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INVOLVEMENT OF THE MACROLIDE ANTIBIOTIC INDUCIBLE CYTOCHROME P-450 LM_{3c} IN THE METABOLISM OF MIDAZOLAM BY MICROSOMAL FRACTIONS PREPARED FROM RABBIT LIVER*

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Abstract—This report characterizes the cytochrome P-450 isozyme involved in midazolam metabolism. This study was undertaken into liver microsomal fractions prepared from untreated rabbits or animals treated with drugs known to specifically induce various cytochrome P-450 isozymes such as form LM_2 by phenobarbital, LM_4 and LM_6 by 3-methylcholanthrene and β-naphthoflavone, LM_{3a} by ethyl alcohol and acetone, and LM_{3c} by macrolide antibiotics (rifampicin, erythromycin and triacetyloleandomycin). Among this library of characterized microsomal preparations, only those obtained from macrolide antibiotic-treated rabbits exhibited a Type I binding spectrum upon addition of midazolam (K_s = 3.2–5.3 μg/ml; 10.6–17.5 μM) and significantly metabolized midazolam to its various hydroxylated metabolites ($K_m = 2.52 \pm 0.22$ μg/ml; 8.32 ± 0.73 μM and $V_{max} = 20$ μg metabolites formed/min/mg proteins; 66 nmoles metabolites formed/min/mg proteins). The following observations further confirmed the specific involvement of the cytochrome P-450 LM_{3c} isozyme: (i) only anti-cytochrome P-450 LM_{3c} isozyme antibodies intensively inhibited midazolam metabolism, (ii) incubation of microsomes, prepared from TAO-treated rabbits, with midazolam in the presence of potassium ferricyanide which restored the functional cytochrome P-450 LM_{3c} isozyme, increased midazolam metabolism to a similar extent, and (iii) in the presence of Cyclosporin A, a specific substrate of the rabbit cytochrome P-450 LM_{3c} isozyme, midazolam metabolism was inhibited in a concentration-dependent manner.

These data demonstrated that the rabbit cytochrome P-450 LM_{3c} isozyme was predominantly involved in midazolam metabolism.

The cytochrome P-450-dependent monooxygenase system is a multigene family of hemoproteins, abundant in the endoplasmic reticulum of hepatocytes. It is involved in the oxidative biotransformation of numerous endogenous substrates (such as steroids) and xenobiotics (drugs). Extensive studies in many animals species indicate that multiple polypeptide forms of these cytochromes can be distinguished on the basis of their structure, substrate specificity or responses to various types of inducers [1–3]. For example, different forms of liver cytochromes P-450 were accumulated in rats treated by a member of one of the three "classes" of inducers [4] typified, respectively, by phenobarbital, 3-methylcholanthrene and macrolide antibiotics or glucocorticoides. We focused our attention on this third "class" of cytochrome P-450 termed LM_{3c} in rabbits, P-450p in rats and HLp in humans. This cytochrome P-450 isozyme(s) has been reported to be inducible by glucocorticoids, anti-glucocorticoids, anti-seizure drugs and by macrolide antibiotics (triacetyloleandomycin, rifampicin or erythromycin) [5–7]. The induced-cytochrome P-450 form(s) was found to exhibit a high turnover for erythromycin and to convert macrolide antibiotics, especially triacetyloleandomycin and erythromycin, into metabolites that bind tightly to the heme moiety of the cytochrome P-450 isozyme, forming a stable spectrally detectable complex [6, 8]. It is exclusively responsible for the *N*-demethylation of erythromycin [9] and for the complex metabolism—mainly hydroxylations at various sites of the molecule—of Cyclosporin A [10]. Based upon differential spectroscopic data, these drugs exhibited high K_s and/or low ΔA_{max} values. Moreover both molecules were only slowly metabolized by this isozyme [10, 11].

In the present report we examined (i) the metabolic pathway of midazolam, a novel imidazobenzodiazepine [12, 13], by liver microsomal fractions prepared from untreated or inducer-treated rabbits, and (ii) the role of cytochrome P-450 isozymes in these biotransformation processes. The specific involvement of the cytochrome P-450 LM_{3c} isozyme in midazolam metabolism is demonstrated.

MATERIALS AND METHODS

Chemicals and materials. Midazolam (Ro 21-3981; m.w. 303), (^{14}C)midazolam (32 μCi/mg) and its

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metabolites including the 1-hydroxy- and 4-hydroxy-midazolam (Ro 21-6347 and Ro 21-5975), the di-hydroxy-midazolam (Ro 21-0284) and the *N*-demethyl-midazolam (Ro 21-5259) were generously given by Hoffman LaRoche Laboratories (Basel, Switzerland). Radiolabeled drug was 97% pure and used without further purification.

Cyclosporin A (m.w. 1204) was a kind gift from Sandoz Ltd (Basel, Switzerland). Inducer chemicals were: phenobarbital (Specia, Paris), β-naphthoflavone, erythromycin and 3-methylcholanthrene (Sigma, St Louis, MO), rifampicin (Merrel Dow-Lepetit, Paris, France), dexamethasone, triacetyloleandomycin, ethyl alcohol and acetone (Merck-Clevenot). Antibodies to highly purified cytochrome P-450 LM_2, LM_{3c}, LM_4 and LM_6 were generous gifts of Doctor P. Maurel (INSERM U 128, Montpellier, France).

Animals and treatments. Male New Zealand rabbits weighing 2.0 to 3.0 kg were used for liver microsomal preparations.

Treatments were as follows: phenobarbital (PB), 0.1% in drinking water (pH 7.0) for seven days; ethyl alcohol (AL), 1% in drinking water for three weeks; acetone (AC), 10% in drinking water for two weeks; β-naphthoflavone (BNF), i.p. injection of 80 mg/kg/day in corn oil for three days; 3-methylcholanthrene (3MC), i.p. injection of 250 mg/kg in corn oil for three days; rifampicin (RIF), i.p. injection of 50 mg/kg/day in water for four days; triacetyloleandomycin (TAO) and erythromycin (ER), 1 nmole/kg/day mixed in the food for seven days.

Preparations of liver microsomes. Liver microsomes were prepared as described previously [8] and stored at −80° in potassium phosphate buffer (0.1 M, pH 7.4) containing 20% glycerol and 1 mM EDTA. Protein concentration was estimated by the Biorad dye reagent protein assay [14]. Total cytochromes P-450 and b_5 concentrations were estimated spectrally according to Omura and Sato [15].

Differential spectroscopic analysis of the binding of midazolam to liver microsomes. Liver microsomes were diluted to 1 mg/ml in 0.1 M potassium phosphate buffer (pH 7.4) and the solution was divided into two aliquots (1 ml each) placed in both cuvettes of an Uvikon 860 spectrophotometer. After adjustment of the baseline, an aliquot of a midazolam solution (prepared in dimethylsulfoxide) was added to the sample cuvette, while the same volume of solvent was added to the reference cuvette. The final dimethylsulfoxide concentration never exceeded 1%. After 3 min, the difference spectrum was recorded between 525 and 350 nm. Sequential additions of midazolam allowed a 1–7 μg/ml (3.3–23.1 μM) concentration range to be covered. These measurements were carried out at 20°.

Assay conditions and HPLC analysis of midazolam metabolism. Liver microsomes preparations were diluted to 0.1–1.0 mg proteins/ml in 0.1 M potassium phosphate buffer (pH 7.4) and appropriate aliquots of midazolam (minimal total radioactivity used = 10,000 dpm per assay) were added to the suspension. After a preincubation period of 3 min at 37°, the reaction was initiated by the addition of 1 mM NADPH and allowed to proceed at 37°. At selected times, the reaction was stopped by addition of methyl alcohol (1/5, v/v). After precipitation and elimination of the proteins by centrifugation (20,000 *g* for 5 min), 0.1–0.2 ml of the supernatant fluid was analyzed by HPLC.*

All analyses were performed on a reversed phase C_{18} column (25 × 0.4 cm; pore size 10 μm) (Knauer, F.R.G.) using a high performance liquid chromatograph (Hewlett Packard 1084 B) equipped with a variable wavelength spectrophotometer and a chromatographic terminal (Hewlett Packard 79850 ALC).

Elution was carried out at 1.2 ml/min along a linear gradient of 50%–50% to 62%–38% methyl alcohol-double distilled water over 30 min. Column temperature was maintained at 25° and absorbance was recorded at 254 nm. Eluent from the column was analyzed by a radioactive flow detector (Radiomatic Instruments). Figure 1 represents an HPLC radiochromatogram of midazolam and its metabolites. Metabolites are identified according to their retention times relative to those of standards.

Inhibition of microsomal midazolam metabolism by anti-cytochrome P-450 antibodies. The assay conditions used in these experiments were the same as those described in the previous section, except that the microsomes were diluted so that the total cytochrome P-450 concentration (according to Omura and Sato [15]) was 0.5 μM. The concentration of anti-cytochrome P-450 antibodies ranged from 0 to 10 mg/nmole total cytochrome P-450.

Microsomes and IgG were first incubated at room temperature for 20 min, midazolam was then added and the reaction initiated with NADPH (1 mM final concentration).

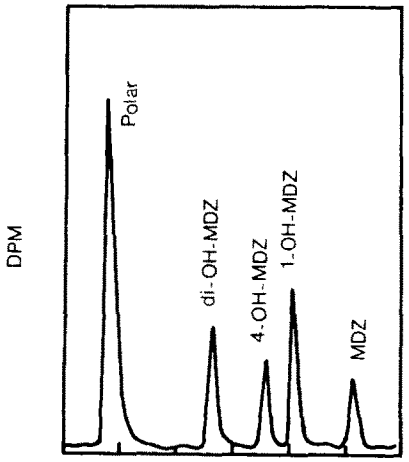

Fig. 1. High performance liquid chromatogram of midazolam and its metabolites. Microsomes (0.5 mg/ml) from erythromycin-treated rabbits were incubated for 5 min with 5 μg/ml (16.5 μM) ^{14}C-midazolam and 1 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.4). After quenching of the reaction with methyl alcohol and removal of proteins by centrifugation, the clear supernatant fluid was analyzed by HPLC.

* Abbreviations used: HPLC, high performance liquid chromatography; DMSO, dimethylsulfoxide.

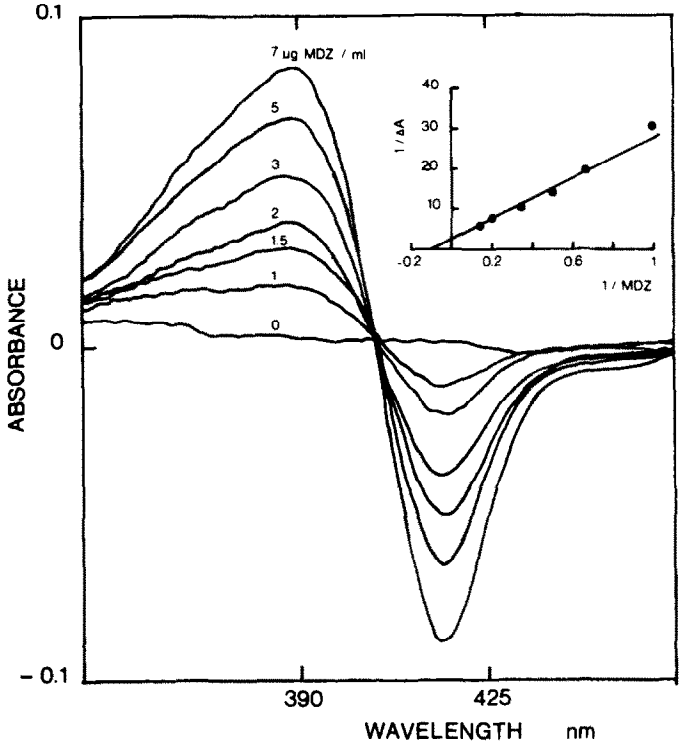

Fig. 2. Type I binding spectra obtained upon addition of midazolam to liver microsomes prepared from erythromycin-treated rabbits. Liver microsomes were diluted to 1 mg/ml in 0.1 M potassium phosphate buffer (pH 7.4). Equal amounts of this solution were placed into the two cuvettes of an Uvikon 860 spectrophotometer. After the baseline was recorded, aliquots of a 1 mg/ml (3.3 mM) midazolam solution in dimethylsulfoxide (DMSO) were added to the sample cuvette while equal volumes of DMSO were added to the reference cuvette. The difference spectra were recorded 3 min after each addition. Insert: reciprocal plot of A (absorbance at 390 nm minus absorbance at 420 nm) vs midazolam concentrations expressed in µg/ml.

TAO complex formation. The amount of the "TAO metabolite–cytochrome P-450 complex" formed *in vivo* was measured by diluting liver microsomes isolated from TAO-treated rabbits (1.97 nmol cytochrome P-450/mg proteins) to 1.0 mg protein/ml in 0.1 M potassium phosphate buffer (pH 7.4). One ml of the suspension was placed into the reference and the sample cuvettes of the spectrophotometer. After a baseline was recorded, 15 µl of 2 mM $K_3Fe(CN)_6$ was added to the reference cuvette. After a 10-min incubation at 25°, the spectrum was recorded to determine the difference in absorbance between 457 and 510 nm. The concentration of the "TAO metabolite–cytochrome P-450 complex" was calculated using the extinction coefficient of 68 mM^{-1} cm^{-1} [6].

RESULTS

Differential spectroscopic analysis of the interaction between midazolam and microsomal cytochrome P-450

The binding of midazolam to rabbit liver microsomes prepared from untreated and inducer-treated animals has been analyzed by differential spectroscopy. Typical difference spectra can be obtained upon addition of increasing amounts of midazolam (1.0–7.0 µg/ml; 3.3–23.1 µM) to liver microsomes prepared from erythromycin- (Fig. 2), rifampicin- and triacetyloleandomycin-treated animals. The minima at 420 nm and the maxima at 390 nm were typical of a Type I binding spectrum resulting from the low to high spin transition accompanying the binding of midazolam to low spin cytochrome P-450. Using microsomes prepared from erythromycin-treated rabbits, the reciprocal plot of the absorbance change (at 390 minus 420 nm) against midazolam concentration (insert of Fig. 2) was linear as expected from a saturation process involving a single binding site or binding site(s) with similar dissociation constants. From such a plot, a spectral dissociation constant, K_s(app), and a maximal absorbance change (ΔA_{max}) could be estimated for each microsomal preparation and are reported in Table 1.

Only microsomes prepared from animals treated with macrolide antibiotics exhibited a Type I binding spectrum upon addition of midazolam. The absence of spectral changes with the other preparations could not be attributed to any kind of denaturation since, in control experiments, these microsomes exhibited Type I or Type II binding spectra with their known substrates, i.e. benzphetamine (LM_2), aniline (LM_{3a}), acetanilide (LM_4 and LM_5) and benzo-*a*-pyrene (LM_6).

Table 1. Interactions between cytochrome P-450 and midazolam: spectral parameters characterizing the Type I binding spectrum and dissociation constant of the complex

Microsomes*	$\Delta A_{max}^{390/420}$†	K_s†		N‡
		(μg/ml)	(μM)	
UT	0	—		2
TAO	0.14 ± 0.01	3.2 ± 0.3	10.6 ± 0.9	2
ER	0.36 ± 0.10	5.3 ± 0.4	17.5 ± 1.3	6
RIF	0.15 ± 0.02	3.8 ± 0.9	12.5 ± 2.9	5
PB	0	—	—	2
BNF	0	-	—	2
3MC	0	—	—	2
AL	0	—	—	2
AC	0	—	—	2

* These measurements have been carried out with liver microsomes (at a protein concentration of 1 mg/ml) from untreated (UT) or inducer-treated rabbits.

† Maximal change in absorbance between 390 and 420 nm, and dissociation constants determined from reciprocal plots of difference in absorbance versus midazolam concentration (see insert Fig. 2 and text).

‡ Number of determinations.

Metabolism of midazolam by rabbit liver microsomes

Liver microsomes prepared from untreated rabbits or from animals treated with various inducers and at a final protein concentration of 0.5 mg/ml, were incubated for 5 min with 5 μg/ml (16.5 μM) ^{14}C-midazolam and NADPH. The amounts of metabolites formed after a 5 min exposure of midazolam to the different microsomal preparations are illustrated in Fig. 3. In agreement with the data presented in Table 1, only the microsomes prepared from rabbits treated with macrolide antibiotics were able to metabolize midazolam significantly.

The incubation of microsomes prepared from TAO-treated rabbits with 20 μM potassium ferricyanide broke down the complex and restored the functional cytochrome P-450 LM_{3c} isozyme [8]. In our preparations, approximately 20% of cytochrome P-450 LM_{3c} isozyme were complexed with the TAO nitroso derivative. In agreement with these data, when microsomes prepared from TAO-treated rabbits were exposed to midazolam in the presence of 20 μM potassium ferricyanide, midazolam metabolism was increased by approximately 26%.

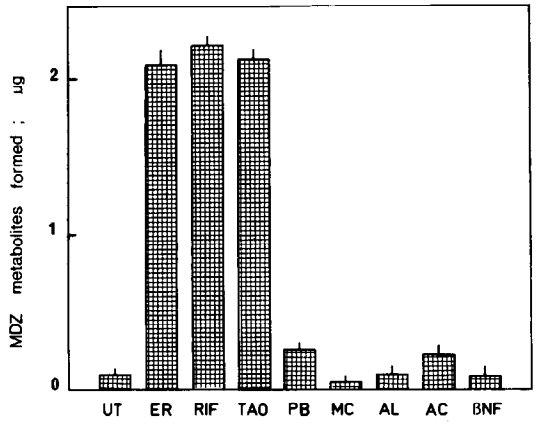

Fig. 3. Effect of various inducers on the *in vitro* metabolism of midazolam by liver microsomes. Microsomes (0.5 mg/ml) prepared from livers of untreated- or inducer-treated rabbits were exposed for 5 min to 5 μg/ml (16.5 μM) ^{14}C-midazolam and 1 mM NADPH, in a total volume of 0.5 ml. After quenching of the reaction with methyl alcohol and removal of proteins, the clear supernatant fluid was analyzed by HPLC. Each result is the mean ± SD of two different experiments.

Liver microsomes prepared from rifampicin-treated rabbits (0.1 mg protein/ml) were incubated with increasing concentrations of ^{14}C-midazolam (0.5–3.0 μg/ml; 1.65–9.9 μM) for 1 min, conditions under which metabolic rates were linear, only 1-hydroxy-midazolam was formed and unchanged midazolam represented more than 70% of drug-related material. Lineweaver–Burk analysis of kinetic parameters allowed the estimation of the Michaelis–Menten constant (K_m) and of the maximum velocity (V_{max}). They were about 2.52 ± 0.22 μg/ml (8.32 ± 0.73 μM) and 20 μg (66 nmoles) metabolites formed/min/mg proteins respectively.

The time courses of midazolam consumption and metabolites production by liver microsomes prepared from erythromycin-treated rabbits are presented in Fig. 4. During the first 2–5 min, the decrease in midazolam concentration was associated with the appearance of the monohydroxylated derivatives, mainly the 1-hydroxy-midazolam, which represented almost 70% of total radioactivity after a 2 min exposure. The level of these monohydroxylated derivatives then decreased and their disappearance was associated with the appearance of other compounds such as the dihydroxylated metabolite and a highly polar derivative. After a 30 min exposure, the polar metabolite(s) of midazolam represented approximately 50% of total radiolabel. This polar derivative did not coelute with any of the available metabolites of midazolam. In a similar experiment, microsomes prepared from rifampicin-treated rabbits were incubated for 30 min with ^{14}C-midazolam in order to synthetize large amounts of this polar derivative (Table 2). Microsomes were removed by centrifugation at 105,000 *g*

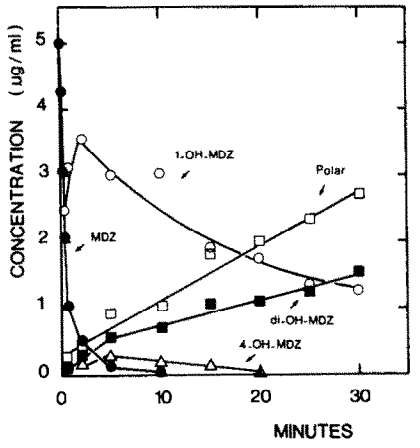

Fig. 4. Kinetics of midazolam consumption and metabolites production by rabbit liver microsomes. Liver microsomes (0.5 mg/ml) from erythromycin-treated rabbits were incubated at 37° in a total volume of 2 ml of 0.1 M potassium phosphate buffer (pH 7.4), in the presence of 5 μg/ml (16.5 μM) ^{14}C-midazolam and 1 mM NADPH. At different intervals of time after initiation of the reaction, incubation mixture was analyzed by HPLC.

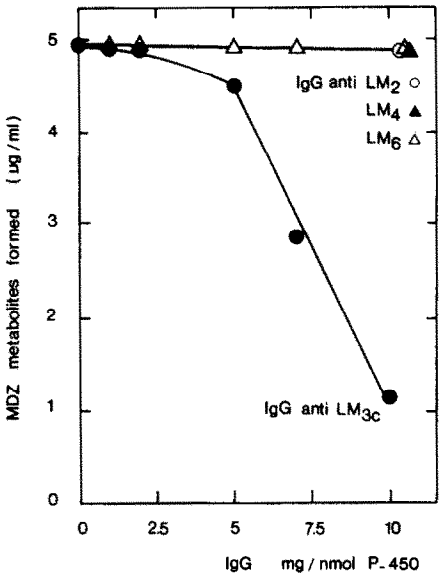

Fig. 5. Immunoinhibition of midazolam metabolism in rabbit liver microsomes. Liver microsomes from erythromycin-treated rabbits were diluted to a final concentration of 0.5 nmoles/ml (according to Omura and Sato [15]) and incubated for 20 min at room temperature in the absence or the presence of increasing amounts of antibodies against various forms of rabbit cytochrome P-450. The reaction was then initiated at 37° by the addition of 5 μg/ml (16.5 μM) ^{14}C-midazolam and 1 mM NADPH successively. After a 20 min period of incubation, reaction was quenched by the addition of methyl alcohol, proteins were removed by centrifugation and the clear supernatant fluid was analyzed by HPLC.

for 1 hr and supernatant fluid containing mainly the polar derivative was incubated overnight with *Helix pomatia* juice (10,000 units β-glucuronidase and 100,000 units sulphatase). While the polar derivative represented almost 65% of total radiolabel in standard conditions, after hydrolysis, 70–80% of this derivative was reverted to a single metabolite co-eluting with 1-hydroxy-midazolam. The preincubation of the polar derivative with β-D-glucuronidase alone remained without effect suggesting a specific sulfoconjugation of the metabolite.

Effect of various anti-cytochrome P-450 antibodies on the microsomal activity

Liver microsomes from erythromycin-treated rabbits were diluted to a final concentration of 0.5 μM cytochrome P-450 and incubated for 20 min at room temperature, without or with increasing amounts of antibodies directed to various isozymes of rabbit cytochrome P-450 including P-450 LM_2, LM_{3c}, LM_4 and LM_6. The reaction was then initiated by the addition of 5 μg/ml (16.5 μM) ^{14}C-midazolam and 1 mM NADPH and quenched after a 30 min incubation at 37°. From data presented in Fig. 5, we showed that only anti-cytochrome P-450 LM_{3c} antibodies inhibited the metabolism of midazolam by approximately 80%. On the other hand this activity was not inhibited by anti-cytochrome P-450 LM_2, LM_4 and LM_6 antibodies used at 10 mg/nmol of cytochrome P-450. In Fig. 6 are illustrated the patterns of midazolam and its different metabolites in the presence of increasing concentrations of anti-cytochromes P-450 LM_{3c} and LM_6 antibodies (Figs. 6A and 6B respectively). For anti-cytochromes P-450 LM_{3c} antibodies concentrations up to 5 mg/nmol total cytochrome P-450, the amounts of both dihydroxy-midazolam and the polar derivatives decreased while the levels of midazolam and of the monohydroxylated derivatives slightly increased. In contrast and for anti-cytochrome P-450 LM_{3c} antibodies concentrations ranging between 5 and 10 mg/nmol, the formation of all the metabolites decreased, resulting in an increase of unmetabolized midazolam.

In the presence of increasing concentrations of anti-cytochrome P-450 LM_6 antibodies, the pattern of midazolam metabolism was not modified.

Table 2. Effect of *Helix pomatia* juice on midazolam metabolic pattern

Compound	Before incubation with *Helix pomatia*	After incubation with *Helix pomatia*
Midazolam	0.8%	1.9%
1-Hydroxy-midazolam	3.4%	49.8%
4-Hydroxy-midazolam	9.1%	8.0%
Di-hydroxy-midazolam	23.1%	22.6%
Polar derivative	63.6%	17.7%

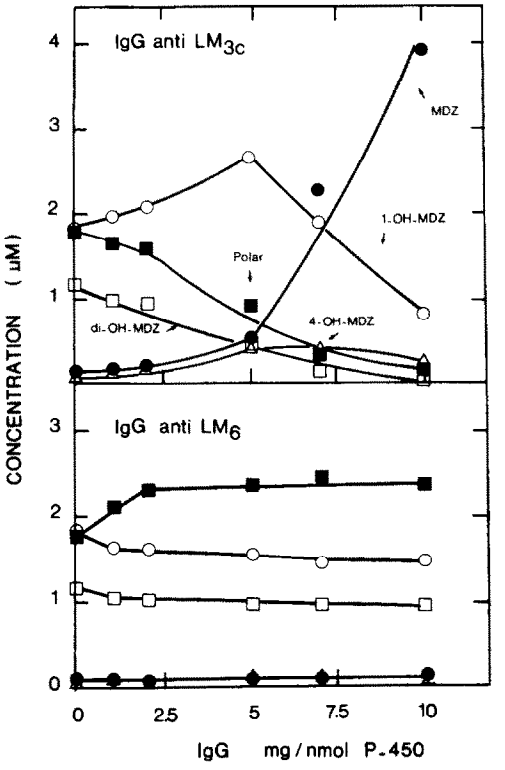

Fig. 6. Effect of different antibodies against cytochromes P-450 LM_{3c} and LM_6 on midazolam metabolism. Liver microsomes from erythromycin-treated rabbits were diluted to a final concentration of 0.5 nmoles/ml and incubated for 20 min at room temperature in the absence or the presence of increasing amounts of antibodies against cytochromes P-450 LM_{3c} or LM_6. The reaction was initiated by the addition of midazolam and NADPH as described in the text. After quenching of the reaction and removal of proteins by centrifugation, the incubation mixture was analyzed by HPLC.

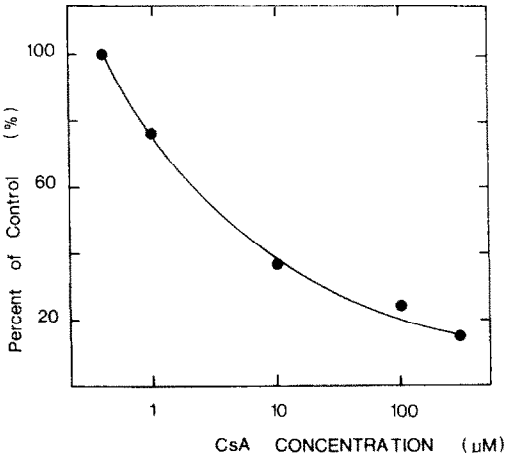

Fig. 7. Effect of Cyclosporin A on midazolam metabolism. Liver microsomes prepared from erythromycin-treated rabbits were incubated at 37° with 5 μg/ml (16.5 μM) ^{14}C-midazolam in the absence or the presence of increasing Cyclosporin A concentrations. Thirty minutes after initiation of the reaction with 1 mM NADPH, the reaction was quenched with methyl alcohol, proteins were removed by centrifugation and the clear supernatant fluid was analyzed by HPLC. The percentage of midazolam metabolism in Cyclosporin A-treated incubates relative to control conditions was plotted against the Cyclosporin A concentration.

Effect of Cyclosporin A on midazolam metabolism by rabbit liver microsomes

Liver microsomes prepared from erythromycin-treated rabbits and at a final concentration of 0.5 mg/ml were exposed for 30 min to 5 μg/ml (16.5 μM) ^{14}C-midazolam in the absence or the presence of various Cyclosporin A concentrations ranging between 1.2 and 241 μg/ml (1 and 200 μM). Cyclosporin A was chosen on the basis of previous studies which demonstrated that its metabolism was predominantly dependent on a single isozyme of cytochrome P-450 [10, 16].

As illustrated in Fig. 7, Cyclosporin A inhibited the metabolism of midazolam in a concentration-dependent manner. The affinity of midazolam for cytochrome P-450 LM_{3c} was compared to those reported for Cyclosporin A and erythromycin (Table 3). Moreover among the various specific substrates of the cytochrome P-450 LM_{3c} isozymes(s), i.e. midazolam, Cyclosporin A [11] and erythromycin [6–9], midazolam exhibits the highest affinity (higher V_{max}/K_m ratio) for cytochrome P-450.

DISCUSSION

Midazolam is an imidazobenzodiazepine which has been intensively studied in our laboratory. Its pharmacokinetic as well as its metabolic behavior have already been reported in the literature [17, 18].

The data presented in this paper allow us to demonstrate that the cytochrome P-450 LM_{3c} form was predominantly involved in the metabolism of midazolam by rabbit liver microsomes. This conclusion was drawn from the following experiments: (i) among various microsomal preparations obtained

Table 3. Comparison of kinetic constants of midazolam, Cyclosporin A and erythromycin as substrates for cytochrome P-450

Substrate	K_m (μM)	K_i (μM)	V_{max} (nmoles/min/mg)	V_{max}/K_m
Midazolam	8.3 ± 0.7	—	66	7.95
Cyclosporin A*	0.4 ± 0.1	—	2.07 ± 0.3	4.81
Erythromycin*	—	156 ± 51	—	—

* From Ref. 11.

from livers of untreated or inducer-treated rabbits, only preparations from animals treated with specific cytochrome P-450 LM_{3c} inducers (erythromycin, triacetyloleandomycin and rifampycin) [19] extensively metabolized midazolam. The other microsomal preparations generated only low levels of hydroxylated derivatives; (ii) similarly among this library of treated and untreated rabbit liver microsome preparations, only microsomes prepared from macrolide antibiotic-treated rabbits exhibited a Type I binding spectrum in the presence of midazolam. The absence of spectral changes with the other preparations could not be attributed to any kind of denaturation since, in control experiments, these microsomes exhibited Type I or Type II binding spectra with their known substrates, i.e. benzphetamine (LM_2), aniline (LM_{3a}), acetanilide (LM_4 and LM_5) and benzo-*a*-pyrene (LM_6); (iii) anti-cytochrome P-450 LM_{3c} antibodies strongly inhibited the metabolism of midazolam in liver microsomes prepared from erythromycin-treated animals. Furthermore anti-cytochromes P-450 LM_2, LM_4 and LM_6 antibodies had no effect on this metabolism. This lack of effect cannot be related to the functionality of these antibodies since, at the concentrations used, they can efficiently inhibit the biotransformation of substrates specifically metabolized by cytochromes LM_2, LM_4 or LM_6 isozymes [10]; (iv) Cyclosporin A, which is a specific substrate of the cytochrome P-450 LM_{3c} isozyme, inhibited midazolam metabolism in a concentration-dependent manner.

Another argument in favor of a direct implication of the cytochrome P-450 LM_{3c} isozyme in the metabolism of midazolam was provided by the effect of potassium ferricyanide treatment on the activity of microsomes prepared from livers of TAO-treated rabbits. Different authors have already shown that in the liver of these animals, the cytochrome P-450 isozyme induced (P-450 LM_{3c} in the rabbit) specifically and irreversibly binds a TAO-metabolite (presumably a nitroso derivative) to form an inactive complex [6, 8]. Treatment of these microsomal preparations with potassium ferricyanide broke down the complex and restored the functional cytochrome P-450 LM_{3c} isozyme. Hence when microsomes prepared from TAO-treated rabbit were incubated with potassium ferricyanide, there was a 26% increase in midazolam metabolism. This percentage agreed well with the amount of cytochrome P-450 LM_{3c} isozyme complexed with the TAO-metabolite, which was spectrally estimated to be around 20%.

The rapid metabolism of midazolam ($V_{max} = 20\ \mu g$ (66 nmoles) midazolam metabolites formed/min/mg microsomal proteins) by microsomal preparations from macrolide antibiotic-treated rabbits correlated well with the short duration of action of this drug [20]. Moreover, the similarity in spectral dissociation ($K_s = 3.8 \pm 0.9\ \mu g/ml$; $12.54 \pm 2.97\ \mu M$) and Michaelis–Menten ($K_m = 2.52 \pm 0.20\ \mu g/ml$; $8.32 \pm 0.73\ \mu M$) constants for midazolam, using microsomes prepared from rifampicin-treated rabbits, led us to the conclusion that the Type I spectral change reflects the formation of a true cytochrome P-450-midazolam complex as mentioned by Shenkman *et al.* [21].

In conclusion, these results demonstrate that cytochromes P-450 LM_2, LM_4 and LM_6 were not involved in midazolam metabolism while cytochrome P-450 LM_{3c} accounted for most of the forms involved in the formation of the different metabolites.

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