Induction Potential of Fluconazole toward Drug-Metabolizing Enzymes in Rats

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The induction of drug-metabolizing enzymes in rat liver was studied after subchronic administration of the new triazole antifungal agent fluconazole. The administered doses were 10, 40, and 160 mg/kg per day for 7 days. Fluconazole behaved as a high-magnitude inducer and significantly increased cytochrome P-450 concentrations already at 10 mg/kg (+42%). Cytochrome P-450 induction by fluconazole was dose dependent and reached a value of 302% of the control value at the dose of 160 mg/kg. The induction effects on cytochrome P-450 were also reflected in the drug-metabolizing enzyme activities in hepatic microsomes of pretreated rats. Fluconazole (160 mg/kg per day) preferentially induced the demethylase activities of N,N-dimethylaniline and p-nitroanisole to 258 and 281% of the control values, respectively. The detoxification enzyme UDP-glucuronosyltransferase was significantly lowered by fluconazole at the highest dose. A possible link between the induction potential and the pharmacokinetic properties of triazole antifungal agents is discussed.

Cytochrome P-450, a heme protein containing one molecule of iron-protoporphyrin IX as its prosthetic group, is the terminal oxidase of the hepatic microsomal mixed-function oxidase system. Cytochrome P-450 plays an important role in the biosynthesis and degradation of many physiologically important substances. Moreover, cytochrome P-450 enzymes hold a key position in the biotransformation of xenobiotics. The activity of many of the cytochrome P-450 isozymes can be substantially increased during treatment of animals and humans with or exposure of animals and humans to a large variety of xenobiotics. The multiplicity of cytochrome P-450 and the independent induction of specific forms of cytochrome P-450 have led to a classification of the inducers of these enzymes into six major families. Prototype inducers for each class are phenobarbital, 3-methylcholanthrene, isosafrole, pregnenolone-16α-carbonitrile, ethanol, and clofibrate. In general, cytochromes P-450 are much more susceptible to induction than are phase II enzymes (e.g., UDP-glucuronosyltransferases, sulfotransferases, and glutathion transferases), which may lead to an imbalance between toxification and detoxication pathways (27). Therefore, the introduction of new drugs with potent inducing properties might have serious clinical consequences for the disposition of coadministered drugs (5, 27). A failure of oral contraceptives after the treatment of women with the antibiotic rifampin (38) illustrates that the knowledge of the induction potential of new drugs is important.

It has been recognized for a long time that imidazoles and N-substituted imidazoles are inhibitors of cytochrome P-450-dependent enzyme activities. This property was exploited in the design of powerful antifungal agents for the management of a variety of fungal diseases and led to the introduction of ketoconazole, the first oral systemic antifungal agent, into clinical therapeutics (9). The mode of action of azole antifungal agents involves the inhibition of cytochrome P-450-mediated ergosterol synthesis, leading to deteriorated fungal membranes (44). The recently developed triazole antifungal agent itraconazole shares the same biochemical mechanism of action as the imidazoles but is

characterized by a much greater affinity for fungal cytochrome P-450 than for mammalian cytochrome P-450, resulting in an improved safety profile (14, 18, 44).

In addition to being inhibitors of cytochrome P-450, imidazole and imidazole derivatives possess inducing properties which generally become apparent after subchronic administration. Unsubstituted imidazole induced cytochrome form cyt P-450 IIE1, the ethanol-inducible isoenzyme, in rabbits but did not induce this form in rats (15, 32). The N-substituted imidazole antimycotic agent clotrimazole (75 mg/kg per day for 3 days) was a high-magnitude cytochrome P-450 inducer in rats and behaved as a mixed-type inducer, possessing inducing characteristics of both phenobarbital- and pregnenolone-16α-carbonitrile-type inducers (34). In a recent elegant study by Hostetler et al. (11), it was shown that various imidazole antifungal agents induce cytochromes P-450 of at least four different families by multiple mechanisms, one of which apparently involves stabilization of cytochrome P-450. In contrast to the high-magnitude induction by clotrimazole, we showed in a previous study that the triazole antifungal agent itraconazole (160 mg/kg per day for 7 days) was completely devoid of inducing or inhibiting properties in rats (17), which suggested a fundamental difference between imidazoles and triazoles in the ability to induce mammalian cytochromes P-450.

In the present study, we investigated the inducing properties of fluconazole, a new triazole antifungal agent. Fluconazole is a water-soluble, orally active bis-triazole, and it was of interest to study its inductive effects because liver weights were reported to increase in rats after subchronic treatment (40).

MATERIALS AND METHODS

Drugs and drug formulation. Fluconazole was from Pfizer and was dissolved in a solution of PEG 400 in 0.22 N HCl (1:4 [vol/vol], pH adjusted to 2.3 to 2.5) at final concentrations of 2, 8, and 32 mg/ml. Phenobarbital was obtained from Fluka, Buchs, Switzerland, and was dissolved in drinking water (0.1% [wt/vol]). Dexamethasone and 3-methylcholanthrene were obtained from Janssen Chimica, Geel, Belgium. Dexamethasone was suspended in water containing
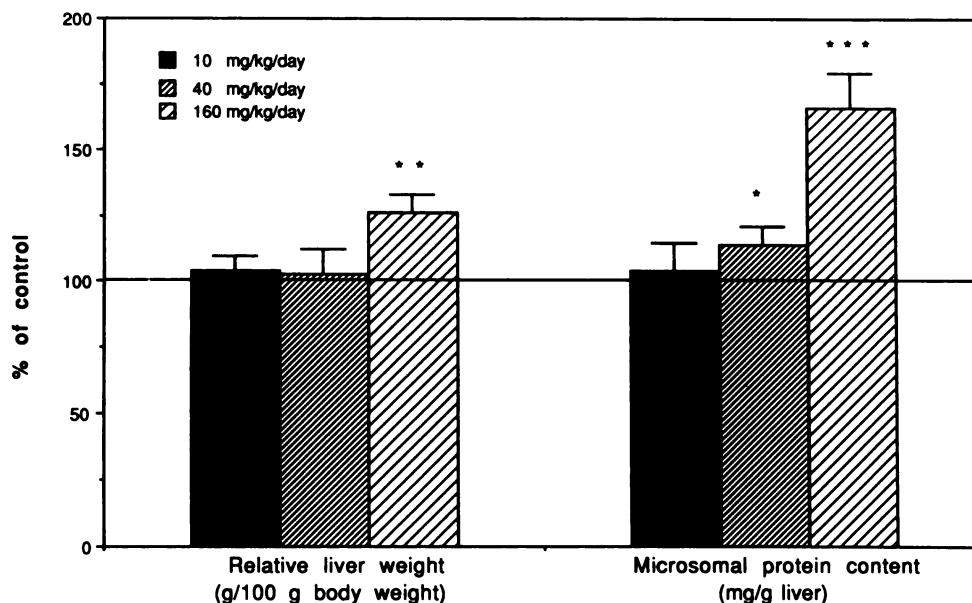
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FIG. 1. Effects of fluconazole treatment of male Wistar rats on relative liver weight and hepatic microsomal protein content. Rats were treated by gastric intubation at the dose level indicated once daily for 7 days. The results are presented as a percentage of the control (plus one standard deviation). Values are the means for four animals. $*$, $P \leq 0.05$; $**$, $P \leq 0.01$; $***$, $P \leq 0.001$.

2% Tween 80 at a final concentration of 30 mg/ml, and 3-methylcholanthrene was dissolved in sesame oil at a concentration of 4.7 mg/ml. The drug formulations were stored at room temperature during the period of drug administration.

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 plastic cages on wood bedding in a facility with a 12-h cycle of light and dark. They were fed a commercially available diet and had access to tap water ad libitum. These conditions were maintained for at least 1 week before treatment. The following doses were administered: fluconazole, 10, 40, and 160 mg/kg per day by gavage for 7 days; dexamethasone, 300 mg/kg per day by gavage for 4 days; and 3-methylcholanthrene, 20 mg/kg per day intraperitoneally for 4 days. Phenobarbital was administered in the drinking water (0.1% [wt/vol]) for 7 days. The dosages for fluconazole were the same as those used in a previous study with itraconazole (17). As is discussed later (see Discussion), it could be deduced from published areas under the curve (12) that in rats, the dose level of 10 mg/kg is comparable to therapeutic dose levels for fluconazole in humans. 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 to 24 h later.

Preparation of liver fractions. Liver microsomes were prepared by differential centrifugation as described previously (17). The suspensions were frozen in liquid nitrogen and stored at −80°C until analysis.

Determination of protein and cytochrome content. Protein concentrations were determined by the Lowry method (19), using the modification of Miller (22), with bovine serum albumin as the standard. Cytochrome P-450 and cytochrome b_5 content were determined by the methods of Omura and Sato (28, 29). 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. On the day of the experiment, glass tubes with microsomes were submerged in cold water (4°C) until melting became apparent and then transferred to the cold room to complete the thawing process. Incubations were done in a Heto shaking water bath at 37°C, under air, at 100 oscillations per min. All spectrophotometric determinations were performed with a Pye Unican SP8800/03 double-beam spectrophotometer equipped with a 4 × 4 automatic sample changer. Fluorimetric determinations were carried out with a Perkin-Elmer LS-SB spectrofluorimeter. Aniline hydroxylase activity was measured by the formation of p-aminophenol (13) as described previously (17). The N-demethylation of N,N-dimethylaniline was measured by the production of formaldehyde with the Nash reagent (24) as described previously (17). The O-demethylation of p-nitroanisole was determined by the method of Netter and Seidel (26), and UDP-glucuronosyltransferase activity with 4-nitrophenol as the substrate was determined by a modification of the method described by Dutton (7), as described previously (17). NADPH-cytochrome c-reductase activity was determined by the method of Mazel (21).

Statistical analysis. The group means and standard deviations were calculated and the statistical significance between treated and control animals was evaluated by using an unpaired Student t test. Differences were claimed to be significant at the $P \leq 0.05$ level, using the two-tailed t distribution.

RESULTS

Effects on hepatic parameters associated with drug-metabolizing enzymes. Administration of seven daily doses of fluconazole (160 mg/kg) resulted in a significant increase of the relative liver weight, up to 126% of the control value

TABLE 1. Effects of oral administration of reference compounds on components of hepatic drug-metabolizing enzyme system in rats[a]

Parameter[b]	Values (mean ± SD for four determinations) obtained with:					
	Control	Phenobarbital (0.1% in drinking water for 7 days)	Control (sesame oil)	3-Methylcholan- threne (20 mg/kg per day for 4 days, i.p.[c])	Control (2% Tween)	Dexamethasone (300 mg/kg per day for 4 days, i.p.)
Relative liver wt (g/100 g of body wt)	3.37 ± 0.15	5.80 ± 0.88***	3.57 ± 0.15	4.50 ± 0.37***	3.48 ± 0.27	8.17 ± 0.95***
Microsomal protein (mg/g of liver)	16.6 ± 0.3	18.8 ± 0.1***	22.7 ± 0.1	25.4 ± 0.2***	25.4 ± 0.2	23.3 ± 0.1***
Cytochrome P-450						
Maximum (nm)	449.1 ± 0.2	449.5 ± 0.3*	449.3 ± 0.4	447.8 ± 0.1***	449.7 ± 0.2	448.3 ± 0.6**
nmol/mg of protein	0.903 ± 0.018	1.438 ± 0.044***	0.914 ± 0.013	1.874 ± 0.014***	0.895 ± 0.015	1.356 ± 0.26***
Cytochrome b_5 (nmol/mg of protein)	0.447 ± 0.003	0.644 ± 0.013***	0.453 ± 0.002	0.659 ± 0.003***	0.449 ± 0.002	0.402 ± 0.006***
NADPH-cyt c-reductase (nmol/mg of protein per min)	90.0 ± 3.9	140 ± 6***	97.4 ± 9.1	69.4 ± 2.4***	90.0 ± 0.0	160 ± 13***
Aniline hydroxylation (nmol/ mg of protein per min)	0.97 ± 0.09	0.99 ± 0.05	0.81 ± 0.05	1.26 ± 0.06***	0.84 ± 0.03	0.69 ± 0.11*
N,N-DMA N-demethylation (nmol/mg of protein per min)	3.33 ± 0.15	9.27 ± 0.76***	2.95 ± 0.48	3.37 ± 0.18	2.85 ± 0.20	4.31 ± 0.26***
p-NA O-demethylation (nmol/ mg of protein per min)	3.35 ± 0.09	6.64 ± 0.46***	3.45 ± 0.17	27.2 ± 1.1***	2.84 ± 0.13	5.01 ± 0.12***
UDP-glucuronyltransferase (nmol/mg of protein per min)	1.76 ± 0.18	4.73 ± 0.13***	3.41 ± 0.07	10.2 ± 0.3***	3.40 ± 0.18	2.81 ± 0.15**

[a] *, $P \le 0.05$; **, $P \le 0.01$; ***, $P \le 0.001$.
[b] N,N-DMA, N,N-dimethylaniline; p-NA, p-nitroanisole.
[c] i.p., Intraperitoneally.

(Fig. 1). Microsomal protein content was increased after treatment with fluconazole at 40 and 160 mg/kg (+14% and +66%, respectively). The relative liver weight was also significantly enhanced by the classical inducers phenobarbital, 3-methylcholanthrene, and dexamethasone (Table 1). On the other hand, hepatic microsomal protein content was elevated after phenobarbital and 3-methylcholanthrene treatment but significantly lowered after dexamethasone treatment (Table 1).

Effects on components of the cytochrome P-450 catalytic cycle. The potential of drug-drug interaction at the level of

drug metabolism does not depend exclusively on changes in the content of the terminal oxidase cytochrome P-450. The activities of other enzymes of the cytochrome P-450-linked drug-metabolizing enzyme system, i.e., cytochrome b_5 and NADPH-cytochrome c-reductase (8), can also alter after subchronic drug administration; therefore, the activities of these enzymes were also determined.

Figure 2 shows the effects of subchronic administration of fluconazole on hepatic cytochrome P-450, cytochrome b_5, and NADPH-cyt c-reductase. Fluconazole behaved as a potent inducer of cytochrome P-450. After seven daily doses
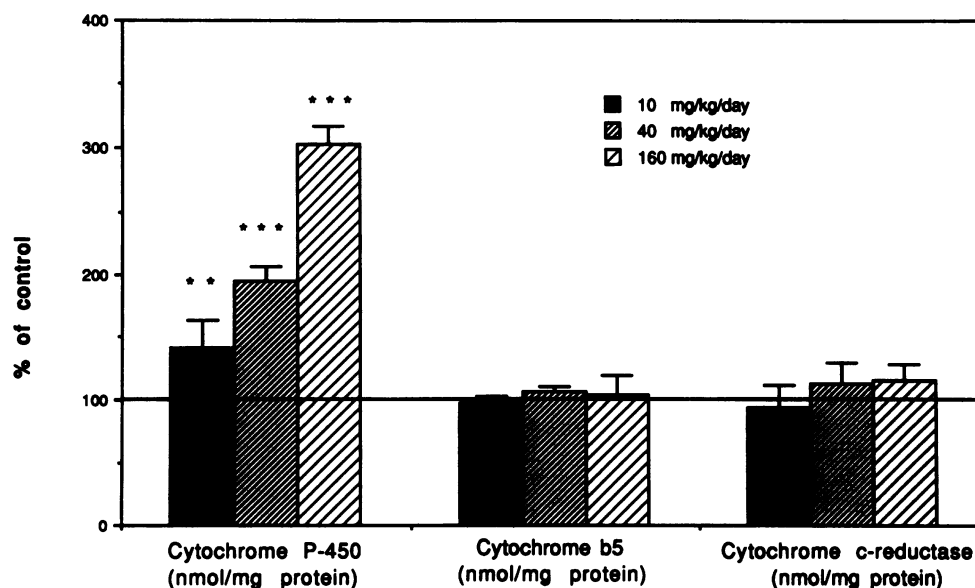

FIG. 2. Effects of fluconazole on cytochrome P-450, cytochrome b_5, and cytochrome c-reductase in hepatic microsomes of rats. Treatment conditions and representation are as described in the legend to Fig. 1.
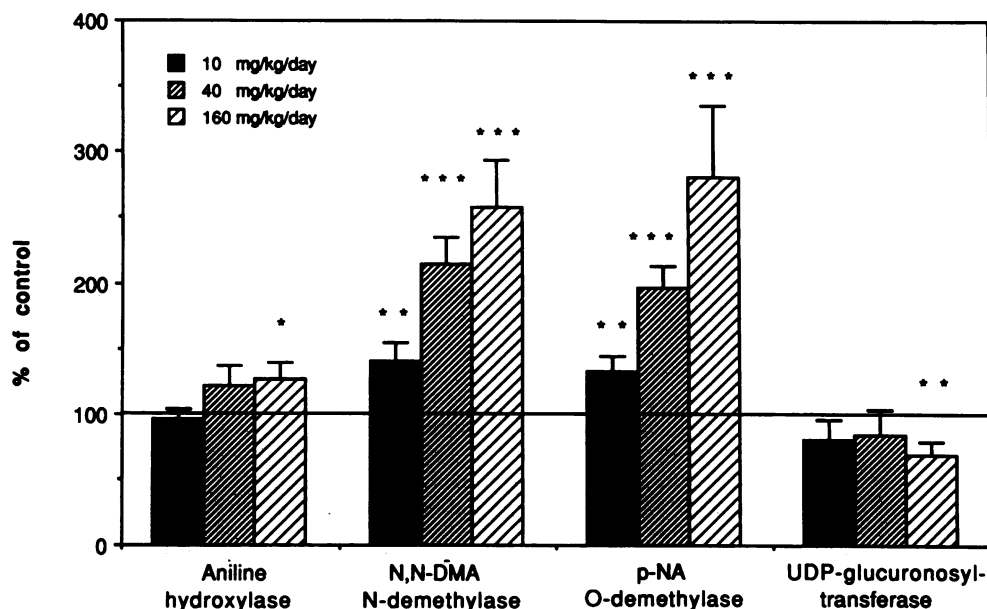
FIG. 3. Effects of fluconazole on enzymatic activities (expressed as nanomoles of product formed per milligram of protein per minute) in hepatic microsomes of rats. Treatment conditions and representation are as described in the legend to Fig. 1.

of 10 mg/kg, cytochrome P-450 was already significantly induced to 142% of the control value. The induction was dose related and augmented up to 302% of the control value at the dose of 160 mg/kg. On the other hand, no induction of the cytochrome b_5 content and the NADPH-cyt c-reductase activity was found. No significant changes were noted on the absorbance maximum of the complex of CO with dithionite-reduced cytochrome P-450 after treatment with fluconazole. In accordance with the literature, 3-methylcholanthrene and dexamethasone caused a blue shift in absorbance maximum (4, 39) and 3-methylcholanthrene did not simultaneously induce NADPH-cytochrome c-reductase (41).

Effects on hepatic drug-metabolizing enzymes. Induction of cytochrome P-450 can be associated with an alteration of the cytochrome P-450 isoenzyme composition in the microsomes. Such a change is reflected in the substrate specificity of the microsomal enzymes after drug pretreatment. The effects of subchronic treatment with fluconazole on enzyme activities are represented in Fig. 3. As expected from the results described above, fluconazole strongly induced in a dose-dependent way N,N-dimethylaniline N-demethylase and p-nitroanisole O-demethylase activities. Significant induction of N-demethylase activity started already at 10 mg/kg per day, and at 160 mg/kg per day, induction augmented to 258 and 281% of the control values for the N-demethylase and O-demethylase activities, respectively. Because of the significant increase in microsomal protein content (Fig. 1), induction even reached values of 425 and 462% when N-demethylase and O-demethylase activities were expressed per gram of liver. Phenobarbital preferentially induced N-demethylase activity, and 3-methylcholanthrene preferentially induced O-demethylase activity, whereas dexamethasone almost equally induced N- and O-demethylase activities and significantly lowered aniline hydroxylase activity (Table 1). UDP-glucuronosyl-transferase, a phase II enzyme, was significantly lowered by fluconazole at 160 mg/kg per day.

DISCUSSION

Previous studies have shown that imidazoles constitute a heterogeneous group of enzyme inducers. Differences between various imidazoles regarding their induction potential (17, 33) and the mechanism of induction (11) as well as the spectrum of enzyme activities which were induced (17, 33) were found. In the present study, it was found that the water-soluble antifungal drug fluconazole behaved as a high-magnitude inducer, which already significantly induced cytochrome P-450 at doses as low as 10 mg/kg per day for 7 days. The present study shows that differences between various triazoles in their abilities to induce cytochrome P-450 also exist. It extends the results of a previous study which showed that itraconazole, another lipophilic triazole, was completely devoid of inducing and inhibiting properties after subchronic oral administration (160 mg/kg per day for 7 days) to rats, even when a broad spectrum of enzyme activities was measured (17). The in vitro results were confirmed by in vivo experiments, showing that itraconazole did not influence the metabolism of a large number of drugs including antipyrine (10), phenytoin, zoxazolamine, tolbutamide, and dicoumarol (6). The in vivo effects of fluconazole on coadministered drugs have rarely been investigated, although a recent study showed that fluconazole (20 mg/kg per day for 5 days) interfered with methohexital hypnosis in rats as a consequence of enzyme induction (2).

The mechanism at the basis of the difference in induction potential between fluconazole and itraconazole is unknown. Induction of hepatic cytochrome P-450 is generally obtained with compounds that attain a high, sustained intracellular concentration in the liver. Multiple biochemical events can initiate a complex cellular mechanism resulting in an elevation of the drug-metabolizing capacity of the cell. These include an interaction with a cytosolic receptor, an adaptive response to an overload or inhibition of the drug-metabolizing enzyme system, or the binding to a cytochrome P-450 form(s) involved in the metabolism of an endogenous sub-

stance that is involved in the regulation of cytochrome P-450 gene expression. Any of these parameters can be affected by differences in the pharmacokinetics of the compounds under study. Both fluconazole and itraconazole are characterized by a good oral and dose-proportional absorption, but the antifungal agents have distinct pharmacokinetic properties. Whereas fluconazole is not extensively bound to tissue or plasma proteins (12), itraconazole has an extensive tissue distribution, with tissue concentrations many times higher than those in plasma and a high plasma protein binding (10). For fluconazole, the volume of distribution approximates that for the total body water; therefore, levels in plasma (approximately 7 μg/ml at 10 mg/kg [12]) are comparable to levels in tissue. For itraconazole, liver concentrations of about 6.5 μg/g of liver were obtained (42) in rats after an oral dose of 10 mg/kg. Therefore, the difference in induction between both antifungal agents does not reside in a difference in hepatocyte concentrations between the drugs. The half-lives in rats are 4.0 h for fluconazole (12) and 5.9 h for itraconazole (42) and thus also cannot account for the differences in induction potential between the antifungal agents. On the other hand, fluconazole is a highly hydrophilic drug, whereas itraconazole is a lipophilic drug. This might result in high cytosolic concentrations of fluconazole, increasing the probability of an interaction of fluconazole with a receptor. Itraconazole is distributed into the cellular membranes, decreasing the probability of interaction with such a receptor. Binding to a cytosolic receptor has been implicated for inducers belonging to the polycyclic hydrocarbon (25) and pregnenolone-16α-carbonitrile (36) families but also in the induction by 1-benzylimidazole (20). Induction of cytochrome P-450$_c$ by the latter compound involved the binding to a receptor with only low efficiency. This could also be valid for fluconazole, which showed only a 2.5-fold induction of the p-nitroanisole O-demethylase activity, whereas with 3-methylcholanthrene, a 7-fold induction was obtained.

Another major difference between both antifungal agents is the extensive metabolism of itraconazole (10), in contrast to fluconazole, which is excreted largely unmetabolized (12). This is in accordance with another hypothesis which states that inducers are poor substrates of constitutive cytochrome P-450 isoenzymes and, as a consequence, can uncouple the monooxygenase reaction and generate activated molecular oxygen, which in fact represents the true inducer (1). On the other hand, the high-magnitude inducers clotrimazole and 1-benzylimidazole are both also capable of potent, long-term inhibition of cytochrome P-450, suggesting that a sort of feedback mechanism is operative. Itraconazole was found to be devoid of inhibitive properties in rat liver microsomes against a variety of cytochrome P-450-catalyzed enzymic reactions in vitro (17, 18) and in vivo (6, 10). Although some investigators claimed that fluconazole had only weak inhibitive effects in rats (31), others have shown a potent inhibition of the antipyrine metabolism in mice (16) and a strong inhibition of tolbutamide hydroxylase activity in human liver microsomes (3). In vivo, fluconazole was also a potent inhibitor of phenytoin disposition in patients (23). Therefore, fluconazole displays the same biphasic effect common to many inhibitors of the mixed-function oxidase system: an inhibitory phase followed by a phase of induction. The clinical interactions with such compounds are difficult to predict. If the coadministered drug is a substrate for the induced cytochrome(s) P-450, its concentration in plasma will decrease. However, if the cytochrome(s) P-450 involved in the metabolism of the drug is decreased or if the inducer

is also an inhibitor of the induced cytochrome(s) P-450, the levels of the coadministered drug in plasma may rise. In the latter case, any inductive effect in the clinical situation might be obscured as long as the concentration of the inducer remains high (34). Differences in the inhibitive properties of fluconazole and itraconazole might be related to structural differences between both molecules. For itraconazole, the combination of the triazole moiety, which can interact with the heme group of cytochrome P-450, and the bulky lipophilic substituent, which can bind to the apoprotein and influence the orientation of the triazole versus the heme center, was thought to contribute to its low inhibitory effect on cytochrome P-450 (18). Thus, the induction potential of fluconazole could also originate from its potent inhibitive properties towards cytochrome P-450. Whether the profound inhibition results in a simple adaptive response to the overload of the enzyme system or whether, in this mechanism, the inhibition of the metabolism of an endogenous factor that initiates the biochemical process of enzyme induction is involved remains speculative. Ritter and Franklin (33) failed to observe a clear relationship between the inductive and inhibitive properties of different imidazole antifungal agents. However, differences in the extent of absorption and rate of metabolism of the different imidazoles under study could have obscured any relation between both interactions with the cytochrome P-450 system. For clotrimazole, it was shown that the antifungal agent decreased the degradation of cytochrome P-450$_p$, probably by binding to the cytochrome by a mechanism which did not involve the formation of a stable metabolite complex (11). Fluconazole and itraconazole can both bind to cytochrome P-450 and form type II difference spectra (18, 37). Therefore, induction of cytochrome P-450 by fluconazole as a result of stabilization of cytochrome P-450 through heme binding seems unlikely.

Apart from the existence of large differences in the potential of drug-inducing properties, various imidazoles also possess distinct properties regarding the type of enzyme activities (both phase I and phase II) they induce (17, 33); so miconazole, in contrast to ketoconazole, behaved predominantly as a phenobarbital-like inducer (17). Tioconazole, on the other hand, preferentially induced UDP-glucuronosyltransferase activity towards 4-nitrophenol (33). Clotrimazole was classified as a mixed-type inducer, displaying characteristics of both the P-450IIB (phenobarbital-inducible) and P-450III (pregnenolone-16α-carbonitrile-inducible) families of cytochrome P-450 inducers (34, 35), whereas 1-benzylimidazole exhibited both polycyclic aromatic hydrocarbon- and phenobarbital-type induction (20, 30). In the present study, it is shown that the triazole fluconazole also behaves as a mixed-type inducer that strongly induced N,N-dimethylaniline N-demethylase activity, preferentially induced by phenobarbital (Table 1), as well as p-nitroanisole O-demethylase activity, preferentially induced by 3-methylcholanthrene (Table 1). In addition, fluconazole significantly lowered UDP-glucuronyltransferase activity, a feature shared with dexamethasone (Table 1) but not with clotrimazole, which had little effect on UDP-glucuronosyltransferase but decreased sulfotransferase (33). The imbalance between toxification and detoxification enzymes created by fluconazole might have consequences for the toxicity of coadministered drugs.

Significant induction of cytochrome P-450 and cytochrome P-450-dependent enzyme activities in rats already occurred at a fluconazole dose as low as 10 mg/kg per day. The area under the curve in rats after an oral dose of 20 mg/kg was 152

$\mu g \cdot h/ml$ (12). In humans, it was 43 $\mu g \cdot h/ml$ after a dose of 1 mg/kg (12), which means that the dose of 10 mg/kg in rats, used in the present study, is comparable to therapeutic dose levels in humans with respect to the exposure of the liver to the drug. Furthermore, the half-life of fluconazole in humans (22 h) exceeds by far that in rats (4 h) (12); this might amplify any inducing effect. Therefore, the present findings might have therapeutic implications, especially since fluconazole seems to have an effect on the balance between phase I and phase II enzymes.

It may be concluded that fluconazole induced cytochrome P-450 and cytochrome P-450-dependent enzyme activities in rats at doses comparable to therapeutic doses in humans. The present study shows that fluconazole at 10 mg/kg behaves as a potent inducer of cytochrome P-450, unlike itraconazole, which does not induce with doses of up to 160 mg/kg per day (17). This indicates that itraconazole is much more selective than fluconazole in its in vivo effects against cytochrome P-450, since itraconazole does not affect mammalian cytochrome P-450 but at the same time strongly interacts with fungal cytochrome P-450, leading to deteriorated fungal membranes (43, 44). This can be attributed to its high specificity for fungal cytochrome P-450 and its unique pharmacokinetic properties.

ACKNOWLEDGMENTS

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