Fluconazole: A Potent Inhibitor of Cytochrome P-450-Dependent Drug-Metabolism in Mice and Humans *in Vivo*. Comparative Study with Ketoconazole

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The inhibitory effect of fluconazole (FCZ), a bis-triazole antimycotic, on mouse hepatic microsomal cytochrome P-450-mediated drug-metabolizing enzyme system was compared with those of ketoconazole (KCZ) *in vivo* and *in vitro*. Additionally, the change in the hepatic oxidative drug-metabolizing capacity in humans treated with FCZ was followed. The pentobarbital sleeping time in mice given a single dose of 1—10 mg/kg of FCZ or 30—50 mg/kg of KCZ was prolonged significantly, and the potency of FCZ for the prolongation of sleeping time was greater than that of KCZ. In contrast, *in vitro* the affinity and the inhibitory potency of FCZ for cytochrome P-450 and aminopyrine *N*-demethylation were 4- to 6-fold smaller than those of KCZ. However, the order of the inhibitory potencies among antimycotics for this enzyme system *in vitro* was reversed by the addition of albumin into the reaction mixture. These results indicate that the difference in the plasma protein binding properties between FCZ and KCZ is an important factor which leads to a reverse in the order of their inhibitory potencies for this enzyme system *in vitro* and *in vivo*. The ratio of 6β-hydroxycortisol (6β-OHF) to cortisol (F) in urine, used as an indicator of oxidative drug-metabolizing capacity in humans, decreased to 50% of the original level during treatment with 200 mg/d of FCZ. These findings indicate that the inhibitory effect of FCZ on hepatic microsomal cytochrome P-450-mediated drug-metabolism *in vivo* was unexpectedly potent from its potential obtained *in vitro* experiments in the absence of albumin, and that FCZ could be a potent inhibitor for hepatic oxidative drug-metabolism in humans.

Keywords fluconazole; ketoconazole; antimycotic; cytochrome P-450; drug-metabolizing enzyme

Introduction

Recently, many azole derivatives have been developed to inspect their systemic antifungal efficacies. Ketoconazole (KCZ), an imidazole derivative, is a typical agent administered orally. It has been considered that KCZ is a universal inhibitor of cytochrome P-450 enzymes,[1] since it inhibits a number of cytochrome P-450-dependent steroidogeneses in adrenal and testicular tissues.[1–4] In addition, it has also been shown that KCZ inhibits the hepatic microsomal cytochrome P-450-dependent enzyme system[5–9]: that is, KCZ affects the clearance of some co-administered drugs in humans, *e.g.*, cyclosporin, antipyrine and warfarin.[10–14]

Fluconazole (2-[2,4-difluorophenyl]-1,3-bis[1*H*-1,2,4-triazol-1-yl-propan-2-ol]) (FCZ) is a new bis-triazole systemic antimycotic. Although it has been confirmed that FCZ is considerably more potent in model fungal infection than KCZ,[15,16] there is no agreement in the literature as to the relative inhibitory potency between FCZ and KCZ for the hepatic microsomal cytochrome P-450-mediated drug-metabolizing enzyme system *in vitro* and *in vivo*: that is, Houston *et al.*[17] reported that FCZ had a lower propensity to interact with rat hepatic cytochrome P-450 than KCZ, and in contrast, Delfa *et al.*[18] reported that the inhibition of FCZ on this enzyme system *in vivo* in mice was more potent than that of KCZ.

In this paper, we focus on the objects as follows: to confirm the potency of the inhibitory action of FCZ on hepatic microsomal cytochrome P-450-mediated enzyme system in comparison with KCZ *in vivo* and *in vitro*; and to determine whether or not treatment with FCZ affects the hepatic oxidative drug-metabolizing capacity in humans.

Materials and Methods

Materials FCZ and KCZ were kindly supplied by Pfizer Pharmaceutical Co., Ltd., Tokyo and Kyowa Hakko Co., Ltd., Tokyo, respectively. FCZ capsules (Diflucan® capsule, 100 mg/capsule) were obtained commercially. Aminopyrine and pentobarbital sodium were purchased from Nacalai Tesque Inc., Kyoto and human serum albumin (essentially fatty acid free grade) was purchased from Sigma Chemical Co., Ltd., St. Louis, MO. All other chemicals and solvents were of analytical grade.

Treatment of Animals Male ddY mice (4—6 weeks old) were used. FCZ and KCZ were dissolved in saline containing 0.1 N HCl (final pH adjusted to 3.0 with 0.1 N NaOH), and 0.3—10 mg/kg of FCZ or 10—50 mg/kg of KCZ was injected intraperitoneally in a single dose. At the same time the control animals were treated with the vehicle alone. Except for the groups in animals used for the measurement of pentobarbital sleeping time, the mice were killed by decapitation of 6 h after the administration and the livers were perfused with ice-cold 1.15% KCl solution *in situ* to remove the blood.

Measurement of Pentobarbital Sleeping Time Pentobarbital sodium dissolved in saline (40 mg/kg) was injected intraperitoneally 0.5, 2, 4, or 6 h after treatment with a single dose of FCZ, KCZ or vehicle alone. Sleeping time was determined as the delay between loss and recovery of the righting reflex.

Preparation of Mouse Hepatic Microsomes Livers from the intact mice or the antimycotics-pretreated mice described above were homogenized in ice-cold 1.15% KCl (1 : 4, w/v) and the homogenate was centrifuged at $10000 \times g$ for 15 min. The supernatant fraction was centrifuged at $105000 \times g$ for 60 min to obtain a microsomal pellet. The microsomes were suspended in 0.1 M sodium/potassium phosphate buffer, pH 7.4, to a concentration of 2—5 mg protein/ml. Protein was determined by the method of Lowry *et al.*[19]

Biochemical Analysis Spectrophotometric determination of the binding of antimycotics and pentobarbital sodium to cytochrome P-450 was carried out as follows: a microsomal suspension containing 2 mg protein/ml 0.1 M sodium/potassium phosphate buffer, pH 7.4, were divided into a sample and a reference cuvette. After recording the base line using a double beam spectrophotometer (Shimadzu, Model UV-300), the samples were titrated with an ethanol solution of FCZ or KCZ or 0.1 M sodium/potassium phosphate buffer (pH 7.4) solution of pentobarbital sodium. An equivalent volume of the solvents was added to the reference cuvette. Spectra were recorded between 360 and 500 nm and the absorbance peak minus trough values were used to construct double-reciprocal plots.

Aminopyrine *N*-demethylation was determined by measurement of formaldehyde according to the procedure of Nash.[20] The assay mixture contained a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (0.5 mM NADP, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$ and 2 units of glucose-6-phosphate dehydrogenase), 77.5 mM sodium/potassium phosphate buffer (pH 7.4), microsomal preparation (0.5 mg/ml), 5 mM aminopyrine as a substrate, and in some tubes human serum albumin was added to make a final concentration of 4%. Antimycotics were dissolved in ethanol and added to the incubation

mixture to make the final concentration of 1—100 μM. The amount of ethanol added to the incubation mixture was 0.1% (v/v), and the same volume of solvent was added to the control. The reaction was started by adding the microsomal suspension and was carried out at 37 °C with shaking for 5 min. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid. It was confirmed that the antimycotics or albumin did not interfere in the spectrophotometric analysis of HCHO by the method of Nash.[20]

Cytochrome P-450 was determined in a microsomal suspension containing 2 mg protein/ml 0.1 M sodium/potassium phosphate buffer, pH 7.4, by the method of Omura and Sato.[21]

NADPH–cytochrome c reductase activity was determined by the method of Peters and Fouts.[22]

Measurement of the Ratio of 6β-Hydroxycortisol (6β-OHF) to Cortisol (F) in Urine as an Indicator of Hepatic Oxidative Drug-Metabolizing Capacity in Humans Five healthy subjects, 4 male and 1 female hospital pharmacists aged from 24 to 45 years old, were employed in the study. All subjects had normal hepatic and renal functions, and they were taking no medication. All subjects received an oral dose of FCZ (Diflucan® capsule, 200 mg once a day) for 3 d. On day 0 (just before treatment) and days 1, 2, 3, 5, 8 and 10, random urine samples were taken for the evaluation of oxidative drug-metabolizing capacity. All urine samples were collected during the time period from 12:00 to 16:00 h. Urine samples were stored frozen at −80 °C until assay. The oxidative drug-metabolizing capacity of each subject was evaluated in terms of the ratio of 6β-OHF to F in urine. Urinary 6β-OHF was determined by the method described previously,[23] and urinary F was determined by the fluorescence polarization immunoassay technique (TDX® system, Dinabot kit).

Results

Changes in Pentobarbital Sleeping Time in Mice by Treatment with Antimycotics As shown in Fig. 1A, when pentobarbital sodium was injected in mice 0.5 h after treatment with a single dose of 0.3—10 mg/kg of FCZ or 10—50 mg/kg of KCZ, pentobarbital sleeping time was prolonged in a dose-dependent manner. The sleeping time was prolonged significantly up to 5.6- and 2.8-fold control value by treatment with 10 mg/kg of FCZ and 50 mg/kg of KCZ, respectively, while no change was observed for KCZ at a dose of 10 mg/kg. The changes in sleeping time with the passage of time after treatment with a single dose of 10 mg/kg of FCZ or 50 mg/kg of KCZ were shown in Fig. 1B. When pentobarbital sodium was injected in mice 6 h after the treatment of FCZ, a significant prolongation of the sleeping time was still observed, but not for KCZ.

Table I shows the changes in parameters associated with hepatic drug metabolism such as cytochrome P-450 and NADPH–cytochrome c reductase activity in the microsomes prepared 6 h after treatment with a single dose of 10 mg/kg of FCZ or 50 mg/kg of KCZ. None of the significant changes in these parameters were found.

Spectral Binding Interactions of Antimycotics with Cytochrome P-450 in Mouse Hepatic Microsomes A binding study was performed in order to assess the ability of the antimycotics to interact with microsomal cytochrome P-450. FCZ and KCZ gave a peak at 428 nm and a trough at 392 and 405 nm, respectively, characteristics of a type II spectral change.[24] These dose-dependent spectral changes were converted to double-reciprocal plots and spectral dissociation constants (K_s) were calculated. The plots for KCZ were biphasic and two K_s (K_{s1} and K_{s2}) values were obtained, while the plot for FCZ was a single line. These data are summarized in Table II. The result indicated that

TABLE I. Changes in the Parameters Associated with Hepatic Microsomal Monooxygenase Activity in Mice 6 h after Treatment with a Single Dose of FCZ or KCZ

Treatments	Cytochrome P-450 (nmol/mg protein)	NADPH–cytochrome c reductase (nmol/mg protein/min)
Control (vehicle alone)	0.99±0.10	140.0±24.2
FCZ 10 mg/kg	1.10±0.08	134.3±14.1
KCZ 50 mg/kg	0.95±0.13	136.1±14.7

Each value is the mean±S.D. of four determinations.

TABLE II. Data Derived from Difference Spectra of Mouse Hepatic Microsomes Recorded in the Presence of FCZ and KCZ

	Absorbance		Dissociation constants (μM)		Inhibition constants for pentobarbital-induced spectral change (μM)
	Maximum (nm)	Minimum (nm)	K_{s1}	K_{s2}	K_i
FCZ	428	392	0.83	ND	0.63
KCZ	428	405	0.22	5.4	0.11

K_s and K_i values were calculated from double-reciprocal plots. Each value is the mean of three measurements. ND: Not detected.

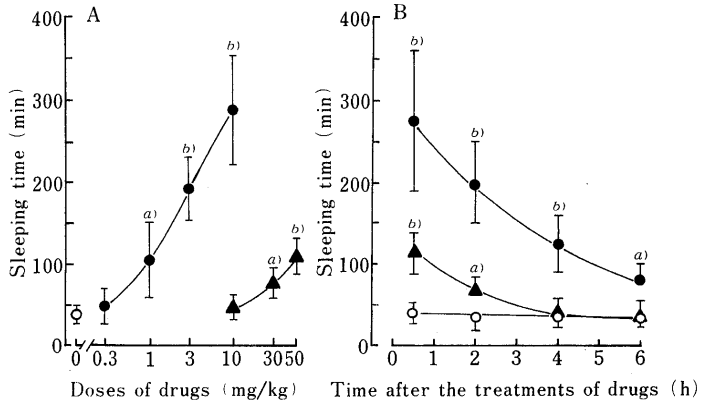

Fig. 1. Dose-Related Prolongation in Pentobarbital Sleeping Time (A) and Its Changes during Time Course (B) after Treatment with FCZ and KCZ

Mice were treated with single doses of 0.3, 1, 3 and 10 mg/kg of FCZ (●) or 10, 30 and 50 mg/kg of KCZ (▲), or the vehicle alone (○). Pentobarbital sodium dissolved in saline (40 mg/kg) was injected i.p. 0.5 h after the i.p. injection of each drug or vehicle alone (A). The same dose of pentobarbital sodium was injected i.p. 2, 4 or 6 h after the injection of 10 mg/kg of FCZ or 50 mg/kg of KCZ or the vehicle alone (B). Value is the mean±S.D. from 4 mice. *a*) Significantly different from the control ($p<0.05$). *b*) Significantly different from the control ($p<0.01$).

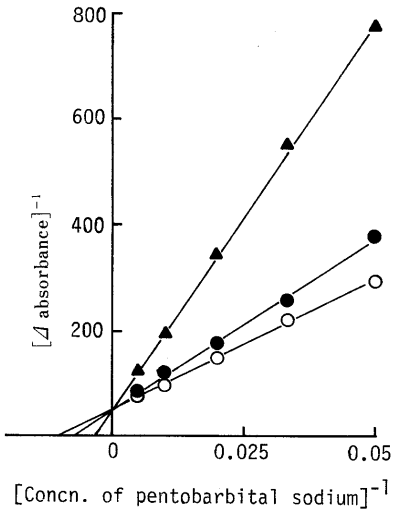

Fig. 2. Double-Reciprocal Plots of the Pentobarbital-Induced Difference Spectra in the Absence and the Presence of Antimycotics

Pentobarbital-induced difference spectra were recorded after the addition of 0 μM (ethanol alone, ○), 0.2 μM of FCZ (●) or KCZ (▲) into both cuvettes and the absorbance peak (383 nm) minus trough (416 nm) values were used to construct double-reciprocal plots. Each value is the mean of experiments with three different mouse hepatic microsomal preparations.

the affinity of FCZ for cytochrome P-450 in hepatic microsomes was lower than that of KCZ, because the K_s value of the former was about 4-fold higher than the K_{s1} (K_s for the high affinity site) value of the latter.

To determine the inhibitory effects of antimycotics on the binding of pentobarbital to hepatic microsomal cytochrome P-450, the measurement of pentobarbital-induced difference spectral change was performed in the absence and the presence of antimycotics. Pentobarbital gave a peak at 383 nm and a trough at 416 nm, characteristics of type I spectral change.[24] The intensity of the spectral change was depressed by the presence of antimycotics. The double-reciprocal plots of the spectral change are shown in Fig. 2. The kinetic data indicated that the inhibition of the binding of pentobarbital to cytochrome P-450 active site by these antimycotics was of a competitive type. The inhibition constants (K_i) for the binding of pentobarbital to cytochrome P-450 which were calculated from the data are also shown in Table II. Nevertheless, these K_i values were apparent because the spectral modifications owing to the replacement of antimycotics as the inhibitors from binding site(s) on cytochrome P-450 by the addition of pentobarbital sodium could not be corrected. In agreement with the order of their affinities for cytochrome P-450, the inhibitory potency of FCZ for the binding of pentobarbital sodium to cytochrome P-450 was smaller than that of KCZ, since the apparent K_i value of FCZ was about 6-fold higher than that of KCZ.

Inhibitory Effects of Antimycotics on Hepatic Microsomal Aminopyrine *N*-Demethylation *in Vitro* in the Absence and the Presence of Albumin The inhibitory potencies of antimycotics for monooxygenase activity in hepatic microsomes *in vitro* were determined by the measurement of aminopyrine *N*-demethylation as a model reaction. Particularly, this experiment was performed by considering the difference in the plasma protein binding in the systemic circulation between FCZ[25] and KCZ.[26] As shown in Fig. 3, although each drug inhibited aminopyrine *N*-demethylase

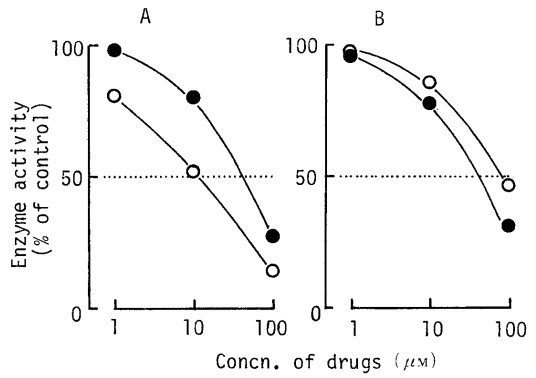

Fig. 3. Dose-Related Inhibition of Aminopyrine *N*-Demethylation in Mouse Hepatic Microsomes by Antimycotics in the Absence (A) and the Presence (B) of Human Serum Albumin

Each value is the mean of experiments with three different mouse hepatic microsomal preparations. Standard deviations for different data points were between 2 and 9 percent. The control activities for the production of HCHO (nmol/mg protein/min; mean ± S.D.) were 9.5 ± 1.4 and 9.7 ± 1.3 in the absence (A) and the presence (B) of 4% albumin, respectively. Symbols: ●, FCZ; ○, KCZ.

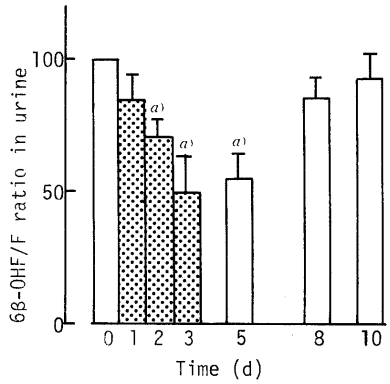

Fig. 4. Change in the Ratio of 6β-OHF to F in Urine Obtained from 5 Healthy Subjects, before, during and after 3 d of FCZ Treatment

The value of the ratio on day 0 (mean ± S.E.) was 2.73 ± 0.18. The ordinate shows the percentage to the ratio on day 0 in each subject. Shaded areas indicate the periods during administration. Each column represents the mean ± S.E. *a*) Significantly different from day 0 ($p < 0.05$).

activity in a dose-dependent manner, a reverse in the order of the inhibitory potencies between FCZ and KCZ was found by the addition of 4% albumin into the incubation mixture: that is, the 50% inhibition concentration (IC_{50}) of FCZ for the enzyme activity (42 μM) was higher than that of KCZ (12 μM) in the absence of albumin (Fig. 3A), while in the presence of 4% albumin, IC_{50} value of FCZ (43 μM) was lower than that of KCZ (85 μM) (Fig. 3B).

Change in the Drug-Metabolizing Capacity in Humans by the Administration of FCZ The change in the ratio of 6β-OHF of F in urine from 5 healthy subjects, before, during and after 3 d of FCZ treatment is shown in Fig. 4. The ratio decreased significantly up to 50% of the original level during treatment with a dose of 200 mg/d of FCZ. The ratio increased slowly with the cessation of treatment with FCZ and returned to its original level 5 to 7 d after the cessation.

Discussion

In the present study, we demonstrated that the inhibitory potency of FCZ for cytochrome P-450-mediated drug-metabolism in hepatic microsomes "*in vivo*", but not *in vitro*, was greater than that of KCZ. The pentobarbital

sleeping time test is a sensitive and reliable *in vivo* method of assessing this enzyme system, since the prolongation or the shortening of the sleeping time reflect the change in the rate of hepatic oxidative metabolism of pentobarbital.[27] The result shown in Fig. 1 indicates that FCZ exhibits a more potent reducing effect on pentobarbital metabolism *in vivo* than KCZ does, because no effect of FCZ on the central nervous system in mice is found.[28] This agrees well with the result reported by Delfa *et al.*[18]: that is, a single dose of 1 to 10 mg/kg of FCZ, but not KCZ, inhibited antipyrine metabolism in mice *in vivo*. In addition, none of the significant changes in the cytochrome P-450 content and NADPH–cytochrome c reductase activity in the microsomes prepared 6 h after treatment with antimycotics were found (Table I). These results indicate that the reduction of pentobarbital metabolism by FCZ may be due to its direct interaction with cytochrome P-450 active site(s). In contrast to the result obtained *in vivo* study, *in vitro* the affinity and the inhibitory potency of FCZ for cytochrome P-450 and its mediated drug-metabolism were 4- to 6-fold smaller than those of KCZ (Table II and Fig. 3A). These agree well with the results reported by Houston *et al.*[17]: that is, KCZ is 5 to 15-fold more effective than FCZ in the inhibition of some monooxygenase reactions in rat hepatic microsomes *in vitro*. On the other hand, it has been recognized to be the significant difference in the pharmacokinetic parameters between FCZ and KCZ: that is, FCZ has much lower plasma protein binding (approximately 10%)[25] and much longer elimination half-life (approximately 30 h)[29] than KCZ does (approximately 90% or more[26] and 3 h,[30] respectively), although the therapeutic plasma peak levels among FCZ (6.2—26.1 μM)[29] and KCZ (3.0—18.9 μM)[30] are similar to each other. Thus, in order to solve the discrepancy between the results obtained *in vivo* and *in vitro* studies described above, we focused on the difference in the plasma protein binding among antimycotics and examined whether the order of the inhibitory potencies of them for monooxygenase activity *in vitro* were altered by the addition of albumin into the reaction mixture. The reasons for a reverse in the order of the inhibitory potencies between FCZ and KCZ induced by the addition of albumin (Fig. 3) may be as follows: the decrease of the free concentration of KCZ due to the binding of its large portion to albumin leads to the decrease of its inhibitory potency; no change in the inhibitory potency of FCZ is due to that the free concentration of FCZ is scarcely altered because of its lower binding property to albumin. Although the distribution properties of these antimycotics to the liver need to be clarified, these findings suggest strongly that the difference in the plasma protein binding properties between FCZ and KCZ is an important factor which leads to a reverse in the order of their inhibitory potencies for cytochrome P-450-mediated drug-metabolism *in vitro* and *in vivo*.

Measurement of the ratio of 6β-OHF to F or 17-hydroxycorticosteroids in urine is useful as a nonincasive method for evaluating changes in human hepatic monooxygenase activity such as enzyme inhibition[31-35] and induction,[9,36-39] because 6β-OHF is a polar metabolite formed by the oxidative metabolism of F in hepatic microsomes. Furthermore, there is no circadian change in 6β-OHF/F ratios, that is, the ratio in randomly obtained urine specimens from subjects may be used to detect the changes in drug-metabolizing capacity in the same manner as in 24-h urine.[32] By the use of this method we also found that FCZ exhibited potent inhibitory action for hepatic oxidative drug-metabolism in humans. The result indicates that the oxidative drug-metabolizing enzyme activity responsible for the formation of 6β-OHF from F in human liver is inhibited by treatment with a therapeutic dose of FCZ (Fig. 4). We demonstrated previously that the ratio of 6β-OHF to F or 17-hydroxycorticosteroids in urine decreased to 65—85% of the original levels during treatment with a relatively high dose (800 mg/d) of cimetidine[33,34] or ozagrel hydrochloride,[35] imidazole derivatives, and the ratios returned to their original levels within 2 d after the cessation of treatment. The present results, in which the ratio decreased to 50% of the original ratio and a longer period was required for the return to the original level by treatment with only 200 mg/d of FCZ (Fig. 4), indicate that the inhibitory effect of FCZ on hepatic oxidative drug-metabolizing capacity in humans may be more potent and longer than those of the imidazole derivatives described above. The duration of the inhibitory action of FCZ for this enzyme system may be due to its longer elimination half-life.[29] In recent clinical studies, FCZ has been reported to be interactive with phenytoin,[39] but not with cyclosporin.[41] Furthermone, of several azole antimycotics, miconazole has been shown to exhibit the inducing action on the hepatic microsomal drug-metabolizing enzyme system by consecutive treatments for a longer period[9] while it is not clear whether or not FCZ does. Therefore, further detailed study is needed to examine the selectivity in the inhibitory and inducing actions of FCZ for various cytochrome P-450 isozymes in hepatic microsomes.

In conclusion, the present study demonstrated that the inhibitory effect of FCZ for hepatic microsomal cytochrome P-450-mediated drug-metabolism *in vivo* was unexpectedly potent from its potential obtained *in vitro* experiments in the absence of albumin, and that FCZ could be a potent inhibitor for certain cytochrome P-450 isozymes-mediated drug-metabolism in humans.

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