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In-vivo kinetics of the interaction between midazolam and erythromycin in rats, taking account of metabolic intermediate complex formation

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Abstract

To predict, quantitatively, the extent of drug interaction during repeated administration of a metabolic inhibitor, we analysed the effects of erythromycin treatment under several regimens on the area under the concentration curve (AUC) of midazolam in rats. Midazolam was administered into the portal vein 12 h after erythromycin treatment for 1, 2 or 3 days, or 12, 24, 36, 48, 72 and 96 h after erythromycin treatment for 4 days, and the plasma-concentration profiles of midazolam were analysed to assess the AUC. Moreover, the contents of total cytochrome P450 and inactive metabolic intermediate (MI) complex were simultaneously quantitated. While the AUC value of midazolam was not affected by the administration of erythromycin for 1 day, repeated administration of erythromycin evoked an increase in AUC ratio (AUC in erythromycin-treated rats/AUC in vehicle-treated rats), which reached a maximum value of 1.99 at 12 h after 4 days' treatment with erythromycin. The total content of cytochrome P450 in liver microsomes was unaffected by erythromycin treatment. Although the MI complex was undetectable after 1 day's treatment with erythromycin, its content increased with duration of erythromycin treatment, and the complex disappeared after the end of erythromycin treatment with a half-life of 12.3 h. In conclusion, the interaction between erythromycin and midazolam could be well predicted when the formation of MI complex in the liver was taken into account.

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Introduction

Most pharmacokinetic drug interactions are caused by inhibition of metabolism in the liver, and this may lead to serious adverse reactions. The mechanisms of these interactions in the liver can be classified into three types: competitive inhibition by metabolic inhibitors; mechanism-based inhibition evoked by the formation of a stable inactive complex of enzyme and inhibitor (or its metabolite); and change in enzyme content during chronic treatment with an inhibitor. We have attempted to predict, quantitatively, the extent of interaction based on competitive inhibition. We have reported, using histamine H_2 antagonists and midazolam as inhibitors and a probe, respectively, that it is essential to employ the unbound concentration of inhibitor in the liver as an input parameter for the accurate prediction of drug interaction (Takedomi et al 1998; Yamano et al 1998, 2000). However, mechanism-based inhibition is also clinically significant since many interactions based on this mechanism have been reported (Pessayre et al 1982; Larrey et al 1983; Byatt et al 1984; Hiller et al 1990; Olkkola et al 1993; Spinler et al 1995; Thomas et al 1999).

Macrolide antibiotics are metabolized by cytochrome P450 3A to produce a metabolic intermediate, which binds to cytochrome P450 and forms a stable and inactive complex (metabolic intermediate (MI) complex) (Delaforge et al 1983; Babany et al 1988). Recently, calcium antagonists such as diltiazem were reported to inhibit cytochrome P450 activity via mechanism-based inhibition in human liver microsomes (Jones et al 1999; Ma et al 2000). For mechanism-based inhibition, it may not be rational to estimate the extent of drug interaction simply from the inhibitory constant (K_i) and the unbound concentration of inhibitor in the liver by using a model based on competitive inhibition.

In this study, using midazolam as an inhibited drug and erythromycin as an inhibitor, we aimed to estimate, quantitatively, the extent of drug interaction and the contents of total cytochrome P450 and MI complex in the liver during and after repeated administration of erythromycin. We also assessed the feasibility of predicting the drug interaction in-vivo from the change in the content of MI complex.

Materials and Methods

Chemicals

Erythromycin was purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). Injection solution of erythromycin was prepared with corn oil by the method described by Frankline (1991). Midazolam injection (Dormicum Injection 10 mg/2 mL) was purchased from Yamanouchi Pharmaceutical Co. (Tokyo, Japan) and given by intraportal-venous administration. Anti-rat cytochrome P450 3A2 antibody was purchased from Daiichi Kagaku Yakuhin Co. Ltd (Tokyo, Japan). All other reagents used were of reagent grade or HPLC grade.

Animals

Male Sprague-Dawley rats (7–8 weeks) were purchased from Seac Yoshitomi Co. (Fukuoka, Japan). Rats were allowed free access to water and food pellets.

The effects of erythromycin on the pharmacokinetics of midazolam

Erythromycin (500 mg kg^{-1}, once a day) was intraperitoneally administered for 1, 2, 3 or 4 days in groups 1, 2, 3 or 4–9, respectively. Control rats in each group received vehicle (corn oil) for the same period. In groups 1, 2 and 3, midazolam (10 mg kg^{-1}) was administered into the portal vein by bolus injection 24 h after the last administration of erythromycin. In groups 4, 5, 6, 7, 8 and 9, midazolam (10 mg kg^{-1}) was administered into the portal vein by bolus injection 12, 24, 36, 48, 72 and 96 h after the last administration of erythromycin. Before the administration of midazolam, rats were cannulated through the portal vein and femoral artery under light ether anaesthesia. Blood samples were collected at 2, 5, 10, 30, 60, 120 and 180 min after the administration of midazolam, and centrifuged at 1000 *g* for 5 min to obtain plasma, which was stored at −20°C until analysed.

Kinetics of erythromycin

Erythromycin (500 mg kg^{-1}, once a day) was intraperitoneally administered for 1, 2, 3 or 4 days. Concentrations of erythromycin in the plasma and the liver were analysed 24 h after erythromycin treatment for 1, 2 and 3 days, and at 24, 48, 72 and 96 h after erythromycin treatment for 4 days. Rats were cannulated through the femoral artery under light ether anaesthesia. The liver was removed after the sampling of blood from the femoral artery. Blood samples were centrifuged at 1000 *g* for 5 min to obtain plasma, which was stored at −20°C until analysed.

Determination of erythromycin and midazolam in plasma and erythromycin in the liver

For determination of midazolam, 0.1 mL of plasma was spiked with 0.1 mL of methanol, 0.5 mL of 1 N NaOH and 3 mL of n-hexane. After having been shaken for 5 min, the mixture was centrifuged at 1000 *g* for 5 min to separate the organic layer. The organic layer (2 mL) was transferred into another tube and dried under a gentle nitrogen stream. The residue was reconstructed with 200 μL of eluent and subjected to high-performance liquid chromatography (HPLC). The HPLC system consisted of a pump LC-9A (Shimadzu, Kyoto, Japan) and a spectrophotometer SPD-10AV (Shimadzu, Kyoto, Japan). A reversed-phase column, Inertsil ODS (4.6 mm i.d. × 250 mm; GL Science, Osaka, Japan), equipped with a guard column (ODS-AM) was maintained at 40°C and used for separation. The eluent was acetonitrile–10mM phosphate buffer (pH 6.5), 8:2 v/v, pumped at a constant rate of 1.0 mL min^{-1}. The absorbance of the eluate was monitored at a wavelength of 220 nm. The detection limit was 50 ng mL^{-1}.

For determination of erythromycin, 0.1 mL of plasma or 0.5 mL of 20% liver homogenate (prepared with

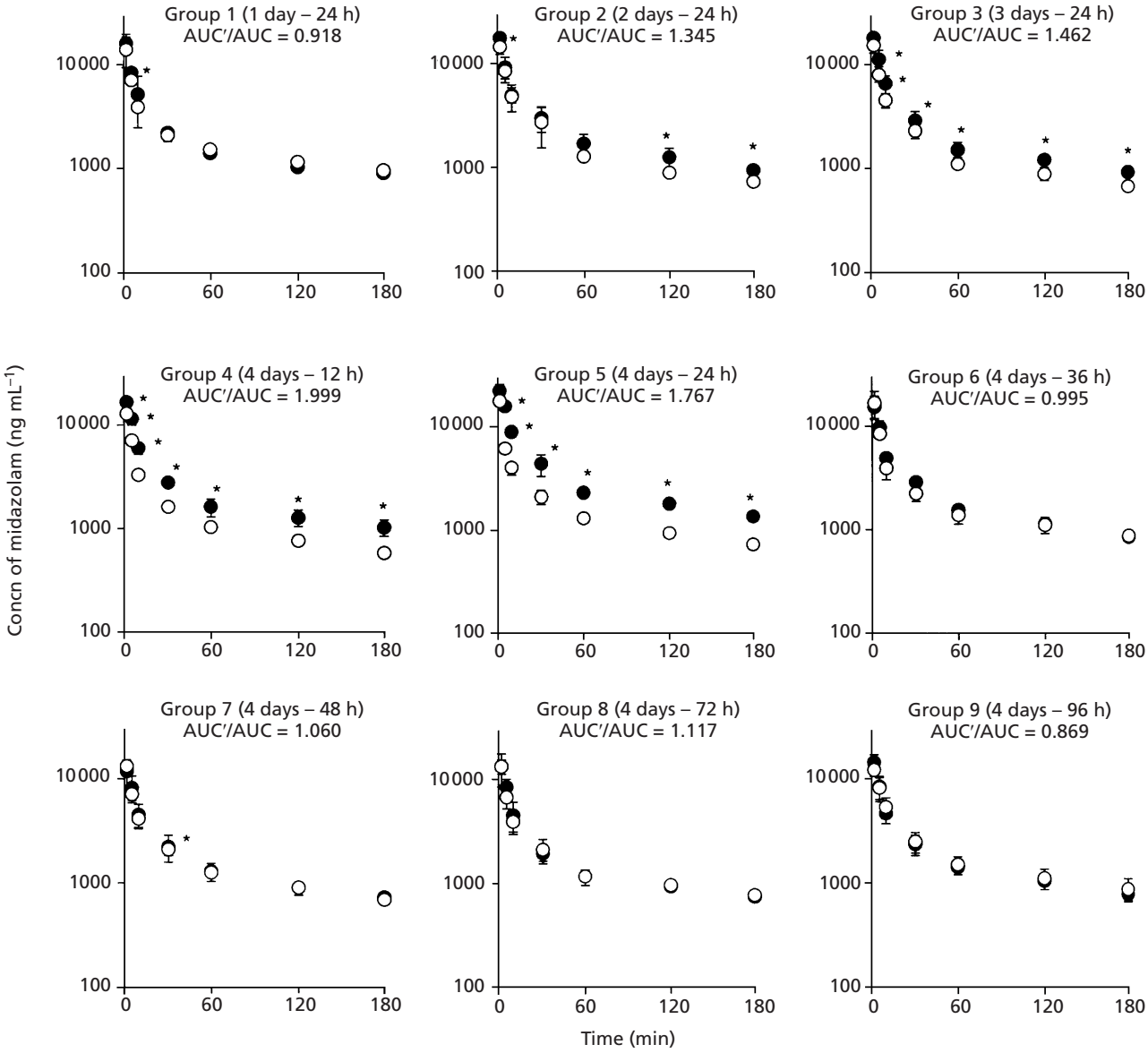

Figure 1 Effects of erythromycin treatment on the kinetics of midazolam in rats. Erythromycin was administered intraperitoneally at a dose of 500 mg kg^{-1}; midazolam was administered through the portal vein at a dose of 10 mg kg^{-1}. AUC′ and AUC indicate the area under the time–concentration curve of midazolam in the presence and absence of erythromycin, respectively. Number of days and h in parenthesis represent the period of erythromycin treatment and the time after the last dose of erythromycin, respectively. ○, Plasma concentration of midazolam in the absence of erythromycin; ●, plasma concentration of midazolam in the presence of erythromycin. Statistical significances were determined by Student's *t*-test (*$P < 0.05$). Each point represents the mean ± s.d., n = 3–7.

saline) was spiked with 0.1 mL of 0.5 M NaOH and 3 mL of *t*-butyl methyl ether. After shaking and centrifugation at 1000 *g* for 5 min, 2 mL of the upper organic layer was transferred to another tube. The organic layer was dried under a gentle nitrogen stream. The residue was dissolved in 50 μL of the eluent and a 20-μL sample was applied to the HPLC system. The HPLC system consisted of a pump LC-10AD (Shimadzu, Kyoto, Japan). The detection potential was set at 1000 mV. A reversed-phase column, Tskgel-80TM ODS (4.6 mm i.d. × 150 mm; Tohso, Tokyo, Japan), was maintained at 30°C and used for the separation. The eluent consisted of acetonitrile–0.1 M phosphate buffer (pH 6.4), 50:50 v/v, pumped at a constant rate of 1.0 mL min^{-1}. The detection limits in the plasma and the liver were 0.2 and 1.0 μg mL^{-1}, respectively.

Preparation of liver microsomes

Rat liver microsomes were prepared by the method of Kremers et al (1981). Briefly, the rat liver was minced and homogenized on ice with three volumes of 1.15% KCl. The homogenate was centrifuged at 9900 *g*, 4°C, for 60 min. The microsomal pellets were further homogenized with 1.15% KCl and centrifuged again at 105000 *g*, 4°C, for 60 min. The obtained microsomal pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing glycerin. The content of protein was determined by the method of Lowry et al (1951). The microsomal suspension was diluted with 50 mM Tris-HCl buffer (pH 7.4) to give a final protein content of 10 mg mL^{-1} and stored at −80°C until the experiment.

Analysis of in-vivo formation of cytochrome P450 metabolic intermediate (MI) complex

The time courses of the contents of total cytochrome P450 and MI complex in rat liver microsomes were analysed in groups 1–9. The liver microsomes were prepared from rats 24 h after the last administration of erythromycin given for 1, 2 and 3 days, and 12, 24, 36, 48, 72 and 96 h after the last administration of erythromycin given for 4 days. The microsomes were suspended in assay buffer (0.01 M phosphate buffer containing 0.15 M KCl, pH 7.4) to give a final protein content of 2 mg mL^{-1} and used for quantitation of the content of MI complex and total cytochrome P450. The contents of MI complex and total cytochrome P450 were determined by the method previously described by us (Yamano et al 2000). This method was based on the following characteristics of MI complex: it does not bind to carbon monoxide (CO); it absorbs at 456 nm; and it is restored to active cytochrome P450 by potassium ferricyanide (Omura & Sato 1962; Danan et al 1981; Delaforge et al 1988; Pessayre et al 1983; Frankline 1991).

Immuno-inhibition study using anti-rat cytochrome P450 3A2 antibody

Immuno-inhibition was carried out using rat liver microsomes. A mixture of 40 μL of microsome suspension, 40 μL of anti-rat cytochrome P450 3A2 antibody solution, 20 μL of 100 mM glucose-6-phosphate, 20 μL of 20 mM nicotinamide adenine dinucleotide-phosphate, 20 μL of 20 U mL^{-1} glucose-6-phosphate, 4 μL of 10 mM di-sodium dihydrogen ethylenediamine, 16 μL of 125 mM magnesium chloride and 200 μL of 0.2 M sodium–potassium buffer was pre-incubated at 37°C for 10 min. The enzymatic reaction was initiated by adding 40 μL of midazolam solution to give an initial concentration of 5 μM. The reaction was terminated by adding 400 μL of ice-cold acetonitrile. The solution was centrifuged at 1000 *g* for 2 min. The concentration of midazolam in the supernatant was determined by the HPLC method described above.

Statistics

Statistical significance was determined by Student's *t*-test or analysis of variance followed by Dunnett's multiple comparisons test, and a P value < 0.05 was considered to be significant.

Results

The effects of erythromycin on the pharmacokinetics of midazolam in-vivo

Figure 1 shows the time courses of plasma midazolam concentration after bolus administration of midazolam in the portal vein in each group of rats. Figure 2 shows the time course of an increase in area under the concentration curve (AUC) ratio (AUC in the erythromycin-treated rats/AUC in the vehicle-treated rats). On the first day (group 1), AUC was not affected

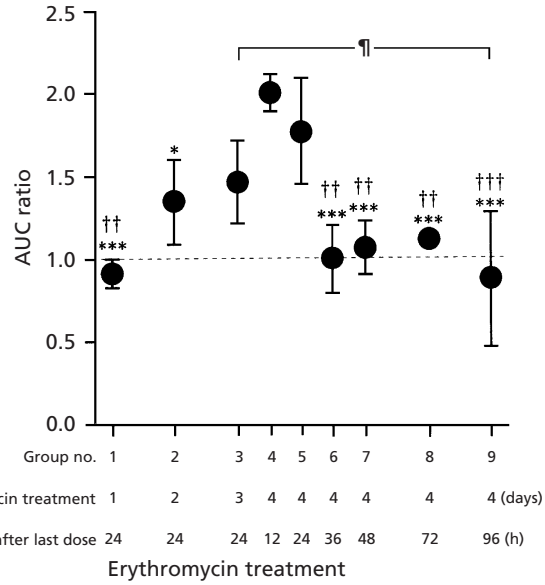

Figure 2 Increase in AUC of midazolam by several treatment regimens of erythromycin. Each point represents the mean ± s.d., n = 3–6. Statistical significances were determined by analysis of variance and Bonferroni's multiple comparisons test. *$P < 0.05$ vs group 4, ***$P < 0.001$ vs group 4, ††$P < 0.01$ vs group 5, †††$P < 0.001$ vs group 5; ¶$P < 0.05$.

by erythromycin treatment. However, with increase in the period of erythromycin treatment, the AUC ratio increased to 1.35, 1.46, 1.77 and 1.79 in groups 2, 3, 4 and 5, respectively (Figure 2). This increase diminished in a time-dependent manner after termination of erythromycin treatment (groups 6–9 in Figure 2).

Kinetics of erythromycin in the plasma and the liver

The mean concentrations of erythromycin in the plasma and the liver increased gradually and reached 0.25 μg mL^{-1} and 4.72 μg mL^{-1}, respectively, after treatment for 4 days (Figure 3). However, at 48 h after the last treatment it fell below the detection limit in both plasma and liver.

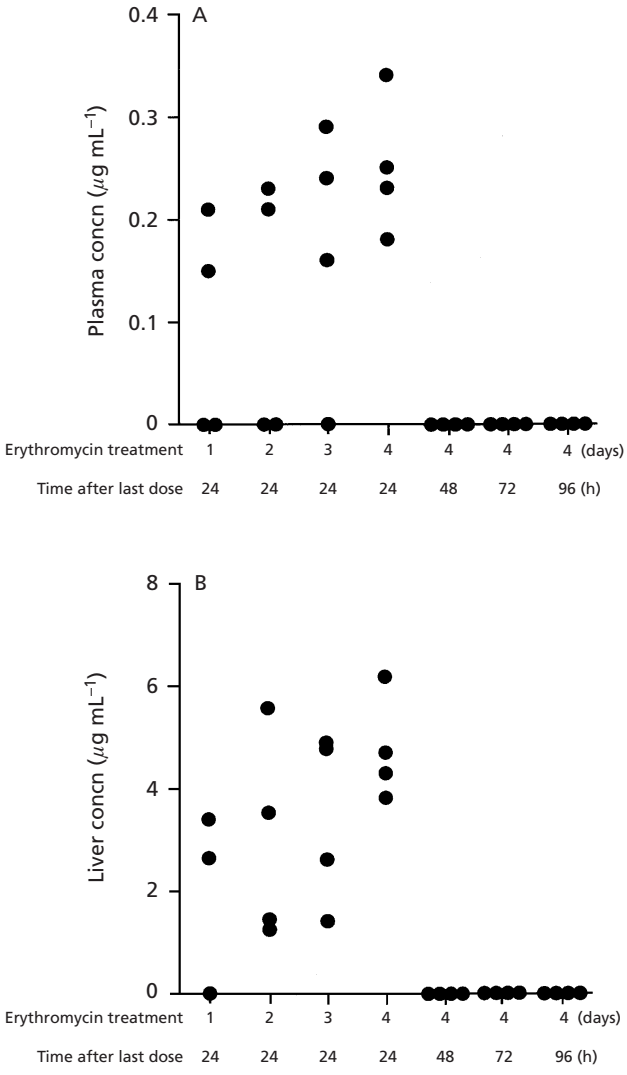

Figure 3 Concentrations of erythromycin in plasma (A) and liver (B) during and after repeated intraperitoneal administration of erythromycin (500 mg kg^{-1}, once a day) for 1–4 days in rats. Each point represents an individual value.

Formation of MI complex in-vivo by erythromycin treatment

Figure 4 presents the time courses of the contents of MI complex and total cytochrome P450 in rat liver micro-

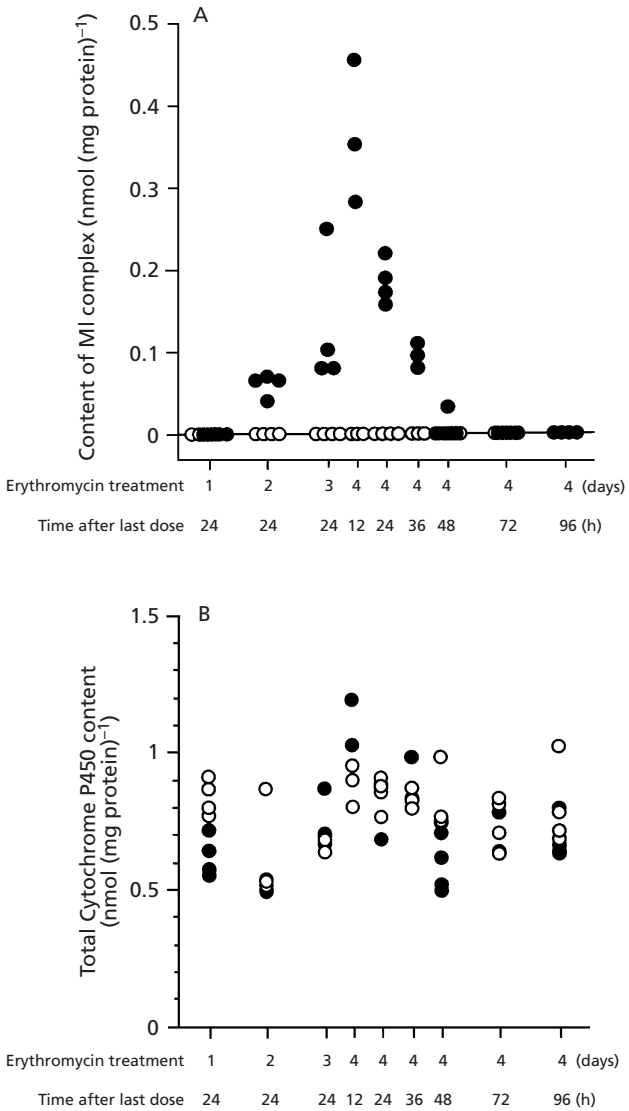

Figure 4 Time-courses of the contents of MI complex (A) and total cytochrome P450 (B) in rat liver microsomes during and after repeated intraperitoneal administration of erythromycin (500 mg kg^{-1}, once a day) for 4 days. The contents of MI complex and total cytochrome P450 were determined by conventional methods. ○, Vehicle-treated; ●, erythromycin-treated rats. Each point represents an individual value.

somes. While formation of MI complex was not detected on the first day, the content of MI complex increased with repetition of erythromycin treatment. MI complex disappeared within 48 h after the last administration of erythromycin. In contrast, the total cytochrome P450 content was not affected by erythromycin treatment.

Immuno-inhibition by anti-rat CYP3A2 antibody

The contribution of cytochrome P450 3A to the metabolism of erythromycin was assessed in microsomes prepared from untreated rats by making use of anti-rat cytochrome P450 3A2 antibody (Figure 5). Since the metabolism of midazolam was inhibited by 61–71%, this reaction is predominantly mediated by cytochrome P450 3A.

Relationship between the AUC ratio and the content of MI complex

Figure 6 presents the relationship between the increase in AUC of midazolam and the content of MI complex throughout the experimental period. There was a linear relationship between the AUC ratio and the content of MI complex ($r^2 = 0.832$).

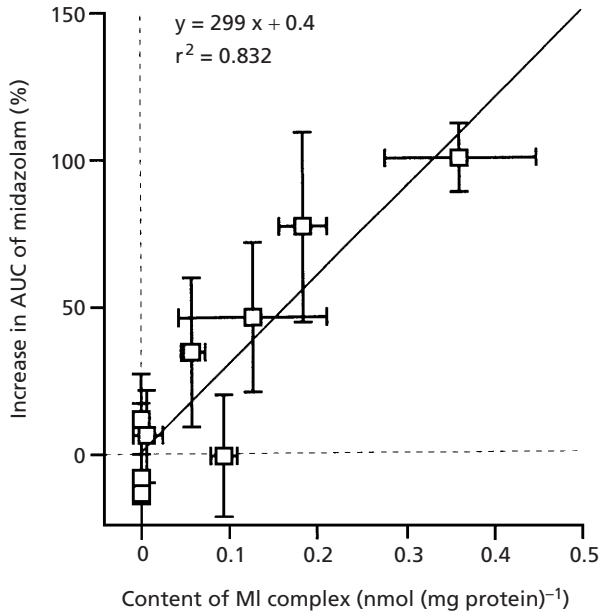

Figure 6 Relationship between the content of MI complex in rat liver microsomes and the increase in AUC of midazolam. Each point represents the mean ± s.d., n = 3–6.

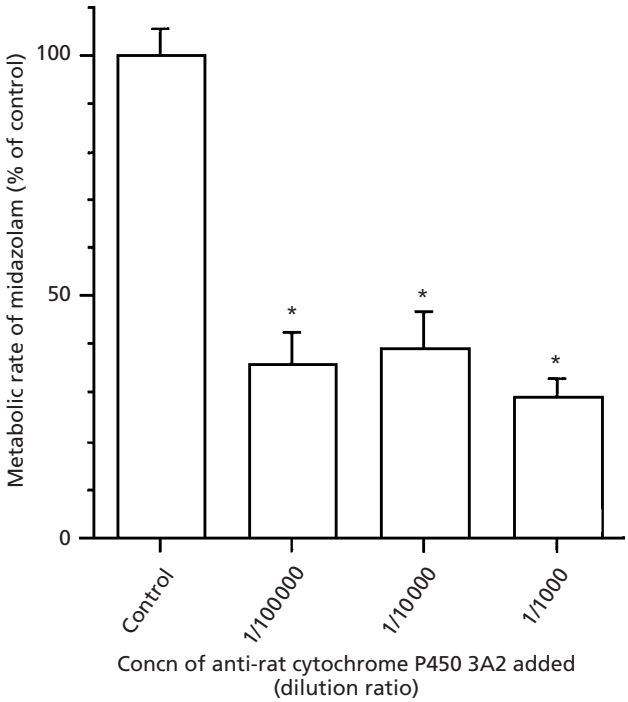

Figure 5 Effect of anti rat-cytochrome P450 3A2 antibody on the rate of metabolism of midazolam in rat liver microsomes. Statistical significances of differences from the control were determined by analysis of variance and Bonferroni's multiple comparisons test (*$P < 0.01$). Each column indicates the mean ± s.e.m. from 3 or 4 experiments.

Estimation of the half-life of MI complex

Figure 7 shows a semilogarithmic plot of the elimination profile of MI complex after the final dose of the 4-day course of erythromycin. The elimination half-life was calculated to be 12.3 h.

Discussion

We have attempted to estimate, quantitatively, the extent of drug interaction evoked by metabolic inhibition in the liver, and demonstrated that, for a competitive inhibitor, the extent of interaction is predictable by integrating the inhibitory constant (K_i value) of the inhibitor and the unbound concentration of the inhibitor in the liver, instead of that in the plasma (Takedomi et al 1998; Yamano et al 1998, 2000). However, some inhibitors are transformed into metabolites which covalently bind to cytochrome P450 and form a stable MI complex, resulting in reduced metabolism (i.e. mechanism-based inhibition). Many cases of drug interaction based on mechanism-based inhibition have

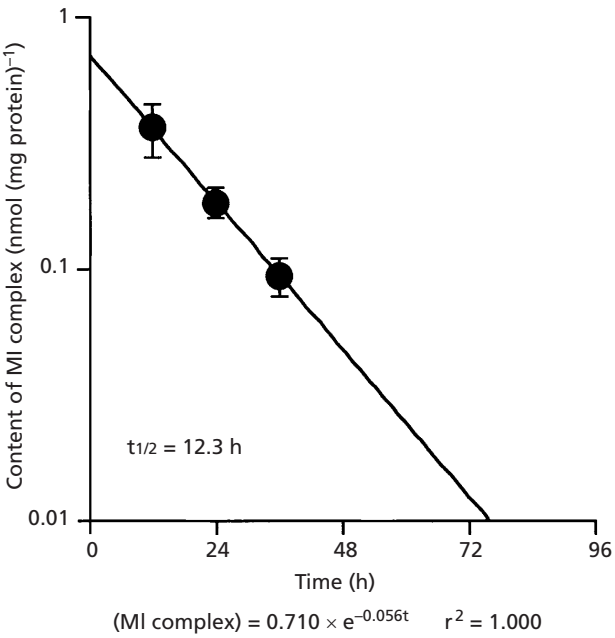

Figure 7 Time-course of the content of MI complex in rat liver microsomes after repeated intraperitoneal administration of erythromycin (500 mg kg^{-1}, once a day) for 4 days. The elimination rate constant was calculated to be 0.056.

been reported (Pessayre et al 1982; Larrey et al 1983; Byatt et al 1984; Hiller et al 1990; Olkkola et al 1993; Spinler et al 1995; Thomas et al 1999). For mechanism-based inhibition, the extent of interaction can not be predicted from the K_i value and the concentration of inhibitor, and the change in the content of MI complex also has to be taken into consideration.

As shown in Figures 1 and 2, the AUC of midazolam administered into the portal vein of rats was not affected by a single (one day) administration of erythromycin (group 1). However, the AUC ratio was increased by repeated administration (groups 2–5) and decreased after the termination of the administration (groups 6–9). In rat liver microsomes in-vitro, under conditions where mechanism-based inhibition did not occur, erythromycin inhibited the metabolism of midazolam competitively with a K_i value of 179 μM (Yamano et al 2000). The estimated maximum unbound concentration of erythromycin in the liver during this study was 3.37 μM, far lower than the above K_i value, suggesting that the effects of competitive inhibition are negligible in our study. In fact, the AUC of midazolam was not affected on the first day (group 1). Therefore, the altered kinetics of midazolam during the repeated administration of erythromycin is attributable to the inactivation of cytochrome P450. Indeed, erythromycin is known to be metabolized by cytochrome P450 and converted into a metabolite, which binds to cytochrome P450 and forms the stable MI complex, thereby impairing the enzymatic activity of cytochrome P450 (Delaforge et al 1983, 1988; Babany et al 1988). The MI complex appeared with a distinct lag period after the exposure of the enzyme to the substrate; in this study, it took more than 24 h to appear (Figure 4A). These data suggest that the MI complex may not be formed in a conventional in-vitro study. Indeed, we found in an in-vitro study using liver microsomes, that pre-incubation with erythromycin for 20 min did not affect the maximal rate (V_{max}) or the Michaelis-Menten constant (K_m) (Yamano et al 2000). On the other hand, the total cytochrome P450 contents of microsomes were similar in the erythromycin-treated and vehicle-treated groups, suggesting that differences in the total cytochrome P450 content are unlikely to have been important in this study. As the metabolism of midazolam in the liver was essentially determined by cytochrome P450 3A (Figure 5), we did not separately determine the content of each isoform of cytochrome P450. Therefore, we can not rule out the possibility that a decrease in the cytochrome P450 3A isoform was accompanied with an increase in other cytochrome P450 isoforms.

Furthermore, we analysed the relationship between the AUC ratio of midazolam and the microsomal content of MI complex and found a good correlation (Figure 6). An increase in AUC proportionally reflects a decrease in hepatic intrinsic clearance, because midazolam was administered into the portal vein. Therefore, the impaired hepatic intrinsic clearance is considered to be explained quantitatively by the formation of MI complex.

After completion of the 4-day erythromycin treatment, the content of MI complex in rat liver microsomes declined in a first-order process with an elimination half-life of 12.3 h, which is virtually equal to the value (12.0 h) reported as the turnover rate of cytochrome P450 in rats by Shiraki & Guengerich (1984).

The results of this study may be applicable to man if the turnover rate of human cytochrome P450 is available. Takanaga et al (2000a, b) concluded, from an analysis of the interactions between grapefruit juice and calcium antagonists (felodipine and nisoldipine) that the cytochrome P450 3A in human gut, inactivated by grapefruit juice, disappeared in a first-order process with an elimination half-life of 8.16–30.1 h. Though the turnover rate of cytochrome P450 in gut may possibly be different from that in the liver, time-courses of drug

interaction based on mechanism-based inactivation may be quantitatively predicted if the turnover rate of the enzyme in the liver can be determined accurately. However, it has been reported that there are interspecies differences in the formation of MI complex. Yamazaki et al (1996) reported that liver microsomes in man formed MI complex with erythromycin within 20 min, while formation of MI complex with erythromycin was not detected in rats within 20 min in-vitro. Therefore, formation kinetics of MI complex in rats may be different from that in man, and it may be difficult to predict formation kinetics of MI complex in man from that in rats.

In conclusion, the effects of erythromycin on the AUC of midazolam in rats appeared on the second day of repeated administration of erythromycin and disappeared within 24 h of completing 4 days of erythromycin treatment. Similarly, the content of MI complex in liver microsomes increased during the repeated erythromycin treatment and was eliminated after the end of the treatment with a half-life of 12.3 h. Therefore, the interaction between erythromycin and midazolam in rats can be attributed to the formation of MI complex. The extent of interaction between erythromycin and midazolam can be well predicted by taking into consideration the formation of MI complex in the liver.

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