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Comparative effects of two antimycotic agents, ketoconazole and terbinafine on the metabolism of tolbutamide, ethinyloestradiol, cyclosporin and ethoxycoumarin by human liver microsomes *in vitro*

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Two antimycotic agents, the azole ketoconazole and the allylamine terbinafine, have been examined for their effects on the metabolism of tolbutamide, ethinyloestradiol, cyclosporin and ethoxycoumarin by human liver microsomes ($n = 4$) *in vitro*. Ketoconazole caused marked inhibition of all enzyme activities with mean IC_{50} values (concentration producing 50% inhibition) of 17.9 μM (tolbutamide hydroxylase), 1.9 μM (ethinyloestradiol 2-hydroxylase), 2.0 μM (cyclosporin *N*-demethylase), 2.1 μM (cyclosporin hydroxylase) and 25 μM (ethoxycoumarin *O*-deethylase). At 50 μM terbinafine concentration, inhibition was less than 5% for tolbutamide and ethoxycoumarin, approximately 12% for both cyclosporin pathways and 35% for ethinyloestradiol. Terbinafine does not have the same inhibitory potential for cytochrome P-450 isozymes as ketoconazole.

Keywords antimycotics ketoconazole terbinafine human liver cytochrome P-450

Introduction

Both the azole (e.g. ketoconazole) and allylamine (e.g. terbinafine) antimycotics are potent inhibitors of fungal ergosterol synthesis. The mode of action of ketoconazole involves inhibition of a cytochrome P-450 mediated enzyme, lanosterol 14-demethylase (Van den Bossche *et al.*, 1978, 1983) whereas terbinafine exhibits strong inhibition of a non-cytochrome P-450 enzyme, squalene epoxidase (Schuster, 1985). Many of the azole antimycotics, including ketoconazole, in addition to inhibiting fungal P-450, also inhibit hepatic oxidative enzymes. This is because these compounds have readily accessible non-bonded electrons on a nitrogen atom, the imidazole 3-N, enabling them to bind (Type II interaction) to the ferric form of the haemoprotein as a sixth ligand (Sheets & Mason, 1984; Back & Tjia, 1985; Meredith *et al.*, 1985; Brown *et al.*, 1985; Sheets *et al.*, 1986; Lavrijson *et al.*, 1987; Back *et al.*, 1988). In contrast, terbinafine is a Type I substrate for a small portion of cytochrome(s) P-450 of hepatic microsomes (Schuster, 1987).

The present study was undertaken to compare the inhibitory potential of terbinafine and ketoconazole on the hepatic metabolism of tolbutamide (TOL), ethinyloestradiol (EE_2), cyclosporin A (CSA) and ethoxycoumarin (EC) using human liver microsomes *in vitro*.

Methods

Histologically normal livers were obtained from kidney transplant donors (three males; three females). Ethical approval for the study was granted and consent to removal of the liver was obtained from donors' relatives. Donor 1 was known to be a smoker and donor 6 to have received both phenobarbitone and phenytoin for management of epilepsy. Washed microsomes (105,000 *g* pellets) were prepared using the classical differential sedimentation method as previously described (Purba *et al.*, 1987). Cytochrome P-450 was assayed by the method of Omura & Sato (1964) and microsomal protein by the method of Lowry *et al.* (1951).

Tolbutamide 4-hydroxylase and ethinyloestradiol 2-hydroxylase activities were measured

using initial velocity conditions as previously described (Back *et al.*, 1988; Purba *et al.*, 1987).

Cyclosporin *N*-demethylase (to metabolite M21) and hydroxylase (to metabolite M17) activities were determined as follows: Incubations containing cyclosporin (CSA; 40 μM, a gift from Sandoz Pharmaceuticals, Basle), $MgCl_2$ (5mM), EDTA (1mM), KCl (1mM), NADPH (1mM), microsomal protein (3 mg) and 0.067M phosphate buffer (pH 7.4) to a final volume of 2.5 ml, were performed in Erlenmeyer flasks at 37° C with agitation for 10 min. CSA and metabolites were extracted into ether (6 ml) and quantified by h.p.l.c. Metabolites (M17; M21) were identified according to the retention times of the authentic standards (Sandoz). Separations were performed at 76° C on a Partisil ODS-3 (25 cm × 0.46 cm) column protected by an in-line guard column. The mobile phase used was acetonitrile:water (67:33) and the flow rate was 1.5 ml min^{-1}. The eluate was monitored by UV detection at 210 nm (Spectra-Physics UV/Vis detector). Initial velocity conditions were established for both metabolic pathways which were linear over 20 min and up to 4 mg protein. In experiments involving the antimycotics and CSA metabolism, radiolabelled CSA (^3H; 17 Ci $mmol^{-1}$, 0.2 μCi per incubation; 40 μM; Amersham International) was used and the metabolites were measured by determining the radioactivity in 0.5 min eluate fractions in 4 ml of scintillant (Aqua Luma Plus; LKB).

7-Ethoxycoumarin *O*-deethylase (ECOD) activity was determined under conditions previously shown to be linear with respect to time and protein concentration. Incubations containing ethoxycoumarin (10 or 100 μM), NADPH (1 mM), microsomal protein (150 μg) and 0.067 M phosphate buffer (pH 7.4) to a final volume of 2.5 ml were performed in glass tubes at 37° C with vigorous agitation for 15 min. Extraction and measurement of the product 7-hydroxycoumarin was performed as reported by Greenlee & Poland (1978).

Ketoconazole (a gift from Janssen) and terbinafine (a gift from Sandoz) in the concentration range 0.5–100 μM were dissolved in methanol which was evaporated to dryness before the addition of other reaction constituents. The percentage inhibition produced by the potential inhibitor was determined and where appropriate an IC_{50} value calculated (IC_{50} = concentration of inhibitor producing 50% inhibition).

Results

The microsomal protein and cytochrome P-450 contents of the six livers used in the study are shown in Table 1. Tolbutamide 4-hydroxylase,

Table 1 Enzyme activity of human liver microsomes

*Liver**	*Microsomal protein* (*mg g^{-1} wt*)	*Cytochrome P-450* (*nmol mg^{-1}*)	*Tol 4-OHase* (*nmol min^{-1} mg^{-1}*)	*EE_2 2-OHase* (*nmol min^{-1} mg^{-1}*)	*CSA NDase* (*pmol min^{-1} mg^{-1}*)	*CSA OHase* (*nmol min^{-1} mg^{-1}*)	*ECOD* (*nmol min^{-1} mg^{-1}*)
1 M 54	14.2	0.29	0.15	0.20	–	–	0.26
2 F 17	11.7	0.42	0.18	0.24	–	–	0.18
3 M 27	11.1	0.56	0.49	0.45	7.44	0.55	0.11
4 M 29	14.4	0.44	0.33	0.44	9.01	0.56	0.08
5 F 66	11.0	0.90	–	–	10.98	0.81	–
6 F 46	16.0	0.87	–	–	18.79	1.32	–

*The sex (M = male, F = female) and age of each donor is given.
Tol 4-OHase = Tolbutamide 4-hydroxylase; EE_2 2-OHase = ethinyloestradiol 2-hydroxylase;
CSA NDase = Cyclosporin *N*-demethylase; CSA OHase = Cyclosporin hydroxylase;
ECOD = ethoxycoumarin *O*-deethylase (low substrate concentration)

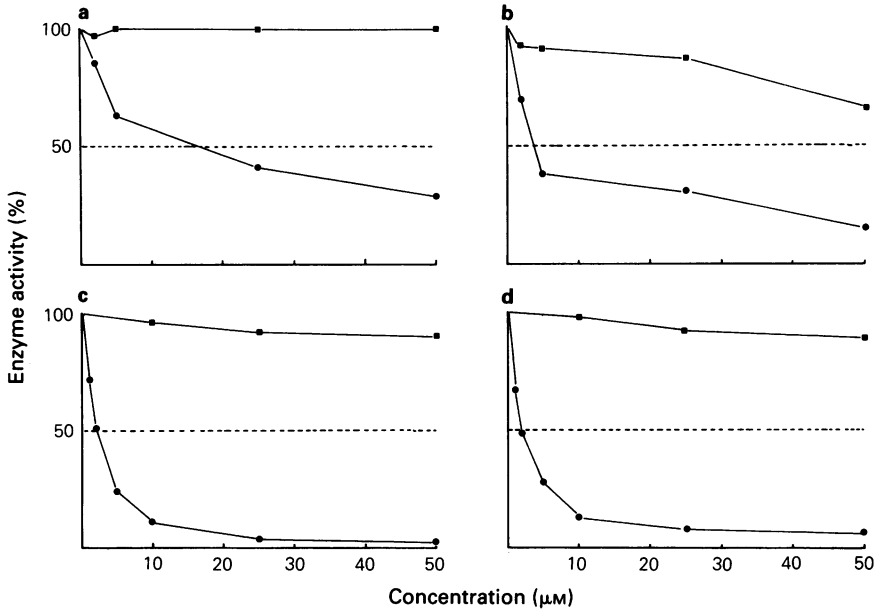

Figure 1 Effect of ketoconazole ●—● and terbinafine ■—■ on the metabolism of tolbutamide (a), ethinyloestradiol (b) and cyclosporin (to metabolite M17, c; to metabolite M21, d). Each value is the mean obtained from four different microsomal preparations.

ethinyloestradiol 2-hydroxylase, cyclosporin *N*-demethylase, cyclosporin hydroxylase and ethoxycoumarin *O*-deethylase activities are also shown. It is interesting to note that cyclosporin metabolism was highest in the liver of the donor who had received phenobarbitone and phenytoin and ethoxycoumarin metabolism (at low substrate concentration) was greatest in the liver from the known smoker. Ketoconazole caused marked inhibition of all enzyme activities with mean IC_{50} values of: 17.9 ± 9 μM (TOL), 1.9 ± 1.8 μM (EE_2), 2.0 ± 0.5 μM (CSA → M21), 2.1 ± 0.3 μM (CSA → M17) and 25 ± 3 μM (ECOD; low substrate). In contrast terbinafine up to a concentration of 50 μM (or 100 μM, CSA study), had a much smaller inhibitory effect. Inhibition was less than 5% for tolbutamide and ethoxycoumarin, approximately 12% for both CSA pathways and 35% for EE_2 (Figure 1).

Discussion

In this study ketoconazole has shown marked inhibition of a number of metabolic pathways which are probably catalysed by several different cytochrome P-450 isozymes. Whilst definitive proof i.e. activity of purified human P-450s or use of specific inhibitory antibodies with the microsomal fraction is the ideal approach to characterize the form of enzyme catalysing a particular oxidation, other lines of evidence are important. Based on inhibition studies it has been shown that tolbutamide hydroxylase is distinct from isozymes involved in the metabolism of theophylline, debrisoquine, nifedipine and antipyrine (Back *et al.*, 1988; Miners *et al.*, 1988). Knodell *et al.* (1987) have reported that tolbutamide is a substrate for the purified human liver cytochrome P-450 isozyme responsible for S-mephenytoin 4-hydroxylation. Recently Guengerich (1988) has argued that the enzyme responsible for EE_2 2-hydroxylation is $P\text{-}450_{NF}$. Evidence for this conclusion included results of studies using enzyme reconstitution, immuno-inhibition, correlation of activities and inhibitors. Purified $P\text{-}450_{MP}$ did not have catalytic activity towards EE_2. We have attempted to characterise the selectivity of the forms of P-450 towards both oestradiol (E_2) and EE_2 by using purified rat isozymes (Ball *et al.*, 1988). EE_2 appeared to be mainly metabolised by P450 isozymes from family P450IIC. Kronbach *et al.* (1988) have examined the metabolism of CSA to its three primary metabolites (M17, M21 and M1) and found metabolism to be inhibited in a dose-dependent manner by an antibody against a steroid-inducible P-450 (cytochrome P-450PCN) of rat liver. They concluded that the human isozyme must be similar or identical to $P450_{NF}$ ($P450IIIA_3$). The

final substrate we used, ethoxycoumarin, is metabolised (at least at high concentrations) substantially by isozyme(s) distinct from those responsible for the metabolism of ethoxyresorufin. Thus ethoxyresorufin metabolism, but not that of ethoxycoumarin, is inhibited by an antibody to a 3-MC-induced rat hepatic cytochrome P-450 and is also increased in the livers of cigarette smokers (Pelkonen *et al.*, 1986). Ryan *et al.* (1984) found that ethoxycoumarin was only poorly metabolised by purified rat cytochromes P-450, g and h. It is not clear what the major isozyme(s) responsible for ethoxycoumarin dealkylation are.

The findings of the present study with ketoconazole are therefore wholly consistent with this antimycotic being a potent general inhibitor of cytochromes P-450 due to the binding of the imidazole-3-N to the ferric form of the haemoprotein. The interaction of ketoconazole and cyclosporin has important clinical implications for the management of transplant patients (Cockburn, 1986; Ferguson *et al.*, 1982; Dieperink & Moller, 1982; Kronbach *et al.*, 1988). The elevation of CSA blood concentrations in transplant recipients given ketoconazole concurrently is clearly the result of inhibition of the major metabolic pathways (to M21 and M17, data shown; to M1, data not shown).

Terbinafine does not appear to have a general inhibitory effect on cytochromes P-450. The enzyme most inhibited was EE_2 2-hydroxylase (by 35% at 50 μM). It is possible that terbinafine is a substrate for the same isozyme(s) metabolizing EE_2 and the observation reflects competition for the substrate binding site. Further studies are necessary to clarify this point. The maximum plasma concentration following a 250 mg oral dose of terbinafine is 3 μM (unpublished observation).

The results of this work indicate that it is unlikely that the allylamines will cause clinically significant pharmacokinetic drug interactions *in vivo*.

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