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THE ACUTE AND SUBCHRONIC EFFECTS OF KETOCONAZOLE ON HEPATIC MICROSOMAL MONOOXYGENASES IN THE RAT

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Abstract—Female adult Wistar rats were treated with single or repeated doses of ketoconazole ranging from 10 mg/kg to 100 mg/kg. Single dose treatment produced inhibition of hepatic microsomal ethoxyresorufin O-deethylation (EROD) and aldrin epoxidation (AE) 2 hr after oral dosing. Twenty-four hours after a single dose, inhibition was still demonstrable after the low dose of 10 mg/kg, but at higher doses increased microsomal activity was apparent. After 7 days repeated dosing liver weight and microsomal protein content were increased in a dose-dependent fashion. EROD and AE were induced at all doses after repeated treatment when the increase in liver size was considered. These effects were seen at doses within the antimycotic therapeutic range and add support to the suggestion that reported drug interactions with ketoconazole in man are due to the effects of this drug on hepatic microsomal activity.

Ketoconazole, an orally active broad spectrum systemic antifungal drug, is rapidly absorbed and widely distributed within the body [1]. However, with widespread use in man, hepatotoxicity [2] has been described, as well as the demonstration that ketoconazole may interact with drugs undergoing microsomal monooxygenase metabolism.

N-Substituted imidazoles, including ketoconazole, depend partly for their antimycotic activity on the inhibition of P-450-dependent ergosterol synthesis in the fungal cell well [3]. Imidazoles also have inhibitory effects on mammalian cytochrome P-450 [4, 5]. *In vitro*, ketoconazole binds to cytochrome P-450 producing a type II difference spectrum and inhibits cytochrome P-450 activity as measured by a range of substrates with induced and non-induced hepatic microsomes [6–9]. *In vivo*, it prolongs methohexital-induced hypnosis [10] and inhibits aminopyrine and caffeine demethylation [11]. Four to seven days oral dosing in male Wistar rats has been shown to produce microsomal induction, but these studies showed that this occurred only with high doses, using non-specific substrates, and with no induction at dose levels comparable to those during therapy [12].

In man, the effects of 5 days treatment with ketoconazole on antipyrine clearance have varied [13, 14], and the reported decrease in chlordiazepoxide clearance was not mirrored by change in the rate of theophylline metabolism [15]. There are, however, clinical case reports of drug interaction with ketoconazole consistent with an effect of the drug on hepatic microsomal metabolism. Ketoconazole appears to inhibit cyclosporin A metabolism causing elevated serum levels and an increased risk of renal toxicity in man [16–18], as well as in both rats [19] and mice [20]. There has also been a report of potentiation of warfarin anticoagulation in man [21], although studies in two healthy volunteers had indicated no such appreciable change [22].

In this study the effect of single doses and 7 days repeated daily dosing with ketoconazole on rat hepatic microsomal monooxygenase activity was assessed *ex vivo* by the use of the specific substrates aldrin and ethoxyresorufin and including low dose treatment regimes.

MATERIALS AND METHODS

Chemicals. Ketoconazole was a gift from Janssen Pharmaceuticals. Aldrin and dieldrin were supplied by Shell Research Limited. 7-Ethoxyresorufin and resorufin were a gift from Dr. M. D. Burke (Marischal College, Aberdeen). Polyethylene glycol (PEG), molecular weight 200 was obtained from Sigma Chemicals Ltd.

Animals. Adult female pathogen free Wistar rats (150–200 g) were used in all experiments, housed in groups of four to six, exposed to a 12-hr artificial light cycle, fed a standard diet and allowed free access to food and water. Animals were killed by cervical dislocation prior to removal of the liver. Female rats were used as these appear to be more susceptible to the toxic effects of oral ketoconazole [23], an observation confirmed in our hands (unpublished) in both rats and hamsters.

Dosage. Ketoconazole was administered as a solution in PEG by gavage at 0900 hr as a single dose or daily for 7 days. Four to eight animals in each group were treated with ketaconazole at doses of 10 mg/kg, 50 mg/kg or 100 mg/kg and controls were untreated or given PEG alone. Livers were removed after 2 hr or 24 hr from the acutely dosed animals, and on the eighth day (24 hr after the oral dose) from those treated for 7 days and rapidly frozen at −80° and stored until analysis.

No difference was found in any of the measured

Table 1. Effects of ketoconazole in acute single doses and repeated daily doses for 7 days on liver weight and microsomal protein content

	10 mg/kg			50 mg/kg			100 mg/kg		
	2 hr (N = 4)	24 hr (N = 4)	7 days (N = 6)	2 hr (N = 6)	24 hr (N = 6)	7 days (N = 6)	2 hr (N = 6)	24 hr (N = 6)	7 days (N = 8)
Liver wt as % of body weight[a]	3.85 ± 0.22	3.45 ± 0.17	4.48 ± 0.13†††	3.48 ± 0.24	3.84 ± 0.10	4.84 ± 0.18†††	3.72 ± 0.16	3.97 ± 0.07	5.56 ± 0.39†††
Microsomal protein content in mg/g wet weight of liver as % of control	94.2 ± 10.7	90.1 ± 5.1	102.1 ± 1.7	98.6 ± 1.0	110.5 ± 2.6	99.6 ± 2.4	84.0 ± 3.3†	113.1 ± 5.1	120.1 ± 7.2†

[a] Control values for single dose groups = 3.74 ± 0.22% (N = 8) and for repeated dosage groups 3.31 ± 0.14% (N = 8).
[b] Control value for single dose groups = 17.8 ± 0.5 mg/g wet weight except 10 mg/kg acute dose when = 23.2 ± 1.0 mg/g wet weight, and for repeated dosage groups = 21.5 ± 1.0 mg/g wet weight.
† $P < 0.05$.
††† $P < 0.001$.
Values are mean ± SEM.

parameters between control rats given PEG alone or untreated control animals. The results of these two groups have therefore been amalgamated.

Microsome preparation. Prior to analysis a portion of liver was thawed at +4°, chopped finely with scissors and homogenised in ice-cold potassium chloride phosphate buffer (pH 7.25) using a Polytron homogenizer. The homogenate underwent differential centrifugation (5 min at 1000 *g* followed by 10 min at 8000 *g*) and the resulting supernatant was centrifuged at 105,000 *g* for 60 min to sediment the microsomal pellet. The pellet was resuspended in homogenisation buffer using a glass to glass homogeniser, and resedimented at 105,000 *g* for 60 min before resuspension in phosphate buffer containing 30% glycerol to a final protein concentration of approximately 3 mg/ml. Aliquots were stored separately at −80° until activity assessed. Protein content was determined by a modification of the method of Lowry [24] using bovine serum albumin as standard.

Activity measurement. Ethoxyresorufin O-deethylation (EROD) was measured by the continuous monitoring fluorimetric method of Burke and Mayer [25] with the following modifications. Reactions were carried out in a microfluorimetry cuvette at 37° in a final volume of 500 μl containing 250 μl of buffer (10 mM potassium dihydrogen phosphate pH 7.5 containing 0.8 mM NADPH), 250 μl 30% glycerol buffer pH 7.5 (containing 20–50 μg microsomal protein) and 2 μM ethoxyresorufin in dimethyl sulphoxide (1 μl) as substrate. Resorufin formation was continuously monitored and protein quenching of resorufin fluorescence was assessed. All assays were performed in duplicate. Rates of product formation were linear with respect to time and protein concentration.

Aldrin epoxidation (AE) was determined by the method previously described [26]. Incubations were carried out in triplicate. Five microlitres of microsomal protein (20–50 μg) was added to 95 μl phosphate buffer containing 0.8 mM NADPH and the reaction started by the addition of 2 μl aldrin in methanol. Following a 5 min incubation at 37° dieldrin formed was extracted into hexane and measured by electron capture gas chromatography.

Statistics. Results are expressed as group means and standard errors of the mean. Statistical significance between treated and control animals were assessed by Student's unpaired *t*-test.

RESULTS

Liver weight and microsomal protein content

Following single doses no significant increase was seen in either relative liver weight or microsomal protein content after 2 or 24 hr (Table 1). After 7 days treatment ketoconazole, at all three administered doses, produced dark discolouration of the livers as previously noted in mice [27], with no histological abnormality. There was a dose-dependent increase in both absolute and relative liver weight after one week's treatment with ketoconazole. By contrast, microsomal protein content (expressed as mg/g wet weight of liver) was significantly increased only in the high dose (100 mg/kg) group.

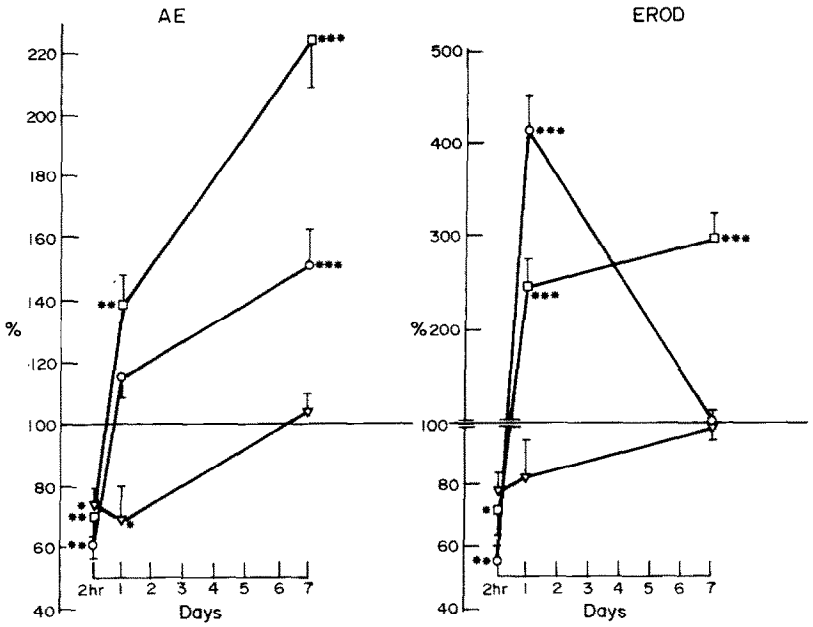

Fig. 1. The time course of the ketoconazole effect on hepatic microsomal activity as percentage of control values. Results are mean ± SEM. ▽, 10 mg/kg; ○, 50 mg/kg; □, 100 mg/kg; *P < 0.05; **P < 0.01, ***P < 0.001. Control activity for the acute animals are AE 2.32 ± 0.18 pmol/g wet wt/min and EROD 3.79 ± 0.46 pmol/g wet wt/min.

Monooxygenase activities

The monooxygenase activities were expressed both in relation to microsomal protein (pmol product formed/mg microsomal protein/min) and to liver weight (nmol of product formed/g wet weight liver/min) and in order to take account of the marked increase in liver weight after repeated dosing, relative to the whole animal (nmol product formed/100 g body weight/min).

Acute dosing of ketoconazole produced inhibition of activity of both EROD and AE at 2 hr at all three administered doses (Fig. 1). At 10 mg/kg EROD and AE activities were reduced to 78.1% and 74.1% of control whilst at the higher doses more pronounced inhibition was seen. Twenty-four hours following a single 10 mg/kg dose AE remained inhibited at 69% of control ($P < 0.05$). Although EROD was reduced to only 83% of control activity after 24 hr, this does not reach statistical significance. Twenty-four hours following doses of 50 mg/kg, EROD is markedly induced, whilst AE is comparable to control values. Both are significantly induced 24 hr following the high dose.

Seven days treatment with ketoconazole significantly induced AE activity at doses of 50 mg/kg and 100 mg/kg when all expressions of activity are considered (Table 2). In relation to microsomal protein the increase was 88% at 100 mg/kg and 51% at 50 mg/kg. However, taking into account liver weight and body weight (as a measure of overall metabolising capacity) the increases were 277% and 121% respectively at these doses. In the low dose group (10 mg/kg) there was no apparent induction when specific activity per mg microsomal protein or per g wet weight of liver was calculated, but when the increase in liver weight compared to body weight was taken into account there was a significant increase (20.9%) in the overall activity ($P < 0.01$). Ketoconazole produced a highly significant increase in EROD activity at the high dose (100 mg/kg). However, at 50 mg/kg and 10 mg/kg a significant increase was only seen when expressed in relation to liver size and body weight (Table 3).

Figure 1 reveals that AE activity shows time and dose dependent inhibition followed by induction. For EROD activity there is the surprising result at 50 mg/kg where induction at 24 hours exceeds that produced by 100 mg/kg although this difference is inapparent after 7 days.

DISCUSSION

This study has investigated the effect of ketoconazole given acutely and repeatedly for 7 days on rat hepatic microsomal activity using ethoxyresorufin and aldrin as substrates. A range of doses of ketoconazole from 10 mg/kg to 100 mg/kg were employed.

Ketoconazole has a biphasic effect on microsomal enzyme activity *in vivo*, with initial inhibition followed by subsequent induction. Inhibition is apparent even at the low dose of 10 mg/kg and persists for at least 24 hr. At higher doses induction becomes apparent after 24 hr. For aldrin metabolism the effect is clearly time and dose dependent. The effect of ketoconazole on ethoxyresorufin metabolism is more complex: at 24 hr induction after 50 mg/kg exceeds that produced by 100 mg/kg, but after 7 days treatment the inducing effect of 50 mg/kg does not persist. Calculation of enzyme activities with reference to

Table 2. Effect of 7 days treatment with ketoconazole on aldrin epoxidase activity

Enzyme activity	Control (N = 8)	10 mg/kg (N = 6)	50 mg/kg (N = 6)	100 mg/kg (N = 8)
nmol Product/g wet wt liver/min	2.82 ± 0.09	2.93 ± 0.20	4.26 ± 0.31†††	6.29 ± 0.41†††
nmol Product/100 g body weight/min	9.36 ± 0.53	13.16 ± 1.11††	20.68 ± 1.76†††	35.21 ± 3.46†††

†† $P < 0.01$.
††† $P < 0.001$.
Results are expressed as mean ± SEM.

Table 3. Effect of 7 days treatment with ketoconazole on ethoxyresorufin *O* deethylase activity

Enzyme activity	Control (N = 8)	10 mg/kg (N = 6)	50 mg/kg (N = 6)	100 mg/kg (N = 8)
nmol Product/g wet wt liver/min	2.36 ± 0.23	2.31 ± 0.10	2.40 ± 0.24	7.08 ± 0.55†††
nmol Product/100 g body wt/min	7.85 ± 0.87	10.41 ± 0.72†	11.73 ± 1.53†	38.95 ± 3.39†††

† $P = <0.05$.
††† $P < 0.001$.
Results are expressed as mean ± SEM.

microsomal protein in the induced state gives only an indirect measure of the induction of particular isozymes, and prominent induction of one isozyme may obscure lesser induction of another or even produce spurious inhibition of substrate metabolism by the lesser isozymes. This may explain the anomaly.

The persistence of inhibition for at least 24 hr at the low dose is probably due to the presence of ketoconazole in the liver at this time. Ritter and Franklin [28] have presented evidence that *N*-substituted imidazoles may persist in microsomal preparation beyond 24 hr after an oral dose. Presumably induction seen as early as 24 hr following a single dose represents a balance between inhibition and induction and may be a further explanation for the anomalous results at 24 hr with EROD. Similarly, levels of induction reported after 7 days treatment may be underestimated if ketoconazole is not cleared from the liver at the time of sacrifice.

Administration of all doses for one week clearly produced a dose-dependent increase in liver weight and microsomal protein content. Whilst significant induction of both ethoxyresorufin and aldrin metabolism is seen at the higher doses when specific activity/mg microsomal protein and per g wet weight of liver is calculated, when calculated with reference to liver size and body weight significant induction is apparent even at the lower does of 10 mg/kg which is within the antimycotic therapeutic range. This could represent a potentially significant pharmacological effect, and shows that analysis of enzyme activities with reference to liver size and body weight reveals effects on overall drug metabolising capacity that are obscured when expressed solely in relation to microsomal protein.

The inhibition demonstrated may be a relatively non-specific effect on all constitutive P-450 isozymes, as *in vitro* imidazole inhibition seems non-selective [4, 5]. However, it has been suggested that antifungal imidazoles produce greater inhibitory effects on phenobarbitone induced microsomes than 3 methylcholanthrene-induced microsomes [29, 30]. Subsequent induction may occur through a completely different mechanism. The substrates used in this study were chosen for their relative specificity, ethoxyresorufin as a marker for the aromatic hydrocarbon inducible fraction of cytochrome P-450 (P-450 *c* + *d*) in the rat [31, 32] and the homologous human isozymes [33], and aldrin as a marker of the phenobarbitone inducible fraction (P-450 *b* and *e*) [34]. Aldrin, however, is also metabolised by a number of additional isozymes to varying degrees [35], and the same is true of ethoxyresorufin [36]. Therefore we may have demonstrated induction of isozymes other than the classical polycyclic aromatic hydrocarbon or phenobarbitone-inducible forms. We have not measured cytochrome P-450 levels in our animals because of the insensitivity of this measure to change in individual isozymes. Preliminary studies in our laboratories using Western blotting techniques have indicated that there is no induction of P-450c after 7 days treatment with ketoconazole whereas immunoreactive P-450d is induced within 24 hours by higher doses. Studies of a wider range of isozymes is required to clarify the process further.

The reported interactions of ketoconazole with cyclosporin and warfarin are suggestive of inhibition of their hepatic metabolism. However, it has been claimed that ketoconazole has little if any effect on hepatic cytochrome P-450 at therapeutic doses in man [1]. Our results suggest that microsomal cyto-

chrome P-450 inhibition may be responsible for such interactions, whilst recognising the problems of extrapolating animal data to man. The usual recommended human dose is 4–8 mg/kg/day, although higher doses (up to 24 mg/kg/day) are used in severe fungal infections [37] and in the treatment of advanced prostatic carcinoma [38]. Peak serum levels after equivalent doses in man and rat are broadly similar. Thus, after a single 10 mg/kg dose in the rat a peak level is seen at about 2 hr of 12.9 ± 1.27 mg/ml with a plasma half-life of 1.08 hr [39]. In man following a single 200 mg dose (i.e. 4 mg/kg) peak levels range from 2.75 ± 1.78 to 6.90 ± 0.30 mg/ml after 2 hr, with a half-life of 2–3 hours [22, 40]. Cyclosporin metabolism is dependent on isozyme cytochrome P-450 3*c* in the rabbit, inducible by pregnenolone 16α carbonitrile [41]. The homologous isozyme in the rat (P-450*p*) is also an effective catalyst of the 10 hydroxylation of *R*-warfarin [31]. Whilst P-450*p* is age and sex dependent in the rat, not being expressed in the uninduced adult female, limited studies to date on human subjects have revealed the presence of an immunochemically related isozyme (HLP) and corresponding mRNA in female as well as male subjects [42, 43]. Inhibition of this isozyme might then explain the reported human interactions.

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