrifuged again at 105,000 *g* for 1 hr to obtain the microsomal pellet. Aliquots of the supernatant were taken and stored for glutathione *S*-transferase (GST) assays. The microsomal pellet was rinsed and vortexed gently to separate it from the glycogen pellet, then resuspended in storage buffer to a protein concentration of 20–50 mg/ml. The storage buffer was made up of the perfusion buffer at pH 7.4 with the addition of 20% glycerol. The cytosolic and microsomal samples were stored at −80° until required.

Protein and enzyme assays. Protein content was determined by the method of Lowry as modified by Chaykin [10], using bovine serum albumin as the standard. Cytochrome P-450 content was determined by the method of Omura and Sato [11] using a buffer containing 0.2 M potassium phosphate, 1 mM EDTA and 20% glycerol (pH 7.5). Ethoxyresorufin and ethoxycoumarin-*O*-deethylation were determined by spectrofluorimetric quantification of the product at 37° according to the method of Prough *et al.* [12]. Aldrin epoxidation was determined according to the procedure of Wolff *et al.* [13], with modifications. The 5 ml incubations contained 0.5 mg microsomal protein, cofactors (0.1 mM NADP, 20 mM isocitric acid, 5 mM $MgCl_2$, 2.5 mM nicotinamide, and 0.5 units isocitric dehydrogenase), 274 nmol aldrin in 0.1 ml methyl cellosolve and buffer (pH 7.6 at 25°). Incubation time was 10 min at 37°. Aminopyrine *N*-demethylation was determined following

Table 1. Dose-dependent effects of propiconazole on hepatic xenobiotic biotransformation in the male rat

	Dose				
Enzyme parameter	Control*	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg
Cytochrome P-450 (nmol/mg protein)	0.73 ± 0.06^{B}	0.75 ± 0.03^{B}	0.73 ± 0.05^{B}	0.81 ± 0.04^{B}	0.96 ± 0.05^{A}
Ethoxyresorufin-*O*-deethylase (pmol/min/mg protein)	81 ± 7^{B}	91 ± 7^{B}	83 ± 6^{B}	108 ± 10^{B}	261 ± 26^{A}
Ethoxycoumarin-*O*-deethylase (pmol/min/mg protein)	339 ± 46^{B}	282 ± 34^{B}	275 ± 25^{B}	304 ± 30^{B}	868 ± 41^{A}
Aldrin epoxidase (nmol/min/mg protein)	2.21 ± 0.11^{B}	2.16 ± 0.22^{B}	2.14 ± 0.15^{B}	2.81 ± 0.22^{B}	3.82 ± 0.32^{A}
Aniline hydroxylase (nmol/min/mg protein)	0.21 ± 0.01^{A}	0.21 ± 0.02^{A}	0.21 ± 0.02^{A}	0.23 ± 0.02^{A}	0.24 ± 0.01^{A}
Aminopyrine *N*-demethylase (nmol/min/mg protein)	7.07 ± 0.27^{B}	7.17 ± 0.36^{B}	7.80 ± 0.54^{B}	9.43 ± 0.33^{A}	9.90 ± 0.35^{A}
Epoxide hydrolase (nmol/min/mg protein)	16.6 ± 1.5^{B}	18.0 ± 1.3^{B}	20.1 ± 0.9^{B}	20.9 ± 1.2^{B}	29.1 ± 2.7^{A}
Glutathione *S*-transferase (nmol/min/mg protein)	208 ± 24^{A}	N.D.	169 ± 19^{A}	N.D.	225 ± 35^{A}

Data are presented as means ± SEM of 6 rats.
Rats were dosed daily (i.p.) for 1 week.
Means having the same superscript as one another are not significantly different.
* Control is corn oil at 1 ml/kg.
N.D. Not determined.

the method of Mazel [14]. Aniline-*p*-hydroxylation was measured by the method of Mazel [14] with minor modifications. The reaction mixture contained 5 mM aniline, 3–4 mg microsomal protein, cofactors and buffer (pH 7.6) at 25° in a total volume of 4 ml. GST activity was determined by following the method of Kulkarni *et al.* [15] using DCNB as the substrate. Epoxide hydrolase (EH) was determined by the method of Guengerich [16] using ^{14}C-styrene oxide as the substrate. Incubations contained 0.18 mg microsomal protein and 55,000 dpm of ^{14}C-styrene oxide.

Sodium dodecylsulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed on 1 mm thick 7.5% gels prepared according to the procedure of Laemmli [17]. Microsomes were incubated with 4% SDS and 5% 2-mercaptoethanol following Toftgard *et al.* [18]. Samples were boiled for 5–10 min. The gels were calibrated by electrophoresis of phosphorylase B (MW 92,500), bovine serum albumin (MW 66,200), ovalbumin (MW 45,000) and carbonic anhydrase (MW 31,000). Molecular weights of sample proteins were determined by comparison of mobilities with the standards using a Hoefer 3000 Scanning Densitometer, interfaced with an Apple IIe computer.

Statistics. All results were evaluated by an analysis of variance and compared using the least significant difference. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of treatment on enzyme activities

The effect of propiconazole on cytochrome P-450 mediated biotransformations, microsomal EH and GST are shown in Table 1. Cytochrome P-450 was induced 1.3-fold by propiconazole at the highest dose of 100 mg/kg only. Likewise, induction of ethoxyresorufin-*O*-deethylase (EROD), ethoxycoumarin-*O*-deethylase (ECOD) and aldrin epoxide (AE) activities were observed only when animals were treated with the highest dose. Both EROD and ECOD were induced 2.9-fold while AE was induced 1.6-fold. Aminopyrine *N*-demethylase (AD) activity was induced after treatment of the animals with 10 mg/kg and 100 mg/kg. Aniline hydroxylase (AH) activity was not altered by any treatment. EH activity was induced 1.5-fold at 100 mg/kg but there was no significant effect on GST activity.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The appearance of stained protein bands from microsomes of propiconazole-treated rats were compared to bands observed from the microsomes of 3-MC, PB and ISF treated rats. Densitometric scans of these gels suggest that treatment of male rats with propiconazole has resulted in an increase in proteins of approximate molecular weight 54 kD (Fig. 2).

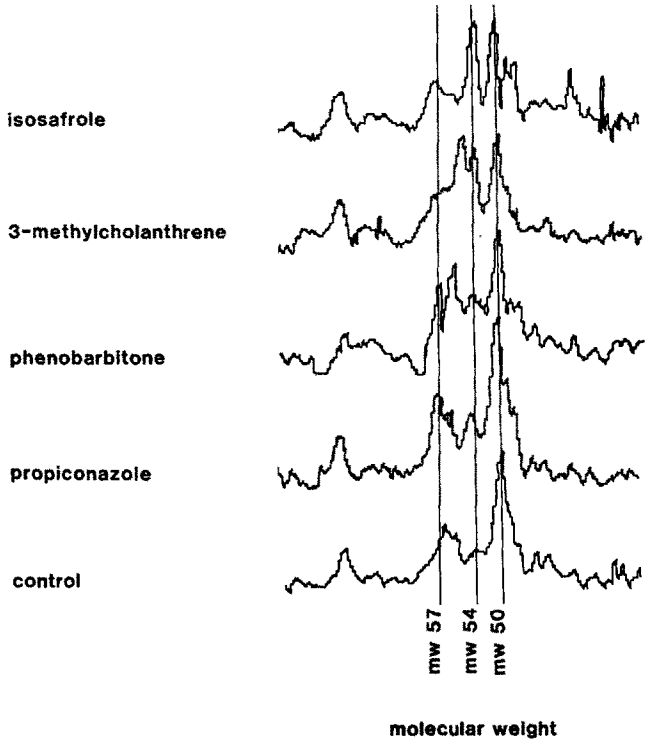

Fig. 2. Densitometer profile from SDS-PAGE of microsomal proteins from male rats. Rats were treated with corn oil control (1 ml/kg for 7 days); propiconazole (100 mg/kg for 7 days); phenobarbitone (80 mg/kg for 3 days); 3-methylcholanthrene (20 mg/kg for 3 days) and isosafrole (150 mg/kg for 3 days).

DISCUSSION

This study shows that propiconazole has an inductive effect on most of the enzyme activities studied. The monooxygenase activities EROD, ECOD, AE and AD as well as overall cytochrome P-450 content were all induced. No change in AH activity was evident. Propiconazole-induced EH activity but had no apparent effect on GST activity.

The results obtained are similar to those obtained for several other *N*-substituted imidazoles. Niemegeers *et al.* [4] for example found that administration of clotrimazole resulted in an initial inhibition of monooxygenase activity but that induction occurred after 5 days of dosing. Murray *et al.* [19] observed an increase in the cytochrome P-450 content and monooxygenase activities after treatment of rats with *N*-phenylimidazole. Miconazole and tioconazole, as mentioned in the Introduction, have also been observed to have an inductive effect on cytochrome P-450 and its associated monooxygenase activities. Ritter and Franklin [5, 7] observed residual *N*-substituted imidazole in the microsomes 24 hr after *in vivo* treatment. Due to the inhibitory interference by imidazoles on cytochrome P-450 [20], it is possible that in our results the activities of cytochrome P-450 mediated reactions have been underestimated. Likewise, imidazoles have been documented to have an enhancing effect on EH activity [6] and therefore our results may show an overestimation of EH activity.

Increases in EROD activity suggest that one or both of cytochrome P-450c or P-450d have been induced as EROD is metabolized almost exclusively by these isozymes [8, 21]. It is most likely that cytochrome P-450d has been induced as cytochrome P-450c is associated with particularly high activity towards both EROD and ECOD [8]. Increases in ECOD activity again suggest the induction of cytochrome P-450d, but could be due to an induction of cytochromes P-450b or $P\text{-}450_{PCN\text{-}E}$ which also have activity towards the ethoxycoumarin substrate [8].

In our experiments AE activity was increased, suggesting the induction of the PB inducible isozymes cytochromes $P\text{-}450_{PCN\text{-}E}$, $P\text{-}450_{PB\text{-}C}$, P-450b and/or P-450e, known to have moderate activity towards the epoxidation of aldrin [22]. The increase in AD activity is consistent with the induction of the cytochrome P-450d and the PB inducible cytochromes, particularly P-450b, both of which are known to have high activity towards the *N*-demethylation of aminopyrine [8]. Aniline-*p*-hydroxylation would also be expected to increase in this study as it is catalysed by both cytochrome P-450b and P-450d. Other researchers, however, have found this assay to be an insensitive indicator of cytochrome P-450d induction even when results from other assays clearly point towards the induction of this isozyme [23].

The absence of any change in GST activity contrasts to results obtained by other authors [7], who observed an increase in activity after treatment by clotrimazole, miconazole and tioconazole using 1-chloro-2,4-dinitrobenzene as a substrate.

Collectively the data from the enzyme assays suggest the possibility of propiconazole inducing one or several of the PB inducible cytochromes $P\text{-}450_{PCN\text{-}E}$, $P\text{-}450_{PB\text{-}C}$, P-450b, or P-450e or the ISF inducible P-450d [21, 24]. Data obtained from the SDS-PAGE experiments support results from the enzyme assays by indicating that propiconazole may have induced cytochromes P-450b or P-450d. Ryan *et al.* [24] have found the minimum molecular weight of cytochrome P-450b, the main isozyme induced by PB, identical to that of the ISF inducible cytochrome P-450d. Research in this laboratory has revealed that ISF and PB both cause an increase in staining of a protein band of molecular weight approximately 54,000. Therefore increased staining of this same protein band after electrophoresis of microsomes from propiconazole treated rats suggest the induction of cytochromes P-450b and/P-450d.

Overall, our results show similarities to those of previous studies on *N*-substituted imidazoles. Induction patterns resembling those of PB were observed after treatment with miconazole [7, 25] phenylimidazole [19] and clotrimazole [6]. In contrast ketoconazole was observed to have a unique pattern of induction [25]. In another study [5] the effects of clotrimazole were attributed to either mixed isozymes or a new isozyme. Perhaps propiconazole is most like clotrimazole in that its induction pattern cannot be attributed to PB inducible isozymes alone. Cytochromes P-450b and P-450d appear to be the main isozymes induced by this fungicide. Since induction occurs at mg/kg levels our data suggest that under the conditions of exposure to propiconazole likely to be encountered in human situations the alterations in cytochrome P-450 monooxygenase activities would be unlikely to have much effect on hepatic xenobiotic biotransformation.

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