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INDUCTION POTENTIAL OF ANTIFUNGALS CONTAINING AN IMIDAZOLE OR TRIAZOLE MOIETY

MICONAZOLE AND KETOCONAZOLE, BUT NOT ITRACONAZOLE ARE ABLE TO INDUCE HEPATIC DRUG METABOLIZING ENZYMES OF MALE RATS AT HIGH DOSES

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Abstract—Male Wistar rats were dosed with miconazole, ketoconazole and itraconazole by gastric intubation once daily for up to 7 days. A dose- and time-dependent induction of the hepatic drug metabolizing enzyme system was observed for miconazole and ketoconazole, while itraconazole proved to be devoid of inductive properties even at the highest dose studied (160 mg/kg). No effect on drug metabolizing enzymes could be demonstrated for either drug at a dose level of 10 mg/kg, which is just above the antifungally active dose. At a dose of 40 mg/kg, miconazole, but not ketoconazole, significantly increased cytochrome P-450 content. At the highest dose of 160 mg/kg, both miconazole and ketoconazole increased the relative liver weight, the cytochrome P-450- and b_5-content and NADPH-cyt *c*-reductase. Furthermore, miconazole, but not ketoconazole, increased specific microsomal aminopyrine and *N,N*-dimethylaniline *N*-demethylase activity, *p*-nitroanisole *O*-demethylase activity and UDP-glucuronyltransferase activity towards 4-nitrophenol while the specific aniline hydroxylase activity was unaffected. Ketoconazole at 160 mg/kg only induced *O*-demethylase activity and UDP-glucuronyltransferase activity, while it lowered the specific activities towards the other substrates. Miconazole was a relatively more potent inducer when compared to ketoconazole. Both drugs displayed biphasic effects on the mixed-function oxidase activities, which were lowered after acute administration (160 mg/kg, 1 hr before death) and were induced when determined after 23 hr had elapsed or after multiple dosage. Both drugs bound strongly to their respective induced cytochromes, giving rise to type II difference spectra, and inhibited the *O*-demethylase activity of the induced microsomes with an I_{50} of 5.2 μM for miconazole and 15.1 μM for ketoconazole. On the basis of a comparison of the enzymatic activities induced by both antimycotics with those induced by PB or 3-MC, it was concluded that miconazole behaved as a PB-type inducer, whereas ketoconazole did not belong to either category of inducers. A comparison of electrophoretograms of microsomes from different origins on SDS–PAGE revealed that miconazole increased the concentration of several proteins, whereas ketoconazole selectively induced a protein with M_r of 47,800. The protein pattern in the 50 kDa region of miconazole-induced microsomes resembled that of PB-microsomes qualitatively.

The imidazole derivatives miconazole or (±)-1-[2-(2,4-dichlorophenyl-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1*H*-imidazole and ketoconazole or (±)-*cis*-1-acetyl-4-[4-[1[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]-phenyl]piperazine (Fig. 1) are potent broad-spectrum antifungal agents. The bioavailability of miconazole after oral administration is poor. The drug is used topically or, for the therapy of systemic fungal infections, intravenously (600 mg/day) [1]. Ketoconazole, on the other hand, is rapidly absorbed and widely distributed following oral administration (normally 200 mg/day) and therefore became the first orally active systemic antifungal agent [2]. Recently, high-dose ketoconazole therapy (3 × 400 mg/day) successfully entered clinical trails for the management of advanced prostatic cancer [3]. Itraconazole or (±)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3*H*-1,2,4-triazol-3-one (Fig. 1) is a new oral antifungal agent (100–200 mg/day) undergoing extensive clinical trials at the moment. The drug has a broad spectrum of activity and is at least four times more active compared to ketoconazole. The inhibitory and inductive effects of imidazoles on microsomal mixed-function oxidase activities have been known for a long time [4–6]. An extensive *in vivo* study on the inhibitory and inductive effect of miconazole and ketoconazole in rats [7] revealed that the methohexital-induced hypnosis time was prolonged significantly at much lower doses for miconazole (3.55 mg/kg) than for ketoconazole (97.0 mg/kg). Similarly, the acenocoumarol-induced prolongation of the prothrombin time was only lengthened at a 12 mg/kg dose for miconazole and 50 mg/kg for ketoconazole. Up to now, no clear evidence has been presented, however, for an inhibitory effect of ketoconazole at the antifungally active dose (10 mg/kg) in the rat [8].

In the mouse, miconazole (50 mg/kg) had a

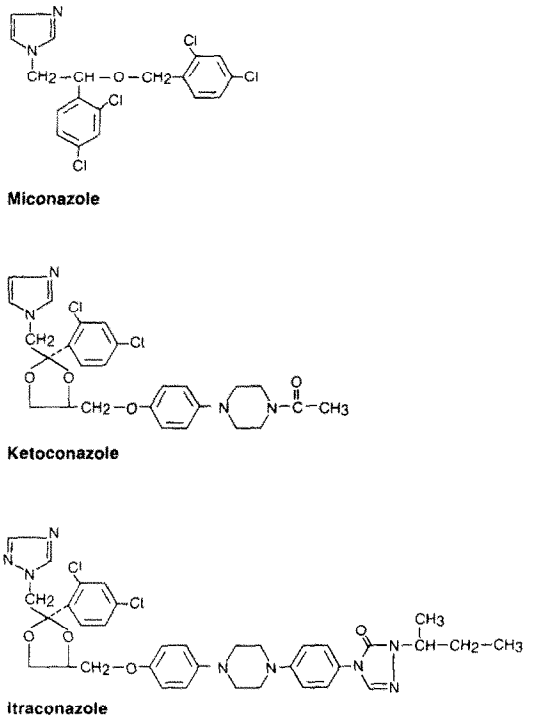

Fig. 1. Chemical structures of miconazole, ketoconazole and itraconazole.

biphasic effect on pentobarbital-induced sleeping time which was prolonged after a one-day treatment while it was decreased after three- or five-day treatments, indicating an inductive effect [9]. Again, differences were found between miconazole and ketoconazole in their ability to induce hepatic drug metabolism. Thus, repeated intravenous administration of miconazole to critical care patients increased the excretion of D-glucaric acid, while therapeutic oral doses of ketoconazole did not [10]. Similarly, even after administration of ketoconazole to patients for periods of longer than one year, no evidence was found that liver enzyme induction occurred [11]. Subchronic treatment of rats with extremely high doses of miconazole or ketoconazole showed, however, that hepatic enzyme activities could be induced by both drugs, and this was demonstrated using *in vivo* [10] or *in vitro* techniques [12].

Recently, it was shown that subchronic ketoconazole treatment (7 days) of rats (50 mg/kg, intraperitoneally) increased caffeine *N*-demethylase, but not aminopyrine *N*-demethylase, suggesting a differential effect on various cytochrome P-450 forms [13]. Up to now, however, no clear picture of the induction process initiated by either antimycotic drug has been presented.

Therefore, the present study was undertaken to define the inductive potential of miconazole and ketoconazole on *in vitro* microsomal hepatic enzymes of the rat, relative to the effects of the new antifungal agent itraconazole and of two classical inducers, PB and 3-MC. Three different doses were selected: 10 mg/kg, which is approximately the antifungally active dose in animals, and 40 and 160 mg/kg, which were included on the ground that they were comparable to doses used in the subacute toxicity studies [14, 15]. At 40 mg/kg, both compounds were minimally toxic, whereas the dose of 160 mg/kg was moderately toxic for miconazole and clearly toxic with fatality for ketoconazole.* In addition, a time relationship at the highest dose level was established, and a primary characterization of the cytochrome P-450 species involved in the induction process was attempted.

MATERIALS AND METHODS

Chemicals. Miconazole (batch C2001), ketoconazole (batch E6201) and itraconazole (batches A04/01 and A1101) were obtained from Janssen Pharmaceutica, Beerse, Belgium. Aniline, 3-MC, *p*-aminophenol, aminopyrine, *N,N*-dimethylamine, *p*-nitroanisole and 4-nitrophenol were obtained from Janssen Chimica (Beerse, Belgium). PB and bovine serum albumin were obtained from Fluka (Buchs, Switzerland) and cyt *c*† (type III, from horse heart) from Sigma (St. Louis, MO). G-6-P and G-6-PDH (yeast, grade II), NADP (disodium salt, 98%, NADPH (tetrasodium salt, 98%), UDPGlUA and UDPGlcNAc were supplied by Boehringer (Mannheim, F.R.G.) All other chemicals were of at least reagent grade quality and were obtained from various commercial sources.

Experimental animals. Male Wistar rats, weighing 220 ± 10 g at the start of the experiment, were obtained from the Janssen Animal Breeding Center (Beerse, Belgium).

Treatment protocol. Rats were housed in groups of five or seven animals in plastic cages on wood bedding in a facility with a 12 hr light/dark cycle. They were fed a commercially available diet (Ref. 010L, Keustermans, Beerse, Belgium) and tap water *ad libitum*. These conditions were maintained for at least one week prior to treatment. Miconazole, itraconazole and 3-MC were dissolved in 37.2 mM HCl in PEG-400. To provide the higher dose levels (40 and 160 mg/kg), miconazole had to be dissolved in a solution of Cremophor EL (11.5%) and lactic acid (29 mM) in water (final pH adjusted to 4.3 with 0.1 N NaOH) and itraconazole in PEG 400 containing 1.38 equivalents hydrochloric acid (final pH adjusted to 2.5–2.7 with 5 N NaOH). Ketoconazole was dissolved in 60 mM HCl (final pH adjusted to 3.0 with 0.1 N NaOH). PB was dissolved in water and immediately before use, the same quantity of HCl with respect to the ketoconazole solution was added, followed by adjustment of the pH to 3.0. Stock solutions of miconazole and ketoconazole were prepared a maximum of 48 hr prior

* H. Van Cauteren, personal communication.

† Abbreviations used: cyt *c*, cytochrome *c*; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; 3-MC, 3-methylcholanthrene; PB, phenobarbital; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; UDPGlUA, UDP-glucuronic acid; UDPGlcNAc, UDP-*N*-acetylglucosamine.

to use. All solutions were stored at room temperature during the period of dose administration. Drugs were given once daily by gastric intubation between 8.00 and 9.00 a.m. The following doses were administered: miconazole, 10 mg/kg (in 2 ml of acidic PEG 400/kg), 40 and 160 mg/kg (in 10 ml of acidic Cremophor EL solution/kg); ketoconazole, 10, 40 and 160 mg/kg (in 10 ml HCl solution/kg); itraconazole, 10 mg/kg (in 2 ml of acidic PEG 400/kg) and 40 and 160 mg/kg (in 2.8 ml of acidic PEG 400/kg); 3-MC, 20 mg/kg (in 2 ml acidic PEG 400/kg); PB: 60 mg/kg (in 10 ml HCl solution/kg). Control animals received the appropriate vehicle only and were treated at the same time as the corresponding experimental animals. Rats were starved immediately after the last dose and were sacrificed 23–24 hr later. Rats receiving a single dose of miconazole or ketoconazole were starved immediately after dose administration and sacrificed either exactly 1 hr or 23 hr after dosing.

Preparation of microsomes. Four or six animals of each group were sacrificed by decapitation, followed by exsanguination. The blotted livers were weighed and homogenized in three volumes of ice-cold KCl (1.15%)–phosphate buffer (0.01 M) pH 7.4 by a Potter-Elvehjem homogenizer. To prepare microsomes, crude homogenates were centrifuged at 12,000 *g* for 20 min at 4° (Kontron TFT 50.38 rotor in a Kontron TGA-50 ultracentrifuge) and from the resulting supernatant, microsomes were pelleted by centrifugation at 110,000 *g* for 1 hr at 4°. The microsomes were washed with and finally resuspended in homogenization buffer, frozen in glass tubes which were submersed in a hexane/solid CO_2 mixture and stored at −70° until analysis.

Determination of protein and cytochrome content. Protein concentrations were determined by the Lowry method [16], using Miller's modification [17], with bovine serum albumin as a standard. Cytochrome P-450 and cytochrome b_5 content were determined according to the methods developed by Omura and Sato [18, 19]. Spectra were recorded with a Pye Unicam SP8800/03 double beam spectrophotometer, using matched quartz cuvettes, which were placed in the turbid sample holder located in the front cell compartment.

Enzyme assays. Enzyme activities were determined under linear kinetic conditions with respect to protein concentration and incubation time. Incubations were carried out in a Heto shaking water bath at 37°, under air at 100 oscillations/min. Reactions were started after a 6-min pre-incubation period as indicated. Aniline hydroxylase activity was measured by the formation of *p*-aminophenol [20]. The incubation mixture contained in 4 ml 50 mM Tris HCl buffer, pH 7.4: 1.5 μmoles NADP, 30 μmoles G-6-P, 15 μmoles $MgCl_2$. 1 unit G-6-PDH, 5 μmoles aniline and 2 mg microsomal protein. The reaction was started by adding the substrate and terminated at the end of 10 min by the addition of TCA. Aminopyrine and *N,N*-dimethylaniline *N*-demethylase activity was measured by the formation of formaldehyde according to the method developed by Nash [21]. The incubation mixture contained in 6 ml 50 mM Tris HCl buffer, pH 7.4: 2.25 μmoles NADP, 45 μmoles G-6-P, 22.5 μmoles $MgCl_2$, 1.5 units G-6-PDH, 21 μmoles semicarbazide, 4 mg microsomal protein and 5 μmoles aminopyrine or 1.7 μmoles *N,N*-dimethylaniline. The reaction was started by addition of the substrate and terminated after 10 min by addition of 2 ml $ZnSO_4$ (15%, w/v) and 2 ml saturated $Ba(OH)_2$. The *O*-demethylation of *p*-nitroanisole was determined by a modification of the method of Netter and Seidel [22]. The incubation mixture contained in 2.5 ml 50 mM Tris HCl buffer pH 7.9: 15 μmoles $MgCl_2$, 15 μmoles G-6-P, 1 unit G-6-PDH, 1.2 mg microsomal protein and 3 μmoles *p*-nitroanisole. The reaction was started by the addition of 0.3 μmoles NADP and terminated after 10 min by the addition of 0.6 ml 20% TCA. Precipitated proteins were removed by centrifugation and after addition of 0.5 ml 20% Na_2CO_3, the absorbance was measured at 420 nm and the amount of 4-nitrophenol formed was determined using a millimolar extinction coefficient of 7.3 [23]. NADPH-cyt *c* reductase was determined essentially as described by Mazel [23]. The incubation mixture contained 23 μmoles nicotinamide, 1.5 μmole NaCN, 52 nmoles NADPH, 5 μg microsomal protein and 0.2 mg cyt *c* in a total volume of 1.05 ml 0.05 M phosphate buffer (1 mM EDTA), pH 7.6. The increase in absorption at 550 nm was measured every 30 sec and the enzymatic reduction rate of cyt *c* was calculated from the initial linear phase using a millimolar extinction coefficient of 21.1. UDP-glucuronyltransferase activity towards 4-nitrophenol was measured by a modification of existing methods [24]. The reaction mixture contained in 0.3 ml 0.05 M phosphate buffer pH 7.1: 0.1 μmole 4-nitrophenol, 57 nmoles $MgCl_2$, 0.6 μmoles UDPGlUA and 0.58 μmoles UDPGlcNAc. To the co-factor mixture, 1.2 mg microsomal protein was added in 0.3 ml KCl (1.15%)–phosphate (10 mM) buffer, pH 7.4. The reaction was started by adding the substrate and terminated by adding 0.8 ml 0.2 M TCA. The precipitated proteins were removed by centrifugation and 0.5 ml of the supernatant was mixed with 1 ml buffer, pH 9.0 (0.27 M glycine, 0.17 M $NaHCO_3$ and 0.12 M NaCl). The extinction was read at 400 mm and the amount of 4-nitrophenol which disappeared from the incubate was calculated using a millimolar extinction coefficient of 18.

Optical difference spectrophotometry. Difference spectra were recorded on a Pye Unicam SP8800/03 double beam spectrophotometer, using 10 mm matched quartz cuvettes, which were placed in the turbid sample holder. The cells contained 3 ml of a microsomal suspension (about 2 mg protein/ml) in KCl (0.25%)–phosphate buffer (0.1 M) pH 7.4. Miconazole or ketoconazole, dissolved in 2.7 mM HCl, were added in microlitre amounts to the sample cuvette and an equal volume of 2.7 mM HCl to the reference cell. Spectra were recorded between 370 and 500 mm and the absorbance peak minus trough values were used to construct an Eadie–Hofstee plot. In the case of non-linear plots, K_s, the spectral dissociation constant, was calculated with an extended least squares non-linear regression program (ELSPLUS, version 2.1) [25] run on a Hewlett–Packard computer type 9816.

Electrophoresis. SDS–PAGE was performed according to the method of Laemmli [26]. Microsomal protein (10 μg) from vehicle-, miconazole-, ketoconazole-, 3-MC- and PB-treated rats were separated

Table 1. Liver weight, concentration of microsomal protein, cytochrome P-450, cytochrome b_5 and NADPH cyt *c*-reductase activity in rat liver microsomes after oral administration of miconazole (160 mg/kg), ketoconazole (160 mg/kg), itraconazole (160 mg/kg), PB (60 mg/kg), 3-MC (20 mg/kg) and vehicle solutions for seven days. Results represent the mean ± S.D. for four (antimycotics) or six (PB, 3-MC) male rats

Treatment protocol	Liver weight (g/100 g body weight)	Microsomal protein (mg/g liver)	Parameter					
			γmax[1] (nm)	Cytochrome P-450		Cytochrome b_5		NADPH cyt *c*-reductase
				nmoles mg protein	nmoles g liver	nmoles mg protein	nmoles g liver	nmoles min mg protein	μmoles min g liver
Control (11.5% cremophor EL, 29 mM lactic acid in water pH 4.3)	3.10 ± 0.35	27.9 ± 2.3	449.6 ± 0.3	0.984 ± 0.093	27.6 ± 4.8	0.448 ± 0.107	12.51 ± 3.28	92 ± 26	2.58 ± 0.87
Miconazole (160 mg/kg)	3.81 ± 0.20*	34.4 ± 3.1*	449.7 ± 0.2	1.988 ± 0.218*	68.6 ± 12.2*	0.716 ± 0.179*	24.36 ± 5.23*	159 ± 8*	5.47 ± 0.65*
Control (HCl, pH 3.0)	3.17 ± 0.23	27.0 ± 1.5	449.6 ± 0.3	1.115 ± 0.013	30.1 ± 2.0	0.548 ± 0.030	14.76 ± 0.31	92 ± 9	2.48 ± 0.31
Ketoconazole (160 mg/kg)	4.71 ± 0.41*	35.4 ± 2.4*	449.1 ± 0.1*	2.066 ± 0.311*	73.6 ± 15.9*	0.702 ± 0.060*	24.88 ± 3.43*	132 ± 5*	4.66 ± 0.50*
Control (HCl in PEG-400, pH 2.5–2.7)	3.33 ± 0.26	23.1 ± 0.4	449.3 ± 0.6	1.013 ± 0.036	23.4 ± 1.3	0.559 ± 0.014	12.92 ± 0.28	97 ± 12	2.24 ± 0.24
Itraconazole (160 mg/kg)	3.44 ± 0.25	22.5 ± 1.4	449.2 ± 0.2	1.049 ± 0.119	23.6 ± 2.2	0.529 ± 0.015*	11.95 ± 1.01	114 ± 13	2.57 ± 0.45
Control (HCl, pH 3.0)	3.19 ± 0.21	26.2 ± 3.4	449.3 ± 0.4	1.020 ± 0.111	26.9 ± 5.8	0.564 ± 0.066	14.87 ± 3.28	N.D.[3]	N.D.
PB (60 mg/kg)	4.03 ± 0.57	36.7 ± 5.4*	449.4 ± 0.4	1.950 ± 0.408	73.0 ± 22.8	0.594 ± 0.142	21.32 ± 4.99*	N.D.	N.D.
Control (37.2 mM HCl in PEG-400)	3.02 ± 0.23	26.1 ± 2.5	449.7 ± 0.2	0.888 ± 0.117	23.2 ± 4.6	0.520 ± 0.032	13.35 ± 1.59	N.D.	N.D.
3-MC (20 mg/kg)	3.27 ± 0.30	30.4 ± 2.3*	448.5 ± 0.4	1.420 ± 0.096*	43.2 ± 3.9*	0.666 ± 0.057*	20.23 ± 1.87*	N.D.	N.D.

* Statistically significantly different from control ($P < 0.05$).
[1] Absorption maximum in CO-difference spectrum.
[2] Mean of five rats.
[3] N.D.: not determined.

using a gel containing 8% polyacrylamide. The gels were stained with Coomassie Brilliant Blue and the molecular weights of the proteins in the microsomal samples were estimated by comparison of their migration distance with those of the molecular weight markers: phosphorylase B (94,000), bovine serum albumin (68,000) and ovalbumin (43,000). In addition, the calibration mixture contained an impurity (M_r about 43,000) and other M_r-markers, which however did not leave the application site.

Statistical analysis. The group means and standard deviations were calculated and statistical significance between treated and control animals were evaluated using an unpaired Student's *t*-test. Differences were claimed to be significant at the $P \leq 0.05$ level, using the two-tailed *t*-distribution.

RESULTS

Effect of subchronic treatment on hepatic parameters associated with drug metabolizing enzymes

Administration of high doses of itraconazole (160 mg/kg) to male Wistar rats for seven days, did not affect the investigated components of the hepatic cytochrome P-450 system to a considerable extent (Table 1).

Treatment with high doses of miconazole or ketoconazole (160 mg/kg), however, significantly increased all the parameters which were studied (Table 1). The relative liver weight was increased by miconazole (+25%) and ketoconazole (+53%) and therefore paralleled the effect of PB but not that of 3-MC (Table 1). For ketoconazole the increase was somewhat accentuated because the mean body weights decreased in ketoconazole treated rats (from 281 ± 22 g in the corresponding controls to 257 ± 22 g) and were virtually constant in miconazole treated rats (270 ± 15 g in controls, 260 ± 35 g in treated rats), while liver weights increased from 8.8 ± 0.9 g in controls to 12.1 ± 1.3 g in ketoconazole treated rats and 8.2 ± 1.1 g to 10.0 ± 1.4 g in miconazole treated rats. In addition, both antimycotic drugs increased microsomal protein content, cytochrome P-450, cytochrome b_5 and NADPH-cyt *c*-reductase activity. The induction of NADPH-cyt *c*-reductase was shared with PB, not with 3-MC [27].

The data for seven daily doses of 40 and 160 mg/kg is described in Figs. 2 and 3. As expected, itraconazole administration had no inductive effect at any of the doses except for some irrelevant deviations from the control group mean. Miconazole or ketoconazole also did not significantly alter the hepatic parameters studied at the dose level of 10 mg/kg (Figs. 2 and 3). At 40 mg/kg the effects of miconazole exceeded those of ketoconazole. Ketoconazole at this dose level did not increase the cytochrome P-450 specific content, nor the NADPH cyt *c*-reductase activity.

At 160 mg/kg, miconazole was again superior in inducing the enzyme components of the cytochrome P-450 catalytic cycle, whereas ketoconazole increased the relative liver weight and the microsomal protein content with greater efficacity.

Effect of subchronic treatment on hepatic drug metabolizing enzymes

Enzyme activities were determined in hepatic

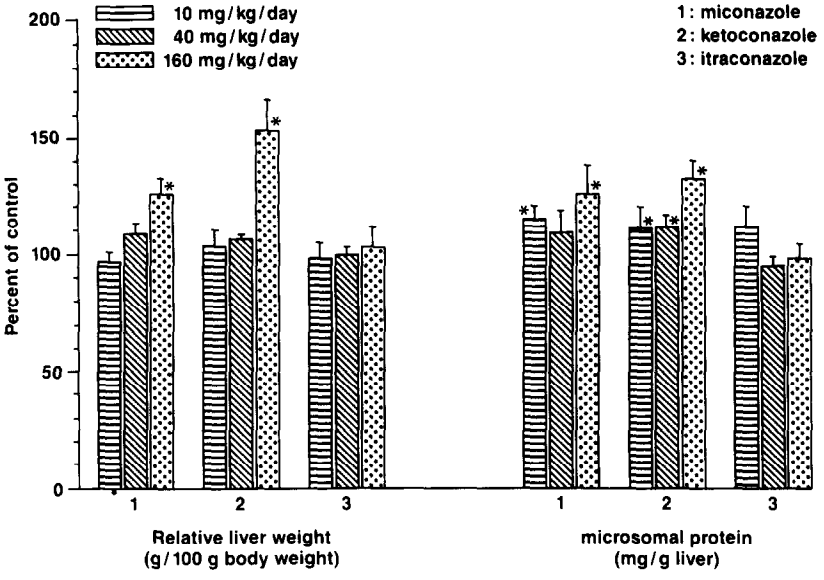

Fig. 2. Effect of treatment of male Wistar rats with different doses of miconazole (1), ketoconazole (2) or itraconazole (3) on the relative liver weight and on the concentration of microsomal proteins in hepatic microsomes. Rats were treated by gastric intubation at the dose level indicated once a day for seven days. Each value is the mean of four (40 and 160 mg/kg) or six (10 mg/kg) determinations and is presented as a percentage of the corresponding activity + 1 S.D. in control animals pretreated with vehicle alone at the same time as the experimental animals.* Significantly different from the corresponding control ($P < 0.05$; Student's t-test).

microsomes prepared from rats which had been treated with miconazole, ketoconazole or itraconazole once daily for seven days at 160 mg/kg (Table 2). Miconazole significantly induced *N*- and *O*-demethylation activities, and to a lesser extent, aniline hydroxylase activity. Ketoconazole only induced the *O*-demethylation of *p*-nitroanisole significantly and reduced *N*-demethylase and aniline hydroxylase activity. The overall effect of ketoconazole was much smaller than that of miconazole, except for the induction of UDP-glucuronyltransferase activity towards 4-nitrophenol, a phase II activity which was similar for both drugs. Itraconazole (160 mg/kg) on the other hand only reduced to a small extent the *N*-demethylase activity but did not influence other enzyme activities. The effects of two classical inducers, PB and 3-MC, on phase I enzyme activities also appears in Table 2. The pattern of enzyme induction by miconazole

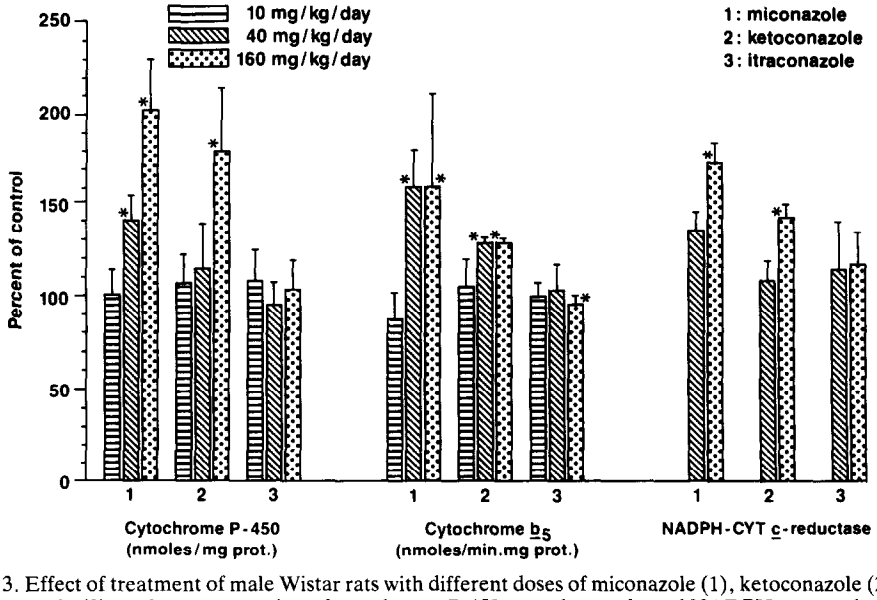

Fig. 3. Effect of treatment of male Wistar rats with different doses of miconazole (1), ketoconazole (2) or itraconazole (3) on the concentration of cytochrome P-450, cytochrome b_5 and NADPH-cyt *c*-reductase in hepatic microsomes. Treatment conditions and representation were identical to those in Fig. 2.

Table 2. Cytochrome P-450 dependent activity in rat liver microsomes after oral administration of miconazole, ketoconazole, itraconazole, PB, 3-MC and vehicle solutions for seven days. Results represent the mean ±S.D. of four (antimycotics) or six (PB, 3-MC) rats

	Enzyme activity									
	Aniline hydroxylation		*N*-demethylation aminopyrine		*N*-demethylation *N*,*N*-di-methylaniline		*O*-demethylation *p*-nitroanisole		UDPglucuronyl-transferase	
Treatment protocol	activity[1] mg protein	activity[1] g liver	activity[2] mg protein	activity[2] g liver	activity[2] mg protein	activity[2] g liver	activity[3] mg protein	activity[3] g liver	activity[4] mg protein	activity[4] g liver
Control (11.5% cremophor EL, 29 mM lactic acid pH 4.3)	0.83 ± 0.12	23.3 ± 5.0	1.69 ± 0.66	47.8 ± 22.1	4.27 ± 1.01	121 ± 37	4.13 ± 0.51	116 ± 21	5.90 ± 0.63	165 ± 29
Miconazole (160 mg/kg)	0.88 ± 0.06	30.0 ± 1.8*	2.94 ± 0.66*	102 ± 29*	10.3 ± 1.3*	355 ± 57*	10.6 ± 1.8*	365 ± 77*	9.82 ± 1.02*	339 ± 56*
Control (HCl, pH 3.0)	0.94 ± 0.08	25.6 ± 3.4	2.54 ± 1.29	69.5 ± 36.0	4.84 ± 1.15	131 ± 36	3.52 ± 0.64	95.5 ± 22.2	6.25 ± 0.38	168 ± 6
Ketoconazole (160 mg/kg)	0.76 ± 0.12*	27.0 ± 5.1	1.80 ± 1.08	65.4 ± 42.7	2.69 ± 0.92*	95.4 ± 34.0	5.19 ± 0.59*	184 ± 28*	9.36 ± 0.32*	331 ± 26*
Control (HCl in PEG-400, pH 2.5–2.7)	0.84 ± 0.03	19.5 ± 0.6	N.D.[5]	N.D.	6.15 ± 0.77	142 ± 17	3.56 ± 0.30	82.2 ± 7.5	3.12 ± 0.72	72.1 ± 0.7
Itraconazole (160 mg/kg)	0.76 ± 0.07	17.0 ± 0.4*	N.D.	N.D.	4.49 ± 0.56*	101 ± 8*	3.23 ± 0.16	72.8 ± 7.1	2.82 ± 0.12	63.6 ± 6.6
Control[6] (HCl, pH 3.0)	0.84 ± 0.06	22.1 ± 3.2	2.84 ± 0.69	74.7 ± 21.9	5.41 ± 1.08	143 ± 38	3.06 ± 0.52	81.2 ± 23.4	N.D.	N.D.
PB (60 mg/kg)	1.00 ± 0.10*	36.6 ± 7.8*	5.24 ± 1.14*	195 ±63*	14.1 ± 3.4*	528 ± 176*	8.46 ± 0.68*[7]	331 ± 55*[7]	N.D.	N.D.
Control (37.2 mM HCl in PEG-400)	0.74 ± 0.13	19.4 ± 4.7	2.66 ± 0.71	70.0 ± 21.1	5.18 ± 1.41	136 ± 42	3.25 ± 0.42	85.4 ± 17.7	N.D.	N.D.
3-MC (20 mg/kg)	0.74 ± 0.11	22.5 ± 3.8	2.00 ± 0.21	60.8 ± 6.3	4.75 ± 0.42	144 ± 23	9.25 ± 1.64*	284 ± 63*	N.D.	N.D.

* Statistically significantly different from control ($P < 0.05$).
[1] nmoles *p*-aminophenol produced/min.
[2] nmoles HCHO produced/min.
[3] nmoles *p*-nitrophenol produced/min.
[4] nmoles glucuronide formed/min.
[5] N.D.: not determined.
[6] Mean of five rats.
[7] Mean of four rats.

resembled, at least qualitatively, that obtained by PB pre-treatment whereas ketoconazole-induction differed from both PB- and 3-MC-induction. The ketoconazole-induced microsomes had low aniline hydroxylase and *N*-demethylase activity on the basis of its cytochrome P-450 content, and only a slightly increased *O*-demethylase activity, whereas 3-MC clearly induced a cytochrome P-450 isoenzyme with a high activity towards the latter substrate.

The dose dependence of the induction of hepatic drug metabolizing enzymes is illustrated in Fig. 4. Miconazole at a dose level of 40 mg/kg induced significantly the *N*-demethylation of *N,N*-dimethylaniline and the *O*-demethylation of *p*-nitroanisole. Ketoconazole was a less potent inducer compared to miconazole and had a significant effect only on the *O*-demethylation of *p*-nitroanisole. Neither of the drugs had a significant effect on microsomal enzymes when given at a dose level of 10 mg/kg, which is comparable or superior to the antifungally active dose. In addition, itraconazole did not induce or inhibit microsomal enzyme activities at the 40 mg/kg dose. Comparison of the data in Fig. 4 and Table 2 clearly reveals that the antimycotic drugs were by far less potent in inducing hepatic drug metabolizing enzymes compared to PB or 3-MC.

Time course of the induction of hepatic parameters and drug metabolizing enzymes

The time course of induction of the cytochrome P-450 dependent enzyme system was studied after single or multiple oral administration of miconazole or ketoconazole at a dose level of 160 mg/kg. Figures 5 and 6 show as a function of time the effects of the drugs on some components of the cytochrome P-450 enzyme system and on the microsomal enzyme activities respectively. The induction of enzyme components of the cytochrome P-450 system occurred faster with miconazole (Fig. 5a) than with ketoconazole (Fig. 5b).

Miconazole, and to a lesser extent ketoconazole, produced a biphasic effect on hepatic microsomal enzyme activities. In rats sacrificed one hour after administration of a single dose of miconazole (day 0 in Figs. 5 and 6), phase I enzyme activities, *N*- and *O*-demethylation, were depressed significantly. The transition inhibition phase is followed by a rapid increase in *N*- and *O*-demethylase activity.

UDP-glucuronyltransferase was only stimulated within seven days of treatment. The effect of ketoconazole was less pronounced, i.e. activities were only moderately inhibited after a single administration or moderately stimulated after repeated dosage.

Kinetic and electrophoretic properties of cytochrome P-450 isoenzymes induced by miconazole and ketoconazole

Miconazole or ketoconazole, when added to microsomal preparations, gave rise to type II difference spectra (Figs. 7a, b). The peak, isobestic and trough wavelengths for the interaction of miconazole with miconazole-induced microsomes were 429 nm, 416 nm and 393 nm respectively, and for the interaction of ketoconazole with ketoconazole-induced microsomes, 430 nm, 417 nm and 393 nm respectively. When the absorbance difference between peak and trough values were used to construct Eadie–Hofstee graphs (Fig. 8), the resulting plots clearly deviated from linearity. Quantitative examination of the data using the strategy outlined in Materials and Methods, indicated a good fit to a two-site binding model, with apparent spectral dissociation constants for miconazole of 1.2 μM (high

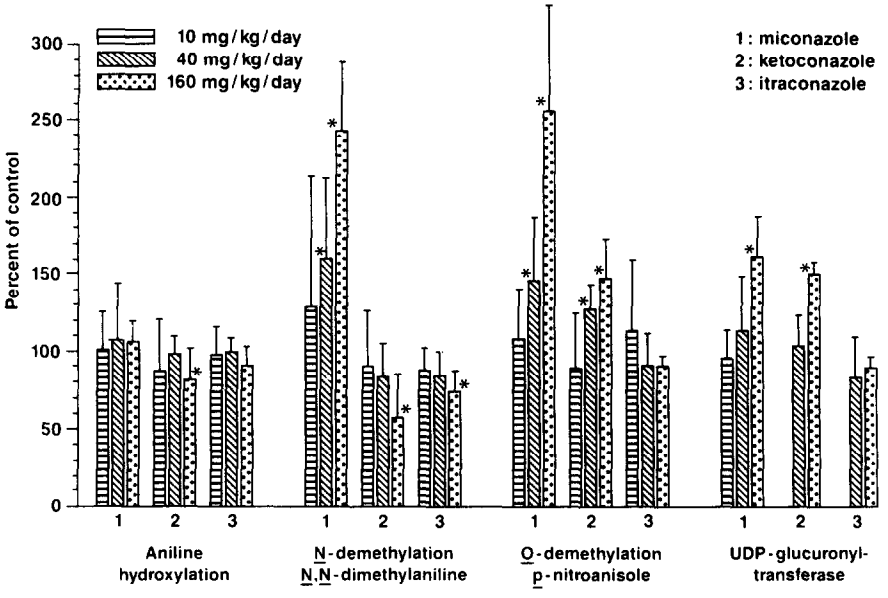

Fig. 4. Effect of treatment of male Wistar rats with different doses of miconazole (1), ketoconazole (2) or itraconazole (3) on mixed function oxidase activities and on UDP-glucuronyltransferase activity in hepatic microsomes. Treatment conditions and representation were identical to those in Fig. 2. The calculations were based on the activities expressed as the amount of product formed min^{-1}.mg $protein^{-1}$.

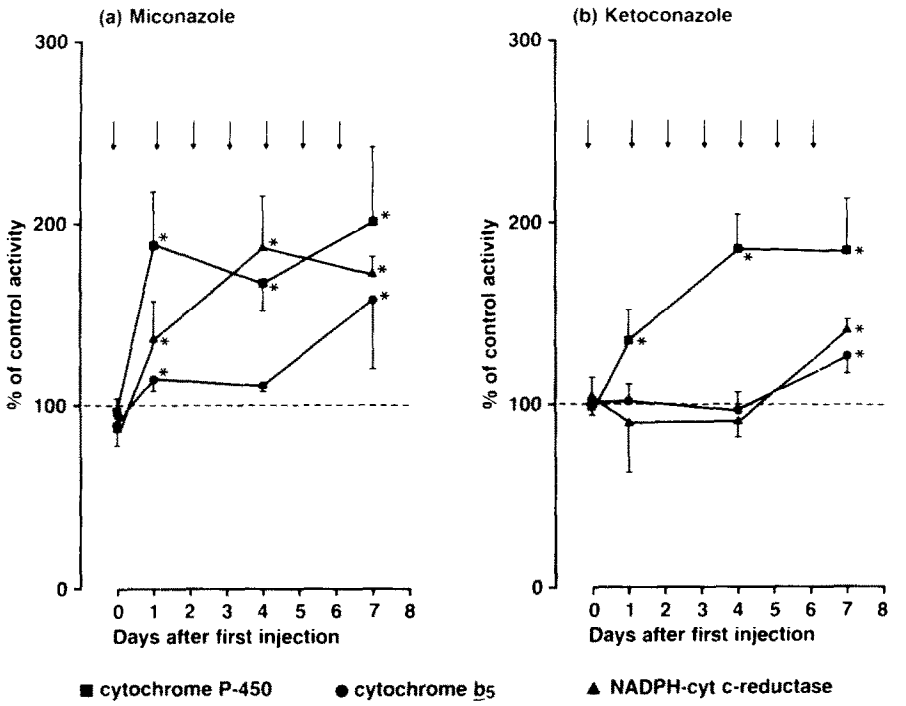

Fig. 5. Effect of multiple dosing of male Wistar rats with miconazole (a) and ketoconazole (b) on the cytochrome P-450, cytochrome b_5 and NADPH-cyt *c*-reductase activity in hepatic microsomes. Rats were treated once daily (see arrows) by gastric intubation at a dose level of 160 mg/kg. Rats were killed 1 hr (day 0) or 23 hr (days 1, 4 and 7) after the last dose. Experimental points represent the percentage of the control value (calculated per mg protein) + 1 S.D.* Significantly different from the corresponding control ($P < 0.05$, Student's *t*-test).

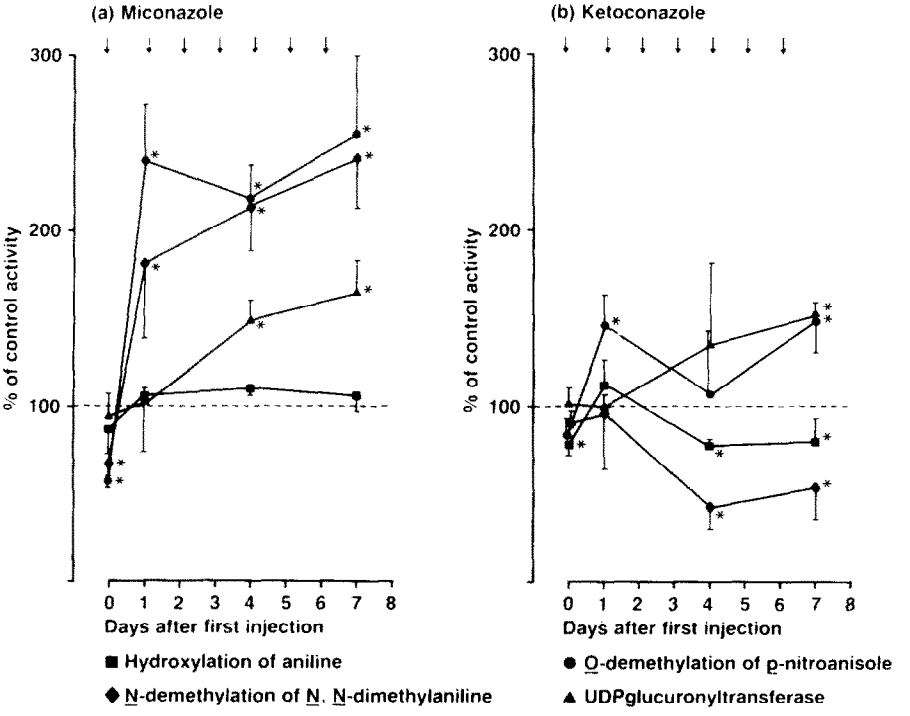

Fig. 6. Effect of multiple dosing of male Wistar rats with miconazole (a) and ketoconazole (b) on mixed function oxidase activities and on UDP-glucuronyltransferase activity in hepatic microsomes. Treatment conditions and representation were identical to those of Fig. 5.

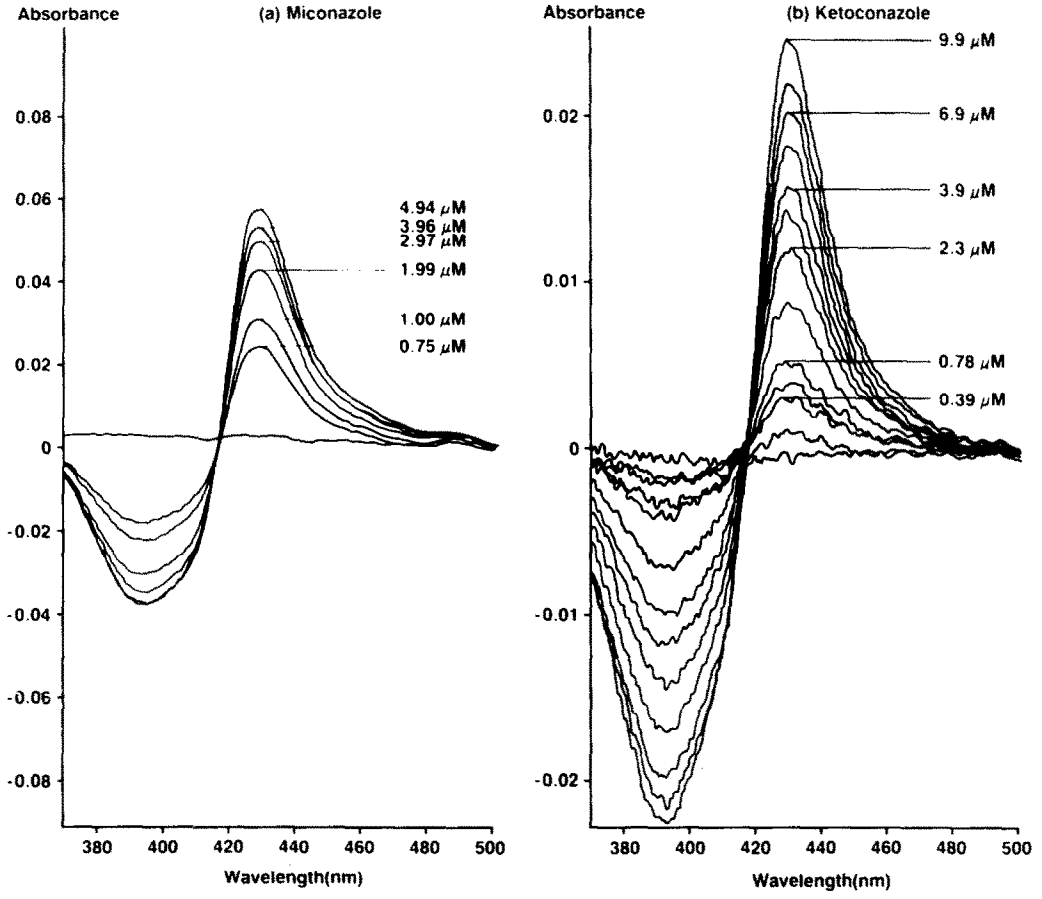

Fig. 7. Difference spectra of liver microsomes from miconazole (a) or ketoconazole (b) pretreated rats obtained in a titration experiment with miconazole and ketoconazole respectively. Each cuvette contained 3 mg of a microsomal suspension in KCl (0.25%)-phosphate buffer (0.1 M) pH 7.4. Protein concentration was 2 mg/ml and cytochrome P-450 concentration 1.829 nmol/mg and 2.521 nmol/mg for miconazole and ketoconazole induced microsomes respectively. Spectra were recorded after addition of small volumes of a drug solution in 2.7 mH HCl to the sample cuvette and the same volume of solvent to the reference cuvette.

affinity) and 25 μM (low affinity) and of 2.3 μM and 27 μM for ketoconazole.

Further information about the isoenzymes induced by the antimycotics was gained by determining the I_{50} (concentration of the inhibitor to obtain 50% inhibition) for the inhibition by miconazole and ketoconazole of the *O*-demethylation of *p*-nitroanisole, which was catalyzed by microsomes from rats treated with the corresponding drug (Fig. 9). The I_{50}-values were 5.2 μM for miconazole and 15.1 μM for ketoconazole. Since striking differences were found in miconazole- and ketoconazole-induced microsomal enzyme activities, which either could reflect differences in the lipid composition of the microsomal membranes, or differences in the occurrence of distinct cytochrome P-450 isoenzymes, microsomes from miconazole-, ketoconazole-, 3-MC- and PB-treated rats were analyzed by SDS–PAGE (Fig. 10). Microsomes from ketoconazole-treated rats contained increased amounts of a protein with an apparent molecular weight of 47,800. In microsomes of miconazole-treated rats, the major protein fractions had molecular weights of 44,300, 47,400 and 49,700. The electrophoretic pattern of PB-microsomes resembled, in general, that of miconazole-induced microsomes, whereas that of 3-MC microsomes clearly showed that the major proteins had larger molecular weights compared to the miconazole-or ketoconazole-induced microsomes.

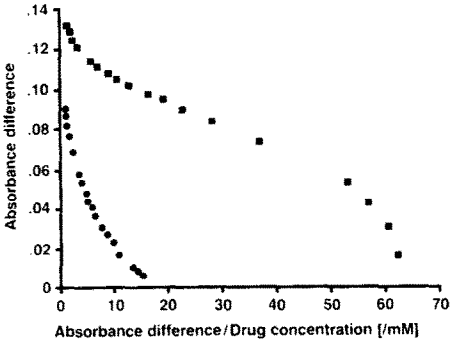

Fig. 8. Eadie–Hofstee plots constructed from data obtained after titration of hepatic microsomes of miconazole (■) and ketoconazole (●) pretreated rats with respectively miconazole and ketoconazole as described in Fig. 7.

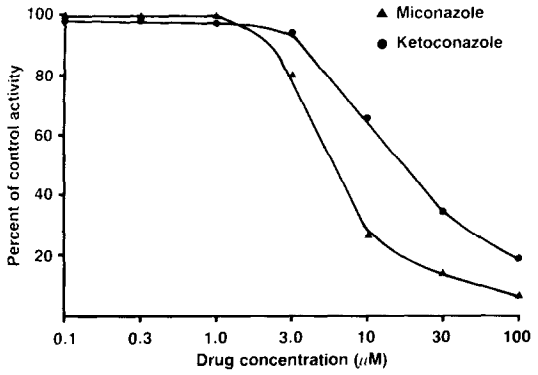

Fig. 9. Dose response curves for the inhibition of *O*-demethylase activity by miconazole (▲) and ketoconazole (●). The inhibitory effects of the drugs on the *O*-demethylase activity of hepatic microsomes prepared from respectively miconazole and ketoconazole pre-treated rats were determined. The control activities of each suspension equalled 12.4 and 5.74 nmoles *p*-nitrophenol produced/min.mg protein respectively. Each point represents the mean of two determinations.

* H. Van Cauteren, personal communication.

DISCUSSION

The data presented in this paper show that after subchronic dosing of male rats with miconazole or ketoconazole at 10 mg/kg, which is in the antimycotic therapeutic range, was without any effect on liver enzymes. Furthermore, itraconazole was devoid of inducing properties, even at the highest dose level of 160 mg/kg. Administration of high doses of miconazole or ketoconazole (160 mg/kg) clearly increased the relative liver weight, cytochrome P-450-content and cytochrome P-450 dependent monooxygenase activities, NADPH- dependent cyt *c*-reductase and UDP-glucuronyltransferase, an enzyme involved in detoxication. For miconazole but not for ketoconazole similar but slighter effects were noted at 40 mg/kg. The inductive effect appeared to be time and dose dependent. It must be emphasized that a daily oral dose of 160 mg/kg of ketoconazole (in food for 3 months) produced clearly toxic effects in rats, including lethality, whereas miconazole was slightly toxic.* Miconazole was the more potent inducer compared to ketoconazole: cytochrome P-450 levels were increased much faster (Fig. 5) and maximal induction exceeded by far the effects of comparable doses of ketoconazole. This agrees well with a previous study

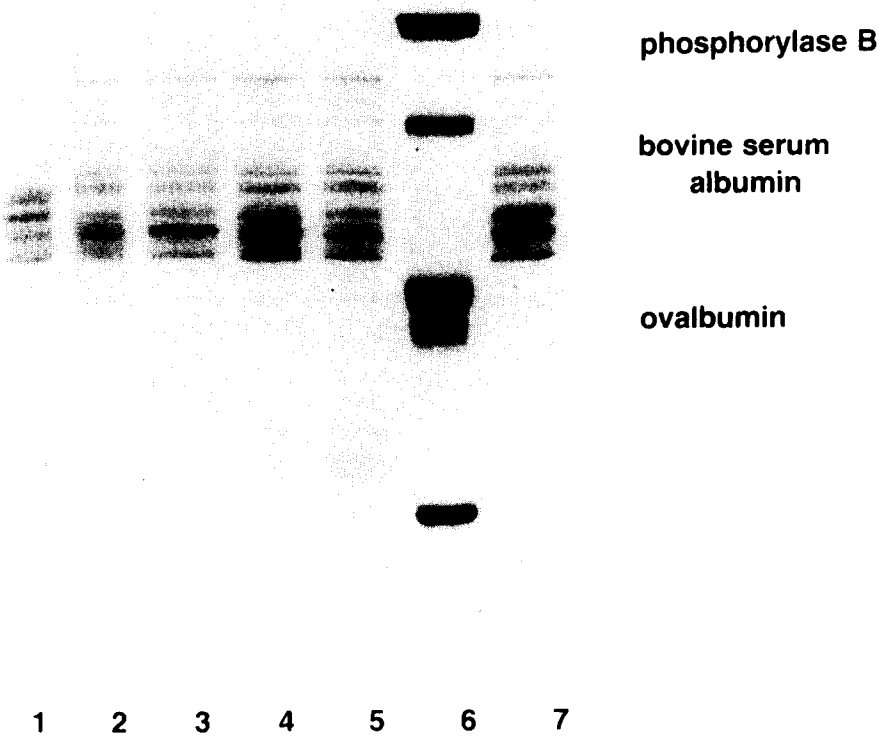

Fig. 10. SDS-PAGE of rat liver microsomes. Of each suspension 10 μg microsomal protein was subjected to electrophoresis as described in Materials and Methods. Microsomes were prepared from livers of rats pretreated with 3-methylcholanthrene (lane 1), control (=HCL, pH 3.0) (2), ketoconazole (3), miconazole (4), control (= acidic Cremophor EL in water) (5) and phenobarbital (7). Lane 6 contains molecular weight markers.

which demonstrated that ketoconazole was less potent than miconazole in inducing the *in vitro* *N*-demethylation of tilidine by female rat liver microsomes [12]. No induction or inhibition was observed at a dose level of 10 mg/kg. This is not exceptional. Greim [29] has already stated that although many xenobiotics are able to stimulate drug metabolism, the number of drugs which have been reported to be inducers when given at therapeutic doses is extremely low. In man, repeated administration of miconazole has been reported to have had an inductive effect [10] whereas multiple administration of ketoconazole has been devoid of inducing effects [11, 30]. The absence of any effect of itraconazole in terms of induction, correlates rather well with the weak interaction of this drug with rat liver cytochrome P-450 as evidenced by the formation of a type II difference spectrum and by the almost complete absence of effects on *in vitro* drug metabolism.*

At 160 mg/kg miconazole, and to a lesser extent ketoconazole, clearly has a biphasic effect on hepatic cytochrome P-450-dependent monooxygenase activities (Fig. 6), a phenomenon which is shared by other compounds such as imidazole itself [4, 6]. The inhibitory properties of miconazole and ketoconazole are well documented [7, 13, 31] and can be attributed to the imidazole moiety of the drug [5]. Since these antimycotic drugs bind to cytochrome P-450 with high affinity (high affinity spectral dissociation constants around 1 μM*), it is possible that at least some drug remains bound to cytochrome P-450 during preparation of the microsomes, especially since high plasma and tissue levels of the drugs are expected one hour after oral administration [32, 33]. For miconazole, similar biphasic effects have been described on microsomal benzphetamine *N*-demethylase activity after acute and subchronic administration to mice [34]. The lowering by ketoconazole of aniline hydroxylation and *N,N*-dimethylaniline demethylation on days 4 and 7 (Fig. 6b) probably reflects the induction of a cytochrome P-450 species with diminished substrate specificity, whereas the enzyme which participates primarily in the metabolism of aniline or *N,N*-dimethylaniline was not induced. It can not be totally excluded, however, that unchanged drug still present in the washed microsomes (results not shown), lowers also the specific activity towards a particular substrate. Similarly, unchanged itraconazole, probably because of its highly lipophilic character, was still present in the washed microsomes and the small reduction of the *N*-demethylase activity after administration of the high 160 mg/kg dose could be the result.

Addition of miconazole and ketoconazole to the corresponding induced microsomes resulted in the formation of type II-difference spectra (Fig. 7), indicative of a ligand interaction of the drugs with oxidized cytochrome P-450 [28]. The biphasic character of the binding (Fig. 8) can probably be attributed to the presence of at least two binding places with distinct characteristics, either two different cytochrome P-450 isoenzymes, or, alternatively, two binding sites with different affinities on a single cytochrome P-450 form [5]. It is also possible that after ligand binding, cytochrome P-450 is stabilized and less susceptible to protein degradation [35] or that microsomal membranes are protected against oxidative damage [36], which leads in both cases to an apparent induction of liver drug metabolizing enzymes. The *O*-demethylase activities catalyzed by the induced microsomes were inhibited by ketoconazole at an I_{50} value comparable to that obtained with microsomes from untreated animals, whereas inhibition by miconazole generated an I_{50}-value intermediate between that of control- and 3-MC microsomes.*

When microsomal enzyme activities were compared, striking differences were found between miconazole- and ketoconazole-induced microsomes. Miconazole induced *N*- and *O*-demethylase activities, whereas ketoconazole only induced *O*-demethylase activity and lowered the specific activities towards the other substrates (Table 2). Previous reports have already shown that miconazole was able to induce *N*-demethylase activity towards benzphetamine after subchronic treatment of mice [34] or towards pethidine after treatment of rats.† Imidazole on the other hand induced *N*-demethylation of aminopyrine but not the hydroxylation of aniline in the rat [6], whereas in the rabbit, it induced *N*-demethylase activity towards *N,N*-dimethylaniline, *O*-demethylase activity towards *p*-nitroanisole [37] and hydroxylase activity towards aniline [38] indicating that imidazole induced the same P-450 species as ethanol in the rabbit, but not in the rat [6, 38, 39]. In the present study, no clear induction of aniline hydroxylase (expressed per mg protein) was observed, and in contrast with the inductive effects of imidazole in rats [6], there was a significant increase in cytochrome P-450 content, which indicates that the antimycotics induced other cytochrome P-450 populations. Furthermore, both antimycotic drugs induced NADPH-cyt *c*-reductase activity which, together with the increase in relative liver weight and microsomal protein content, resembles the inductive effects of PB but not those of 3-MC [35, 40]. On the other hand, 3-MC is also a relatively specific inducer, as is ketoconazole, since only *O*-demethylase activity is increased upon 3-MC-treatment (Table 2). Although the enzymatic activities measured in the present study cannot be used as specific probes to screen PB- or 3-MC-inducible cytochrome P-450 (cytochrome P-448 in the case of 3-MC) isoenzymes, the data indicate that miconazole behaves as a PB-type inducer, whereas ketoconazole does not belong to either class of inducers.

Although the reported differences could originate from differential effects of the two drugs on lipid biosynthesis [41], a number of observations indicate that the cytochrome P-450 populations induced by the drugs are different, either qualitatively, i.e. in isoenzyme nature, or quantitatively with respect to the ratio between identical isoenzymes. First, there was only a hypochrome shift in the CO-difference spectrum of reduced ketoconazole-microsomes (Table 1). Secondly SDS–PAGE of solubilized microsomes (Fig. 10) revealed that in ketoconazole-induced

* K. Lavrijsen, J. Van Houdt, D. Thijs, W. Meuldermans and J. Heykants, manuscript in preparation.

† P. Laduron, G. Aerts and C. J. E. Niemegeers, personal communication.

microsomes, the major protein had a M_r of 47,800, whereas the electrophoretogram of miconazole-microsomes, resembled that of PB-microsomes, with the exception of the occurrence of a double protein band in the 50,000 Da-region. Since most cytochromes P-450 appear to have molecular weights near 50,000 [42], it is valid to conclude that at least some of the above-mentioned protein fractions contain cytochrome P-450. This assumption is strengthened by the fact that 3-MC, indeed, primarily induced cytochromes P-450 (P-448) with higher molecular weights, as compared with PB [42].

Although the data of the present study indicate that distinct cytochrome P-450 isoenzymes are induced by the antimycotics, ultimate identification of these isoenzymes in miconazole- and ketoconazole-induced microsomes can only be given after purification of the cytochromes, followed by determination of spectral, catalytic (in reconstituted systems), immunological and structural properties. These aspects are currently under investigation. Furthermore, the role of the ketoconazole-induced cytochrome, which seems to possess low activity towards the usual model substrates, is subject to further research.

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