*Biochemical Pharmacology*, Vol. 37, No. 3, pp. 401–408, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Journals Ltd.

# COMPARISON OF TWO AZOLE ANTIFUNGAL DRUGS, KETOCONAZOLE AND FLUCONAZOLE, AS MODIFIERS OF RAT HEPATIC MONOOXYGENASE ACTIVITY

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(*Received* 15 *May* 1987; *accepted* 27 *July* 1987)

**Abstract**—The mechanism of action of azole antifungal agents is believed to involve inhibition of fungal cytochrome P-450, and, therefore, an investigation of the interaction of these drugs with mammalian cytochrome P-450 systems should provide some indication of their selectivity as antifungal agents. The ability of ketoconazole and fluconazole, the latter representing a new generation of triazole antifungal agents, to modify rat mixed function oxidase activity has been investigated *in vitro* with hepatic microsomes and *in vivo* using a *N*-methyl-[$^{14}$C] antipyrine breath test. As a measure of selectivity the results have been compared with antifungal potency. Ketoconazole is more potent than fluconazole by an order of magnitude in inhibiting metabolism by O-dealkylation of ethoxycoumarin, methoxycoumarin and ethoxyresorufin ($IC_{50}$ values of 6, 5 and 130 $\mu$M for ketoconazole respectively). The effects on the regio- and stereospecific hydroxylation of [$^{14}$C] testosterone were also measured; the $IC_{50}$ values for inhibition of total testosterone metabolism were 0.1 mM and >3 mM for ketoconazole and fluconazole respectively. Marked selectivity differences were observed for the two drugs as indicated by ketoconazole being a potent inhibitor of 7$\alpha$-hydroxylation of testosterone ($IC_{50}$ 20 $\mu$M) while fluconazole did not inhibit this activity at 3 mM. *In vivo* investigations using a range of doses confirmed their ranking for inhibitory potency; the $ED_{50}$ values for maximum demethylation rate were 17 $\mu$mol/kg and >60 $\mu$mol/kg for ketoconazole and fluconazole respectively. Thus fluconazole has a lower propensity to interact with rat hepatic cytochrome P-450 and can be considered a more selective antifungal agent as its *in vivo* antifungal potency is an order of magnitude greater than ketoconazole.

The mode of action of the azole antifungal agents is believed to involve the inhibition of cytochrome P-450-mediated synthesis of the fungal cell membrane sterol, ergosterol [1, 2]. However, it has been known for some time that *N*-substituted imidazoles are also potent inhibitors of many cytochrome P-450-mediated reactions in other organisms, including those in mammalian liver [3–5] and steroidogenic organs [6, 7]. Thus the introduction of systemically-acting azole antifungal drugs into clinical therapeutics has led to the need for agents which are highly selective inhibitors of the fungal enzyme(s) rather than the various cytochromes P-450 present in mammalian systems. Recent studies with ketoconazole, the first antifungal drug with acceptable oral activity in clinical practice, have shown interactions in man with the metabolism of co-administered drugs [8–10] and of endogenous steroids [11–13].

Fluconazole(2-[2,4-difluorophenyl]-1,3-bis[1H-1,2,4-triazol-1-yl]-propan-2-ol]) is a new antifungal drug which is considerably more potent in model fungal infections than the imidazole, ketoconazole [14, 15]. The purpose of these investigations is to establish the inhibitory potency of these two drugs against rat hepatic mixed function oxidase activity *in vitro*, using standard microsomal preparations with recognised substrate probes, and *in vivo* using the [*N*-methyl-$^{14}$C]antipyrine breath test [16, 17]. Their ability to inhibit mammalian cytochromes P-450 is compared with their *in vitro* and *in vivo* potency against *Candida albicans* [14] and provides some indication of the selectivity of fluconazole and ketoconazole as antifungal drugs.

## MATERIALS AND METHODS

*Microsomal dealkylation investigations.* Hepatic microsomes were prepared from untreated male Sprague–Dawley rats (5 pooled in each group, body weight 230–280 g: University of Manchester) as previously described [18]. The microsomal pellets were resuspended in Tris hydrochloride buffer (20 mM, pH 7.4) containing sucrose (0.25 M) and EDTA (5.4 mM) and stored at −80° before assays were performed. The microsomal cytochrome P-450 content, determined by the method of Omura and Sato [19], was 0.35 nmol/mg protein.

*O*-dealkylation of ethoxycoumarin (Sigma Chemical Co., Poole, Dorset, U.K.), methoxycoumarin (Sigma Chemical Co.) and ethoxyresorufin (Molecular Probes, Junction City, DE) were determined by continuous monitoring of parent phenol pro-

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duction using a Perkin–Elmer Spectrofluorimeter MPF-3. The conditions used were those described by Prough *et al.* [20]. These assays were conducted at 37°, initiated by addition of NADPH and carried out using a microsomal protein concentration of 1 mg/ml. Ketoconazole (Janssen Pharmaceutica, Beerse, Belgium) and fluconazole (Pfizer Central Research, Sandwich, U.K.) were added to the microsomal incubate in dimethyl formamide (maximum vol. 10 μl in 2 ml incubate). The presence of the organic solvent did not influence the *O*-dealkylation activity.

*Testosterone hydroxylation investigations.* Microsomes from untreated male Sprague–Dawley rats (150 g, Charles River, Manston, U.K.) were prepared and stored as described above. Testosterone hydroxylation reactions were carried out using the method of Ballard *et al.* [21]. Briefly, samples of the microsomal suspension containing 1 nmol of cytochrome P-450 were diluted with an NADPH regenerating system comprising isocitrate dehydrogenase (0.5 units, Sigma Chemical Co.), magnesium chloride (0.1 mM), manganese sulphate (0.1 mM), isocitrate (0.17 M Sigma Chemical Co.) and HEPES/sodium buffer (0.05 M, pH 7.4, Sigma Chemical Co.) to yield a final incubation volume of 1 ml. Ketoconazole and fluconazole were added to incubations in dimethylformamide (10 μl). Samples were preincubated for 5 min at 37° prior to the initiation of the reaction by the addition of [4-$^{14}$C]-testosterone (0.1 mM, specific activity 0.9 μCi/μmol, Amersham International plc, Amersham, U.K.). Following incubation for 10 min at 37° the reaction was stopped by precipitation of the proteins with methanol (1 ml). Radiolabelled material was extracted from the supernatant and metabolites separated on thin-layer chromatography plates using the method of Darby *et al.* [22]. Quantitation of the radioactive metabolite pattern was carried out using an ISOMESS I.M. 3000 linear plate analyser (Lablogic Ltd., Sheffield, U.K.).

*Antipyrine CER investigations.* Fifty-four male Sprague–Dawley rats (body weight 280–320 g) were administered [*N*-methyl-$^{14}$C]-antipyrine (25 mg/kg; 10 μCi/kg; i.p.), purchased from the Radiochemical Centre (Amersham, U.K.), with a specific activity of 57 mCi/mmol and a radiochemical purity of 99%). One hour before receiving antipyrine the animals were administered i.p. either ketoconazole (1, 3, 5, 10, 15, 20, 30 or 50 mg/kg), fluconazole (0.1, 0.5, 1.5, 2.5, 4, 8, 12 or 20 mg/kg) or vehicle alone (10% cremophor in 0.1 M HCl, v/v). Groups of 6 animals were tested per day; three received ketoconazole and the others fluconazole. Order of doses was randomised.

Rats were housed in individual all-glass metabolism cages constructed for the collection of exhaled [$^{14}$C]$O_2$ as described previously [23] over a period of 7–10 hr. The trapping fluid was changed every 20 min. [$^{14}$C]$O_2$ exhalation rate (CER) half-lives were calculated from the slope of a plot of the logarithm of the CER (% dose/min) against the mid-point of each collection interval. The maximum CER and the time taken to achieve this maximum were obtained by inspection of these CER vs time profiles.

## RESULTS

### *Inhibition of hepatic microsomal* O-*dealkylation*

The ability of ketoconazole and fluconazole to inhibit the *O*-dealkylation of three substrates, ethoxycoumarin, methoxycoumarin and ethoxyresorufin, was investigated over a concentration range of four orders of magnitude. Ketoconazole was effective at lower concentrations than fluconazole in all cases. The inhibitory effect of ketoconazole is illustrated in Fig. 1 using methoxycoumarin as a substrate; to achieve a similar degree of inhibition fluconazole was added at approximately ten times the molar concentration of ketoconazole. The microsomal drug concentrations required to reduce the dealkylation rate by 50% ($IC_{50}$) are shown in Table 1.

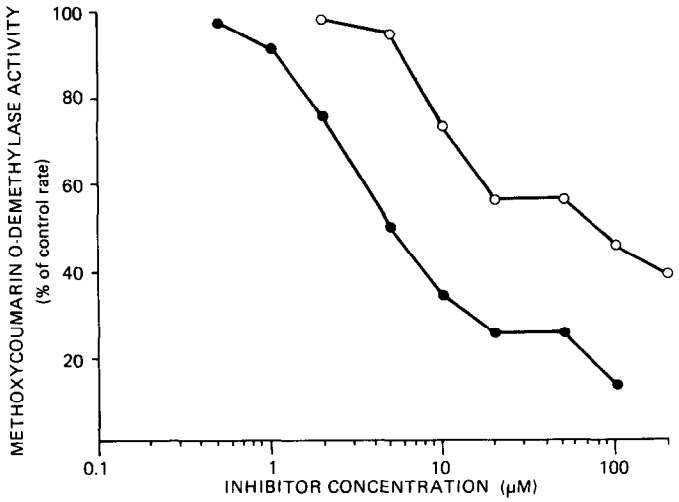


Fig. 1. Relationship between percentage of control activity of methoxycoumarin *O*-demethylase and concentration of either ketoconazole (●) or fluconazole (○). Control activity was 0.208 ± 0.024 nmoles/min/mg protein. Data shown are mean of duplication determinations.

Table 1. *In vitro* inhibition of *O*-dealkylation reactions by antifungal drugs

| Substrate | Enzyme source | Inhibitor $IC_{50}$ (mM) | | Potency difference |
|---|---|---|---|---|
| | | Ketoconazole | Fluconazole | |
| Ethoxycoumarin | Rat hepatic microsomes | 0.006 | 0.031 | 5 |
| Methoxycoumarin | Rat hepatic microsomes | 0.005 | 0.075 | 15 |
| Ethoxyresorufin | Rat hepatic microsomes | 0.130 | >2.0 | >15 |
| Lanosterol* | *Candida albicans* | $5 \times 10^{-5}$ | $5 \times 10^{-5}$ | None |

* Data taken from Hanger *et al.* (1985) where sterol biosynthesis was measured in a cell free extract of *Candida albicans* [24].

Much higher $IC_{50}$ values were obtained using ethoxyresorufin rather than the coumarins, however, in each case the differential between the compounds in inhibitory potency was marked. For comparison, literature data on potency of inhibition for demethylation of lanosterol by *Candida albicans* [24] are shown in Table 1, demonstrating identical activity for the two drugs.

*Inhibition of testosterone hydroxylation*

Incubation of [4-$^{14}$C]-testosterone with rat liver microsomes in the absence of test compounds resulted in the production of eleven radioactive metabolites (Fig. 2). Five of these metabolites, 16$\alpha$OHT; 7$\alpha$OHT; 6$\beta$OHT; 2$\alpha$OHT and A were identified by co-chromatography with standards in two solvent systems. 16$\alpha$OHT and 2$\alpha$OHT were

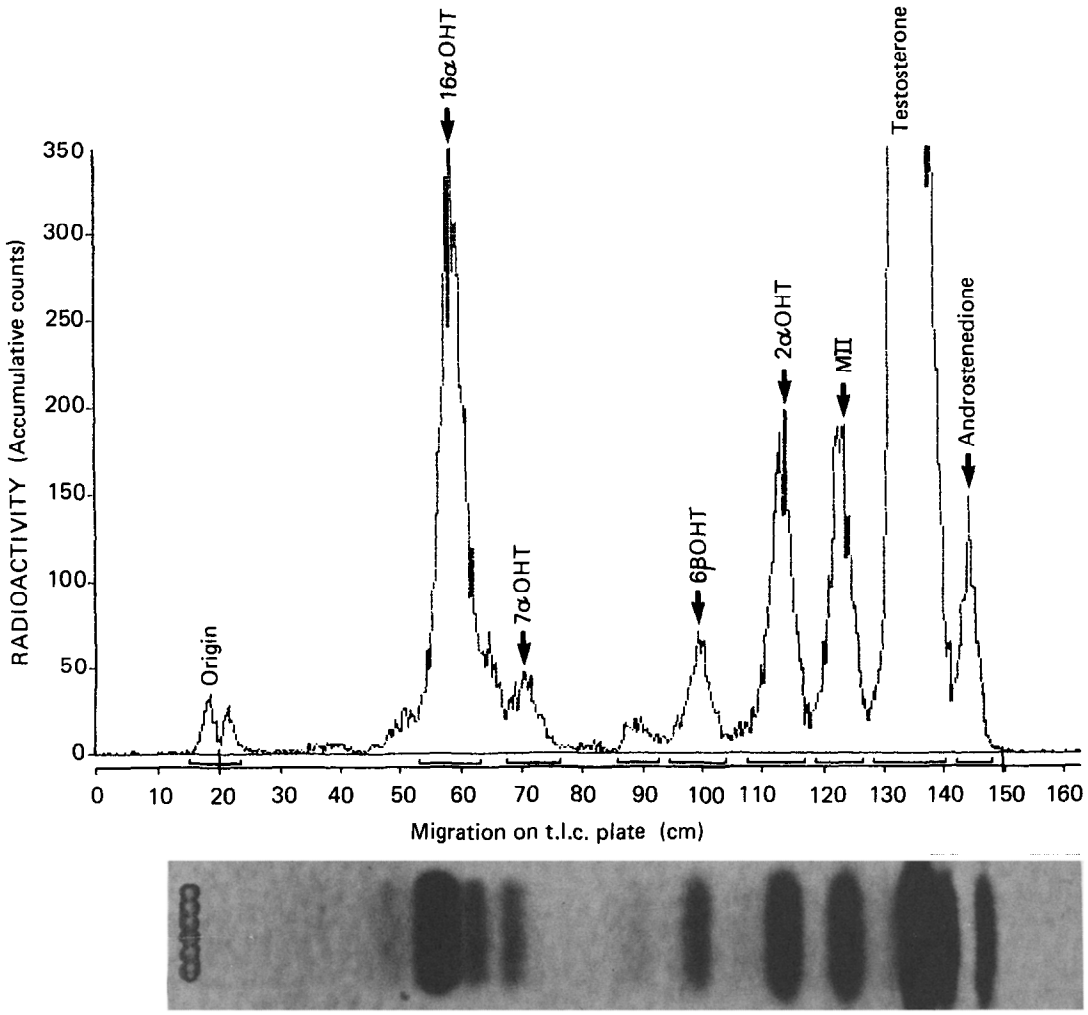


Fig. 2. Autoradiograph and corresponding radiochromatogram of [$^{14}$C] testosterone metabolites produced by rat hepatic microsomes. TLC solvent system was dichlormethane : ethyl alcohol (20 : 1 v/v).

Table 2. *In vitro* inhibition by antifungal drugs of the regio- and stereo-selective metabolism of testosterone by rat hepatic microsomes

| Testosterone metabolism pathway | Inhibitor $IC_{50}$ (mM) Ketoconazole | Fluconazole | Potency difference |
|---|---|---|---|
| 16α-hydroxylation | 0.090 | 1.600 | 18 |
| 7α-hydroxylation | 0.020 | None* | >1000 |
| 6β-hydroxylation | $5.5 \times 10^{-4}$ | 0.105 | 190 |
| 2α-hydroxylation | 0.090 | 0.800 | 9 |
| Oxidation to androstenedione | 0.105 | 2.00 | 19 |
| Total metabolism | 0.100 | >3.0 | >30 |

* No inhibition of this activity seen with fluconazole at concentrations up to 3 mM.

major metabolites representing 38% and 19% of total metabolite radioactivity. 6βOHT (5%), 7αOHT (5%) and androstenedione (11%) were minor metabolites. Unknown metabolite II (18% of total metabolites) was not visible under UV light, suggesting the reductive loss of a double bond. The formation of this metabolite was not affected by the azole drugs. Further chromatography of isolated metabolite bands indicated that the 6βOHT spot contained two minor contaminants accounting for 30% of radioactivity at this $R_F$. Metabolites 16αOHT, 7αOHT, 2αOHT and A were >90% pure. Incubation of [4-$^{14}$C]-testosterone in the presence of the azole compounds showed that both compounds inhibited the conversion of testosterone to metabolites. Mean values for $IC_{50}$ (concentration of inhibitor producing 50% of relative control rate of testosterone conversion) for effects on total testosterone metabolism were 100 μM and >3 mM for ketoconazole and fluconazole respectively. Mean $IC_{50}$ values for effects of the compounds on the production of individual testosterone metabolites are shown in Table 2. The production of 6βOHT was the most sensitive to inhibition by both compounds. However, ketoconazole was two-hundred fold more potent than fluconazole as an inhibitor of the production of this metabolite. There were marked selectivity differences in the action of the two drugs against other activities. Thus, ketoconazole inhibited the formation of 7αOHT, whereas fluconazole exhibited no inhibitory activity against this activity at any concentration up to 3 mM. In addition ketoconazole exhibited similar inhibitory potential for the production of the 16αOHT, 2αOHT and androstenedione metabolites, whereas with fluconazole, which was markedly less potent in each case, the $IC_{50}$ values for the production of these metabolites varied. These data show that ketoconazole is a more potent inhibitor of all testosterone hydroxylations than fluconazole and that the spectrum of inhibition of each compound shows marked differences.

### *Inhibition of antipyrine CER*

CER–time profiles from animals receiving equimolar doses of ketoconazole or fluconazole or vehicle

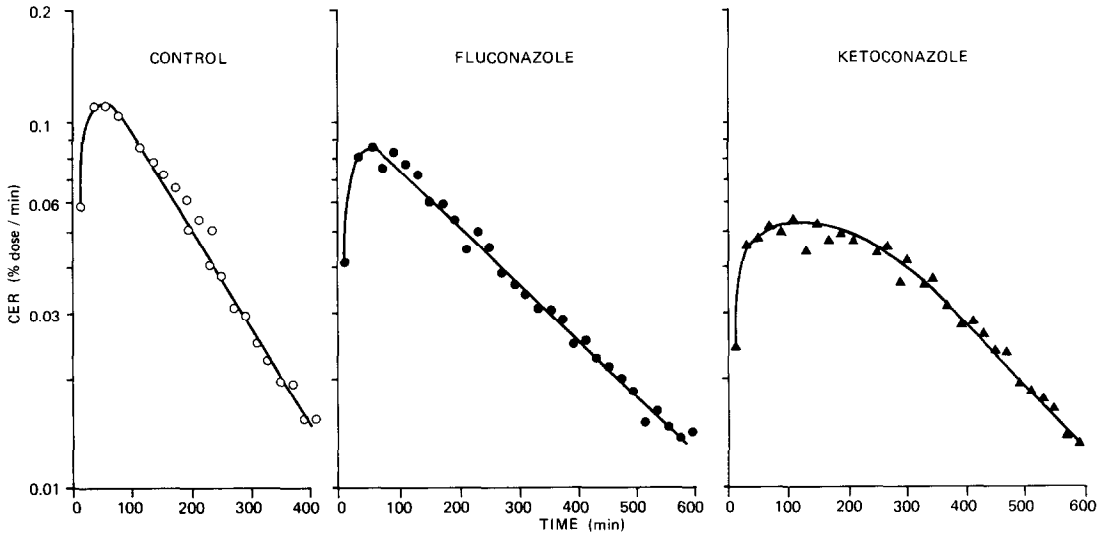


Fig. 3. Effect of approximately equimolar doses of ketoconazole and fluconazole on antipyrine $^{14}CO_2$ exhalation rate (CER)–time profiles. Data were obtained following administration of [*N*-methyl-$^{14}$C]-antipyrine to individual rats under control conditions (○), and after treatment with either ketoconazole, 5 mg/kg (▲), or fluconazole, 2.5 mg/kg (●).

Table 3. Effect of ketoconazole and fluconazole on $^{14}CO_2$ exhalation rate–time profile following administration of [*N*-methyl-$^{14}$C]-antipyrine

| Pretreatment | Dose (mg/kg) | $CER_{max}$ (% dose/min) | CER half-life (min) |
|---|---|---|---|
| Fluconazole | 0.1 | 0.109 ± 0.012 | 132 ± 18 |
| | 0.5 | 0.098 ± 0.005 | 153 ± 5 |
| | 1.5 | 0.081 ± 0.009 | 181 ± 24 |
| | 2.5 | 0.089 ± 0.017 | 181 ± 16 |
| | 4 | 0.086 ± 0.005 | 178 ± 24 |
| | 8 | 0.076 ± 0.006 | 265 ± 21 |
| | 12 | 0.065 ± 0.007 | 274 ± 13 |
| | 20 | 0.076 ± 0.005 | 249 ± 24 |
| Ketoconazole | 1 | 0.086 ± 0.007 | — |
| | 3 | 0.086 ± 0.005 | — |
| | 5 | 0.065 ± 0.008 | — |
| | 10 | 0.049 ± 0.015 | — |
| | 15 | 0.040 ± 0.005 | — |
| | 20 | 0.030 ± 0.001 | — |
| | 30 | 0.032 ± 0.017 | — |
| | 50 | 0.018 ± 0.001 | — |
| Vehicle | — | 0.105 ± 0.009 | 121 ± 7 |

alone are shown in Fig. 3. As described previously [16, 17], in the control state the CER–time profile after [*N*-methyl-$^{14}$C]-antipyrine administration was characterised by a well-defined maximum occurring approximately 50 min after administration and a terminal phase which declines in a mono-exponential fashion with a half-life of approximately 2 hr. Administration of fluconazole did not alter the overall shape of the CER–time profile. However, the maximum CER attained and the half-life of decline were both altered and the extent of both effects became pronounced as the dose of fluconazole was increased. The decrease in $CER_{max}$ and increase in CER half-life with dose are shown in Table 3. This behaviour is consistent with progressive inhibition of antipyrine metabolism over the fluconazole dose range studied.

Ketoconazole also inhibited antipyrine metabolism in a dose-dependent fashion. However, administration of this inhibitor altered the shape of the CER–time profile; not only was the maximum CER attained decreased markedly but the peak broadens considerably. The decline in CER was not log-linear but concave. At the lower doses of ketoconazole (Fig. 3) the CER appeared to approach a mono-exponential decline at later time periods. This type of CER–time profile has been observed with other inhibitors, namely cimetidine and metyrapone [17]. It was proposed [23] that these distinct CER–time profiles are exhibited by inhibitors which are rapidly eliminated and have short half-lives. As the inhibitor is eliminated during the progress of the experiment, the inhibition effect gradually wears off until basal conditions are re-established. This hypothesis would also explain the present observations. Ketoconazole has been reported to have a 1 hr half-life in rats [25] whereas fluconazole has a longer half-life (4 hr [26]) and does not display the usual shaped CER–time profile.

In order to compare the *in vivo* inhibitory potency

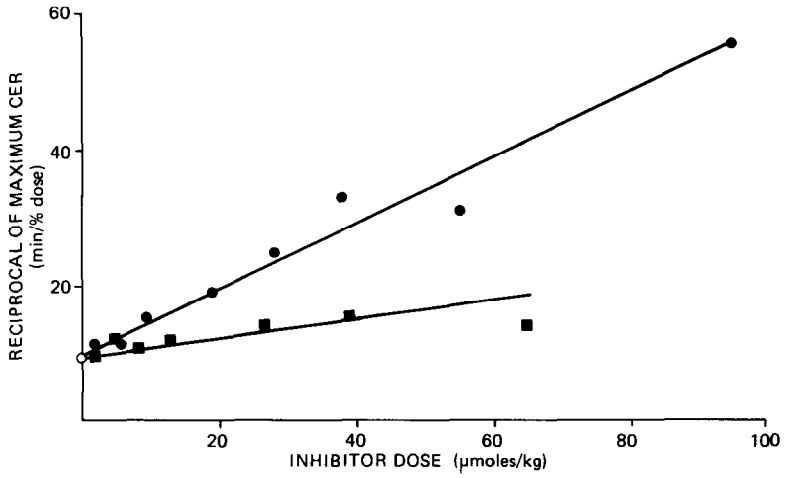


Fig. 4. Relationship between the reciprocal of the maximum $^{14}CO_2$ exhalation rate ($CER_{max}$) and the dose of ketoconazole (●) or fluconazole (■). Data from an animal which received no treatment with inhibitor, i.e. control is also shown (○).

Table 4. Comparison of inhibitory and efficacy potencies of antifungal drugs in rats *in vivo*

| | $ED_{50}$ doses (mg/kg) | | Relative selectivity ratio (inhibition vs fungal efficacy) |
|---|---|---|---|
| | Inhibition of $^{14}C$ antipyrine demethylation rate | Efficacy* against *Candida albicans* infection | |
| Ketoconazole | 9.2 | 4.9 | approx. 2 |
| Fluconazole | >20 | 0.2 | >100 |

* $ED_{50}$ dose at day 2 post-infection mg/kg: data from Richardson *et al.* [14].

of the two compounds the parameter $CER_{max}$ was selected. Previous studies have shown this parameter to be related to hepatic cytochrome P-450 activity [17]. Figure 4 is a plot of reciprocal of $CER_{max}$ vs molar dose. The slope for ketoconazole is steeper than fluconazole reflecting the greater inhibitory potency of the imidazole. The dose required to reduce the $CER_{max}$ by 50% (that is, double the reciprocal $CER_{max}$) was 17 μmol/kg (9.2 mg/kg) for ketoconazole whereas fluconazole produces only 30% inhibition of $CER_{max}$ at 60 μmol/kg (20 mg/kg), the highest dose-level examined. In view of the markedly lower dose-level of fluconazole required for *in vivo* efficacy (see Table 4), this data indicates that fluconazole exhibits a much greater selectivity ratio between antifungal efficacy and inhibition of drug metabolism than ketoconazole.

## DISCUSSION

Inhibition of cytochrome P-450 mediated sterol biosynthesis has been postulated as the mode of action for several antifungal agents [27–29]. However, such a mode of action can have implications for the toxicity profile of a drug since the compound may also inhibit steroid biosynthesis in the host by the same mechanism and inhibit the metabolism of co-administered drugs or endogenous substances. Imidazole antifungal agents, such as ketoconazole and miconazole, have recently been reported to alter mammalian cytochrome P-450 mediated reactions involved in hepatic drug metabolism [30, 31] and steroidogenesis in both adrenals [32, 33] and testis [34, 6, 12].

The present investigations have compared the ability of the imidazole, ketoconazole, and the triazole, fluconazole, to inhibit various rat hepatic cytochrome P-450 reactions. The data reported here demonstrate that relative affinity for fungal and mammalian hepatic cytochrome P-450 can be separated by structural modification of the antifungal agent. Thus whilst fluconazole is equipotent with ketoconazole as an inhibitor of cytochrome P-450-mediated demethylation of lanosterol in *Candida albicans* [24], it is substantially less potent against drug metabolism by hepatic cytochromes P-450 (see Table 1). A similar differential has been reported for the inhibition of cytochrome P-450-dependent C17,20-lyase in rat testis between ketoconazole and another triazole, itraconazole [36].

Studies described here with rat hepatic microsomes show ketoconazole to be approximately 5–15-fold more effective than fluconazole in the inhibition of three widely studied monooxygenases reactions. Methoxycoumarin *O*-demethylase and ethoxyresofurin *O*-deethylase activities are associated with phenobarbitone- and polycyclic aromatic hydrocarbon-inducible isozymes, respectively [37, 38]. Ethoxycoumarin *O*-dealkylase, activity is phenobarbitone and polycyclic aromatic hydrocarbon-inducible [38]. Both antifungal agents are more effective as inhibitors of the isoenzymes responsible for ethoxycoumarin *O*-dealkylase in uninduced rats which may indicate that the inducible isoenzymes are more sensitive to these compounds. These findings with ketoconazole confirm the previous reports of others [30] regarding the potent inhibitory action of this compound.

The relative selectivity of the two drugs to inhibit the different hepatic isozymes of cytochrome P-450 was further evaluated using [$^{14}C$]-testosterone as a substrate. Testosterone has been reported to exhibit regio- and stereo-specificity in the hydroxylations carried out by different hepatic constitutive cytochromes P-450 [39, 40]. Consequently, examination of the quantitative pattern of these metabolites produced in the presence of inhibitors can provide information on the relative selectivity of the inhibitor. These studies confirm that ketoconazole is, overall, a more potent inhibitor of constitutive hepatic cytochromes P-450 than fluconazole. Both compounds were apparently most potent as inhibitors of the isozyme catalysing 6β-hydroxylation of testosterone; ketoconazole being 200-fold more potent than fluconazole. This catalytic activity has been described as being predominantly due to cytochrome P-$450PB_{2a}$ in the rat [41]. Consequently this form may represent the major target for azole inhibition in this species. However, interesting differences in selectivity of action against other isozymes are evident. Ketoconazole showed significant inhibitory activity towards the production of 7α-OHT, which is reported to be catalysed predominantly by cytochrome P-$450_3$ in the male rat [41]. Fluconazole showed no inhibitory activity toward this isozyme. Differences in selectivity against other isozymes were less marked. The production of 16α-OHT and 2α-OHT has been described as being due to one form of P-450 in the male rat, the male-specific cytochrome P-$450_{2C}$ [41], consequently the overlap of inhibitory potential of ketoconazole for these two metabolites could be interpreted as an inhibition of this form of P-450. However, the lack of overlap of inhibition of these activities with fluconazole suggests that another isozyme of cytochrome P-450 is involved, and indi-

cates the need for caution in interpreting and assigning catalytic activities to specific isozymes.

The clear indication from these studies with testosterone is that consideration of overall inhibitory potential of azole drugs, using general substrates for cytochromes P-450, can mask very potent inhibition of specific isozymes. Thus, for instance, the $IC_{50}$ value for ketoconazole against overall metabolism of testosterone (100 $\mu$M) is markedly higher than the concentrations in plasma during therapeutic use of the drug (6–7 $\mu$M) [42]. However, the $IC_{50}$ value for the drug against isozyme P-450 $PB_{2a}$ (0.55 $\mu$M) is exceeded by 10-fold during therapeutic use. In contrast, fluconazole did not inhibit any activities within its predicted therapeutic range (10 $\mu$M, [26]).

The inhibitory action of both antifungal agents is evident *in vivo* using the CER–time profile obtained after [*N*-methyl-$^{14}$C]-antipyrine administration. Previous studies have shown CER–time profiles to be sensitive to a number of classic cytochrome P-450 inhibitors including cimetidine, metyrapone, SKF 525A and $\beta$-naphthoflavone [16]. In these investigations the parameter $CER_{max}$ proved to be consistently the most sensitive to inhibitory action. The CER half-life was also a useful index of inhibition providing that the half-life of the inhibitor was sufficiently long to ensure an adequate body load of drug. The half-lives of ketoconazole and fluconazole differ four-fold. Thus fluconazole, the longer half-life antifungal agent, produces a progressively prolonged CER half-life as the dose is increased. This change correlates well with the decrease in $CER_{max}$. Ketoconazole shows the concave decline in the CER–time noted previously with the short half-life compounds cimetidine and metyrapone. Figure 3 provides a useful comparison of the inhibitory potency *in vivo* of the two antifungal agents based on the $CER_{max}$ parameter. As with the microsomal studies ketoconazole is more potent, with an $ED_{50}$ at least 3 times lower on a molar basis than for fluconazole.

The pharmacokinetic properties of fluconazole, including high concentrations of unbound drug and a long plasma half-life [26], contribute to the superior *in vivo* efficacy of the drug, and thus, antifungal potency and selectivity should also be assessed *in vivo*. Fluconazole is up to 50-fold more potent than ketoconazole in various *in vivo* animal models of fungal infection [14], while the results of these studies using the antipyrine breath test show it to be less potent as an inhibitor of drug metabolism. These data indicate that fluconazole is, therefore, more than 100 times more selective than ketoconazole as an inhibitor of fungal versus mammalian cytochrome P-450. The relevance of these antipyrine studies in rats to clinical use of the azole drugs is illustrated by the observation that ketoconazole prolongs antipyrine elimination in human subjects at doses in the therapeutic range [9]. The results of our investigations comparing the selectivity of fluconazole and ketoconazole indicate that the propensity of fluconazole to inhibit cytochrome P-450-mediated drug metabolism in man would seem low, and indeed, early clinical investigations using an antipyrine test [43] support this view.

*Acknowledgements*—The authors wish to acknowledge the skillful technical assistance of Mr G. Disney in the completion of these studies, and to thank Mrs C. Deeley for careful typing of the manuscript.

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