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Autoinduction of MKC-963 metabolism in healthy volunteers and its retrospective evaluation using primary human hepatocytes and cDNA-expressed enzymes

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Running title:

In vitro evaluation of autoinduction

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Abbreviations used are: AUC, area under the plasma concentration-time curve; C_{max}, maximum plasma concentration; t_{max}, time to reach C_{max}; $t_{1/2}$, terminal half-life; DMSO, dimethylsulfoxide; F, cortisol; 6β-OHF, 6β-hydroxycortisol.

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Abstract

MKC-963, (R)-1-(1-cyclohexylethylamino)-4-phenylphthalazine, a potent inhibitor of platelet aggregation, was synthesized and used in clinical trials in the 1990s. In the process of clinical study, it was found that urinary excretion ratios for 6β-hydroxycortisol and free cortisol increased significantly in parallel with decreases in the plasma concentrations of MKC-963 after repeated oral administration of the compound to healthy volunteers. These findings suggested that MKC-963 caused autoinduction in humans, and clinical studies using the compound were stopped. This experience prompted us to reevaluate the effects of this compound on CYP3A4 using primary human hepatocytes and cDNA-expressed human CYP enzymes in order to determine whether the autoinduction of MKC-963 metabolism in humans could have been predicted if these *in vitro* systems had been used for the evaluation of MKC-963 in the preclinical study. The results of *in vitro* study showed that MKC-963 increased CYP3A4 mRNA expression level and activity of testosterone 6β-hydroxylation to extents similar to those observed with rifampicin in primary human hepatocytes. In addition, approximately 90% of the MKC-963 metabolism in human liver microsomes was estimated to be attributable to CYP3A4. These *in vitro* findings are in good agreement with the results of clinical study, suggesting that studies using human hepatocytes and cDNA-expressed human CYPs are useful for assessing the autoinductive nature of compounds under development before starting clinical studies.

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MKC-963, (R)-1-(1-cyclohexylethylamino)-4-phenylphthalazine (Fig.1), a potent inhibitor of platelet aggregation, was synthesized and used in clinical trials by Mitsubishi Chemical Corp. (Tokyo, Japan) in the 1990s. In the process of clinical trials, the urinary excretion of 6β-hydroxycortisol (6β-OHF) and free cortisol (F) was studied after repeated oral administration of MKC-963 in human volunteers to determine whether this compound induces CYP3A4 or not. This was because the compound would be used for treatment of circulatory disorders together with drugs such as antihypertensives, antihyperlipidemics or antidiabetes. Many of these drugs are metabolized by CYP3A4 (Li et al., 1995; Lehmann et al., 1998; Prueksaritanont et al., 2003; Jerling et al., 2005), a predominant CYP enzyme found in the adult human liver that catalyzes the oxidation of a wide variety of exogenous compounds (Guengerich et al., 1986). In addition, CYP3A4 had been reported to be induced by several drugs, including rifampicin, phenytoin and phenobarbital, that caused clinical drug-drug interactions (Holtbecker et al., 1996; Anderson 1998; Ridtitid et al., 2002). Besides, measurement of the urinary ratio of 6β-OHF and F (6β-OHF/F) had been regarded as a safe and simple method for evaluating induction of CYP3A4 since it is noninvasive and does not require administration of a probe drug to volunteers (Galteau and Shamsa, 2003).

In this clinical study on MKC-963, we found that 6β-OHF/F increased significantly in parallel with decreases in the plasma concentrations of MKC-963 after repeated oral administration of the compound to healthy volunteers. This finding suggested that CYP3A4 is induced by MKC-963 and

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that the compound itself is an autoinducer in humans. Since autoinduction was thought to reduce the therapeutic response of MKC-963 and might cause clinical problems, the clinical study on MKC-963 was abandoned at that time. Thus, we have recently decided to reevaluate the effects of this compound on CYP3A4 using primary human hepatocytes and cDNA-expressing human CYP enzymes in order to determine whether we could have predicted the autoinduction of MKC-963 metabolism if we had used these *in vitro* systems for the preclinical evaluation of MKC-963.

In this manuscript, we describe the results of the clinical study on the pharmacokinetics of MKC-963 and its effects on the urinary excretion ratio of 6β-OHF and F after repeated oral administration of the compound to healthy volunteers and the results of *in vitro* studies on the effects of MKC-963 on the expression and activities of CYP3A4 and identification of CYP enzyme(s) responsible for the metabolism of MKC-963 using primary human hepatocytes and cDNA-expressed human CYP enzymes, respectively. The results suggest that these *in vitro* systems would have been useful for the prediction of the autoinductive nature of MKC-963 in the preclinical study.

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Materials and Methods

Materials.

MKC-963 was provided by Mitsubishi Chemical Corp. (Tokyo, Japan) and its chemical purity was 99.8%. Rifampicin, testosterone and 6β-hydroxytestosterone were purchased from Sigma-Aldrich (St. Louis, MO), Tokyo Kasei Kogyo Co. (Tokyo, Japan) and Sumika Chemical Analysis Service, Ltd (Osaka, Japan), respectively. All other chemicals were of analytical reagent grade.

In Vivo Study.

Subjects

Six healthy male volunteers aged between 20 and 35 years were recruited for the study. They were within ± 20% of their ideal body weight and in good general health according to routine medical history and laboratory data. They did not use any medications for at least two weeks before and were not using any concurrent medications during the study. All of them agreed to refrain from consumption of alcohol and grapefruit or grapefruit juice during the study. Subjects who had clinically significant abnormalities on preliminary examination, those who had a history of drug or food allergies or a history of drug or alcohol abuse, and those who had donated blood or received an investigational drug within 4 months before the start of this study were excluded from this study.

Study protocol

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The subjects received a single oral dose of MKC-963 on day 1 and on day 14 and two oral doses per day with a 12-hour interval for 12 days (from day 2 to day 13). Each dose was 120 mg, and the drug was supplied as tablets (40 mg). The oral doses were administered with 100 mL water at 09.00 AM after breakfast or at 09:00 PM after dinner. Breakfast and dinners were standardized for all the subjects. Blood samples (each 4 mL) were collected by venipuncture at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours after the first administration of MKC-963 on days 1 and 14 and at 0, 1 and 2 hours on days 2, 5, 8 and 11. The blood samples collected were centrifuged at 1,500 g for 10 min at 4°C, and plasma samples were separated and stored at –20 °C until analyses. Urine samples were pooled over a period of 24 hours and collected at the end of designated days: the day before and 1, 2, 5, 8, 11 and 14 days after starting drug administration. The urine was kept cool during collection, and then the total volume was recorded and a 10-mL aliquot was stored at –20 °C until analyses.

The study was conducted at Hohsen Clinic, Research Center for Clinical Pharmacology, The Kitasato Institute (Tokyo, Japan), and the protocol was approved by the institutional review board. The study was conducted in accordance with the guidelines on good clinical practice and the ethical standards for human experimentation established by the Declaration of Helsinki. Each subject gave written informed consent.

Determination of MKC-963 concentrations in plasma

Plasma concentrations of MKC-963 were determined by liquid chromatography-tandem mass

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spectrometry (LC/MS/MS). d5MKC-963 was used as an internal standard. The plasma (0.5 mL) was mixed with 0.4 mL of titrisol buffer (pH 9) and applied on solid-phase extraction column (Extrelut-1, Merck KGaA, Damstadt, Germany). The MKC-963 and internal standard were isolated from the column with 5 mL of diethylether. The organic extract was dried under nitrogen and reconstituted in 1 mL of acetonitrile. The sample was separated by a Waters HPLC system (Waters, Milford, MA) equipped with a Capcell Pak CN column (5 µm, 35 × 4.6 mm in internal diameter, Shiseido, Tokyo, Japan). The mobile phase consisted of acetonitrile/water/acetic acid (90/10/1, v/v/v) and the flow rate was maintained at 0.2 mL/min. MKC-963 and the internal standard were detected by the tandem mass spectrometry using a Finnigan TSQ7000 mass spectrometer (Finnigan Corp., San Jose, CA). For mass spectral detection, the following precursors to product ion reactions were monitored: m/z 332.1 > m/z 222.1 for MKC-963 and m/z 337.0 > m/z 227.1 for d5MKC-963. The standard curves were linear from 0.1 ng/mL to 50 ng/mL. The interassay precision (% CV) assessed from the blank plasma to which known concentrations of the analytes was added (final concentrations of 0.1 ng/mL to 50 ng/mL) ranged from 2.0% to 7.7%. The limit of sensitivity of the assay was 0.01 ng/mL.

Pharmacokinetic parameters

The pharmacokinetic parameters of MKC-963 were estimated by non-compartmental methods with the use of WinNonlin V4.1 (Scientific Consulting, Inc, Apex, NC). The values of C_{max} and t_{max} were determined directly from the plasma concentration-time profiles. The area under the plasma

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concentration-time curve (AUC) from 0 to 24 hours was determined by the linear trapezoidal rule from the beginning of drug administration to the last quantifiable data point. The value of $t_{1/2}$ was calculated by linear regression analysis of the last elimination phase after log transformation of the data.

Determination of urinary 6β-OHF and F

Determination of 6β-OHF and F in urine samples was performed by using enzyme immunoassay kits for urinary 6β-OHF (Stabiligen, Villers-Les-Nancy, France) and F (Biométreux, Marcy l'Etoile, France), respectively, according to the manufacturer's instructions. The cross-reactivity of these kits for urinary F and 6β-OHF were 4.4 and 1.1 %, respectively.

***In Vitro* Study.**

Human primary hepatocytes and treatment with MKC-963

Cryopreserved human hepatocytes (Lot 100, female Caucasian, 74 years old) were obtained from In Vitro Technologies, Inc. (Baltimore, MD, USA). Hepatocytes were suspended in Hepatocyte Culture Medium (Cambrex, Walkersville, MD, USA), centrifuged at 50 × g for 3 min, and resuspended in the same medium. The cells were plated onto Matrigel-coated 24-well plates at a density of 1.5×10^5 cells/well and were maintained in an atmosphere of 95% air and 5% CO_2 at 37 °C. The cell viability was more than 80% assessed by a trypan blue exclusion test. Stock solutions of MKC-963 and rifampicin were prepared in DMSO and were diluted prior to each use.

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Treatments of hepatocytes with chemicals were begun on the fourth day after seeding and continued for 4 days. The hepatocytes were treated with DMSO (final concentration of 0.2%), rifampicin (10 μM), a positive control or MKC-963 (0.25 μM). The concentration of MKC-963 used in the present study was determined considering that C_{max} of MKC-963 was 0.29 μM when 120 mg of MKC-963 was administered orally to human volunteers (Fig. 2). The concentration of rifampicin used in the present study also corresponded nearly to C_{max} of rifampicin after an oral administration of 450-600 mg in patients with tuberculosis (Smith, 2000).

RNA extraction and real-time PCR

Total RNA was extracted using Trizol Reagent (Invitrogen Corp, Carlsbad, CA) according to the manufacturer's instructions. All samples were stored at –80 °C until used for cDNA preparation. One microgram of total RNA was reverse-transcribed into cDNA with random hexamers using a Superscript 2 Transcription system (Invitrogen Corp) according to the manufacturer's instructions. The expression levels of specific mRNAs were determined by using a quantitative real-time PCR method. The primer and fluorogenic probe sets were designed by using Primer Express software (Applied Biosystems, Foster City, CA). The sequences (5' to 3') for the primers and probes are as follows: CYP3A4, forward primer (GCA GGA GGA AAT TGA TGC AGT T), fluorogenic probe (FAM- ATA AGG CAC CAC CCA CCT A- MGB), and reverse primer (CTG AGC GTT TCA TTC ACC ACC); β-actin, forward primer (CCT GGC ACC CAG CAC AAT), fluorogenic probe (VIC-

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ATC ATT GCT CCT CCT GAG- MGB), and reverse primer (CCG ATC CAC ACG GAG TAC TTG). The sequence of fluorogenic probe for CYP3A4 was one base different from that of CYP3A5, which is recognized by MGB-probe according to the supplier's manuals. Cycling conditions of the PRISM 7900 Sequence Detection system (Applied Biosystems) were 50°C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. CYP3A4 mRNA levels in cultured human hepatocytes were expressed as ratio against β-actin mRNA levels.

Determination of CYP3A4 activities in human hepatocytes culture

CYP3A4 activities were determined by the measurement of 6β-hydroxylation activities for testosterone in intact hepatocytes cultured on 24-well plates (Donato et al., 1995). After treatment with chemicals, monolayers were incubated with testosterone (250 μM) for 30 min. Quantification of 6β-hydroxytestosterone was performed by high-performance liquid chromatography (Donato et al, 1993).

Identification of CYP enzyme(s) contributing to the metabolism of MKC-963

Recombinant P450 enzymes expressed in insect cells infected with baculovirus containing human P450 and human NADPH-P450 reductase cDNA inserts were obtained from Gentest (Woburn, MA, USA). Incubation mixtures contained cDNA-expressed CYPs (50 pmol/mL) in potassium phosphate buffer (pH 7.4), an NADPH-generating system and MKC-963. Substrate (2 μM of MKC-963) was incubated at 37 °C for 0, 5, 15 and 30 min with microsomes expressing CYP1A2,

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CYP2C9, CYP2C19, CYP2D6 or CYP3A4, and determined by LC/MS. The remaining percentage of MKC-963 was calculated using the t = 0 value as 100%. Then, *in vitro* clearance of each CYP enzyme (CL) was estimated from the following equation:

CL (μL/min/pmol P450) = - slope (1/min) / CYP concentration (pmol CYP/mL) × 1000,

where slope was determined from linear regression analysis between log percentage of MKC-963 and incubation time (Obach, 1999), and CYP concentration was the concentration of recombinant CYP enzyme in the incubation mixture. CL was corrected with the CYP contents in native human liver microsomes (Rodrigues, 1999) as follows:

Corrected CL = CL × enzyme content of each CYP.

Therefore, the contribution of each CYP enzyme to overall clearance was estimated from the following equation:

Contribution of each CYP enzyme (%) = corrected CL for each CYP enzyme / sum of corrected CL × 100.

Statistics.

Statistical analysis was performed with SAS software (version 8.2, SAS Institute, Cary, N.C.). A P value of < 0.05 was considered statistically significant.

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Results

In vivo study.

Pharmacokinetics of MKC-963

Plasma concentration-time profiles of MKC-963 showed a dramatic change after repeated oral administration of the compound (120 mg) to the healthy subjects. As shown in Fig. 2, the mean (± SD) plasma concentrations of MKC-963 on day 14 at 1 to 8 hours after administration were significantly lower than those on day 1. As a result, C_{max} and AUC values on day 14 had decreased by 77% and 69%, respectively, compared to the values on day 1 (Table 1). There were no notable differences between t_{max} and $t_{1/2}$ values on day 1 and those on day 14 (Table 1).

Figure 3 shows the changes in mean plasma concentrations of MKC-963 at 1 and 2 hours after administration from day 1 to day 14. As shown in this figure, the plasma concentrations of MKC-963 at 1 hour decreased significantly ($P < 0.05$) from day 2 to day 14, and those at 2 hours also showed significant ($P < 0.05$) decreases from day 5 to day 14.

Urinary 6β-OHF/F

Figure 4 shows the mean 24-hour urinary excretion ratios of 6β-OHF and F on the day before the start of administration and from day 1 to day 14. The mean value of 6β-OHF/F increased significantly ($P < 0.05$) from day 2 to day 14 compared to the value on the day before the start of administration, and all subjects showed increases in the urinary excretion ratios of 6β-OHF from day

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2 to day 14.

***In vitro* study.**

Primary human hepatocytes

The effects of MKC-963 (0.25 μM) on the expression of CYP3A4 mRNA and on the activity for testosterone 6β-hydroxylation were investigated using human primary hepatocytes. The effect of rifampicin (10 μM) was also investigated as a positive control. As shown in Fig. 5A, MKC-963 increased the expression level of CYP3A4 mRNA by 6 fold, comparable to the effect of rifampicin (increase of approximately 11 fold). Testosterone 6β-hydroxylation activity was also increased by 9 fold in the presence of MKC-963, which is also comparable to the effect of rifampicin (14-fold increase, Fig. 5B).

Identification of CYP enzyme(s) involved in the metabolism of MKC-963

cDNA-expressed human CYPs were used to estimate the enzyme(s) of CYP mainly responsible for the metabolism of MKC-963 in humans. As shown in Fig. 6A, MKC-963 was metabolized by CYP3A4 extensively, by 2D6 moderately and by CYP1A2 to some extent. However, when the contribution of individual CYP enzymes to the overall metabolic clearance of MKC-963 was estimated by CYP contents in human liver microsomes, approximately 90% of the MKC-963 metabolism in human liver microsomes was estimated to be attributable to CYP3A4 (Fig. 6B).

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Discussion

The results of the *in vitro* study showed that MKC-963 increased CYP3A4 mRNA expression level and activity of testosterone 6β-hydroxylation to extents similar to those observed with rifampicin in primary human hepatocytes (Fig. 5). In addition, approximately 90% of the MKC-963 metabolism in human liver microsomes was estimated to be attributable to CYP3A4 (Fig. 6B). These findings suggest that MKC-963 is a potent inducer of CYP3A4, which catalyzes the metabolism of MKC-963 itself. These *in vitro* findings are in good agreement with the results of the clinical study showing that the urinary excretion ratio of 6β-OHF and F, a marker of CYP3A4 induction, increased significantly (Fig. 4) and that AUC and C_{max} of MKC-963 decreased dramatically after repeated oral administration of the compound to healthy volunteers (Table 1). Therefore, these findings suggest that MKC-963 is a relatively potent autoinducer of CYP3A4 in humans. Moreover, the findings also suggest that *in vitro* studies using primary human hepatocytes coupled with cDNA-expressed CYP enzymes would have been useful to predict or assess the autoinductive character of MKC-963 in the preclinical study.

In the present study, we used 0.25 μM of MKC-963 since C_{max} of MKC-963 was 0.29 μM when 120 mg of MKC-963 was administered orally to human volunteers (Fig. 2). The concentration of rifamicin was also set at 10 μM since it is almost the same as C_{max} of rifampicin after oral administration of 450-600 mg in patients with tuberculosis (Smith, 2000). At that dose, interaction of

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rifampicin with a number of drugs has been reported (Holtbecker et al., 1996; Villikka et al., 1999; Ridtitid et al., 2002). Under these conditions, the effect of MKC-963 on CYP3A4 in the primary culture of human hepatocytes was comparable to the effect of rifampicin, suggesting that MKC-963 is a potent inducer of CYP3A4 similar to rifampicin even *in vivo*. This assumption is supported by the *in vivo* observation that MKC-963 increased urinary excretion ratios of 6β-OHF and F to an extent similar to that reported previously for rifampicin (Ohnhaus et al., 1989; Kovacs et al., 1998; Tran et al., 1999). Therefore, the concentration of the test compound used in the study on human hepatocytes appears to be an important factor for assessing the potential to induce CYP3A4 *in vivo* (Smith, 2000). In fact, it has been reported that thiazolidinediones, including troglitazone, pioglitazone and rosiglitazone, showed the potential to induce CYP3A4 in human hepatocytes but that only troglitazone showed drug-drug interactions due to the induction of CYP3A4 *in vivo* (Sahi et al., 2003). Although the mechanism remains unknown, the authors speculated that the concentrations of pioglitazone and rosiglitazone do not reach concentrations sufficient to induce CYP3A4 in *in vivo* situations (Sahi et al., 2003). In a preclinical study, however, the actual concentrations of test compounds in plasma or other human organs are generally unknown. Thus, prediction of concentrations of the test compound in plasma or other human organs by animal scale up (Mitsuhashi et al., 1990; Izumi et al., 1996) or by extrapolation of *in vitro* clearance obtained from human liver microsomes, human hepatocytes or cDNA-expressed CYPs to *in vivo* clearance in

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humans (Iwatsubo et al., 1997; Ito et al., 1998) appears to be essential to predict the ability of the test compound to induce CYP in *in vivo* situations.

Although the results of the present study clearly showed the induction of CYP3A4 after repeated oral administration of MKC-963, there were some differences in the time courses of changes in the indicators of induction. There was some delay in changes in the urinary ratio of 6β-OHF and F (Fig. 4) compared to those of the plasma concentrations of MKC-963 at 1 hour and 2 hours after administration (Fig. 3). As shown in Fig. 3, the plasma concentrations of MKC-963 decreased dramatically on the second day after starting repeated oral administration of MKC-963, whereas the urinary ratios of 6β-OHF and F did not show a remarkable change on day 2 but showed a considerable change on day 5 (Fig. 4). The reason for this difference in the time courses of these indicators is unknown, but it may be due to the difference in the involvement of intestinal CYP3A4 in the metabolism of MKC-963. CYP3A4 has been reported to be expressed in small intestinal epithelial cells (Watkins et al., 1987; Kolars et al., 1992) as well as in hepatic parenchymal cells (Guengerich et al., 1986; Shimada et al., 1994). Intestinal CYP3A4 has recently been suggested to be a major factor in determining the extent of first-pass metabolism and hence oral bioavailability of drugs (Hall et al., 1999). Since MKC-963 was administered orally in the present study, plasma concentration of MKC-963 should be affected by the induction of CYP3A4 in the small intestine. Although we do not know whether MKC-963 induces CYP3A4 in the small intestine one day after

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oral administration, Kolars et al. (1992) reported that rifampicin induces small intestinal CYP3A4 mRNA within 24 h. Therefore, it is conceivable that induction of intestinal CYP3A4 by MKC-963 occurred very rapidly and reduced the bioavailability of MKC-963 and decreased its plasma concentration within two days after starting drug administration. On the other hand, it has been reported that the formation of 6β-OHF is primarily mediated by the liver and that intestinal metabolism plays a minor role (Galteau and Shamsa, 2003). Therefore, it is possible that the induction of CYP3A4 in the liver is slower than that in the small intestine and that change in the urinary excretion ratio of 6β-OHF and F was therefore delayed compared to that of plasma concentration of MKC-963 after repeated oral administration. In support of this speculation, half-maximal changes in 6β-OHF/F have been reported to be achieved 2 to 3 days after rifampicin administration (Ohnhaus et al., 1989).

It should be noticed that hepatocytes used in this study were derived from one donor, therefore CYP3A4 induction may be less remarkable when other livers with low levels of CYP3A4 were used in the present study. In this case, it is possible that metabolism by CYP2D6 and CYP1A2 could be a predominant route, and autoinduction of MKC-963 metabolism may not be remarkable as observed in the present study.

Finally, CYP3A5 may also be involved in the autoinduction of MKC-963 metabolism. This is because, substrate specificities of CYP3A5 and CYP3A4 are similar and overlapped (Wrighton et al.,

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1990). In addition, CYP3A5 has been reported to be induced by rifampicin in primary human hepatocytes (Zhuo et al., 2004). However, both the catalytic activity and specific content of CYP3A5 in the human liver or small intestine are much lower than those of CYP3A4 (Wrighton et al., 1990; Rodrigues, 1999). Furthermore, the extent of induction of CYP3A5 by rifampicin in human hepatocytes is less than that of CYP3A4 (Zhuo et al., 2004). In addition, primer sets of CYP3A4 used in the present study were specific for CYP3A4 and do not recognize CYP3A5. Therefore, it seems that the contribution of CYP3A5 to the autoinductive nature of MKC-963, if any, is much smaller than that of CYP3A4.

In summary, *in vivo* findings suggesting autoinduction of MKC-963 metabolism prompted us to reevaluate the autoinductive nature of the compound using primary human hepatocytes and cDNA-expressed human CYP enzymes. The results showed that MKC-963 increased CYP3A4 mRNA expression level and activity of testosterone 6β-hydroxylation to extents similar to those observed with rifampicin in primary human hepatocytes. In addition, approximately 90% of the MKC-963 metabolism in human liver microsomes was estimated to be attributable to CYP3A4. These findings are in good agreement with the clinical study. Therefore *in vitro* studies using human hepatocytes coupled with cDNA-expressed human CYPs appear to be useful for assessing the autoinductive nature of compounds under development before starting clinical studies.

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Footnotes.

Autoinduction is defined as the ability of a drug to induce enzymes that enhance its own metabolism, resulting in dispositional tolerance.

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Legends for figures.

Fig. 1. Chemical structure of MKC-963.

Fig. 2. Plasma concentration-time profiles of MKC-963 on day 1 (open circles) and day 14 (closed circles) after oral administration of 120 mg to six healthy subjects. Data are expressed as means ± SD. *$p < 0.05$ and **$p < 0.01$.

Fig. 3. Plasma concentrations of MKC-963 at 1 hour and 2 hours after oral administration of the compound (120 mg) to 6 healthy subjects on days 1, 2, 5, 8, 11 and 14. Data are expressed as means ± SD. *$p < 0.05$.

Fig. 4. Twenty-four hour urinary excretion ratios of 6β-hydroxy cortisol and free cortisol in six healthy subjects on the day before the start of administration and on days 1, 2, 5, 8, 11 and 14. Results are expressed as means ± SD. **$p < 0.01$.

Fig. 5. Effects of rifampicin (10 μM) and MKC-963 (0.25 μM) on CYP3A4 mRNA (A) and activity of testosterone 6β-hydroxylation in primary human hepatocyte cultures (B). Total RNA was extracted, and CYP3A4 and β-actin mRNA levels were measured by real-time PCR methods as

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described in the Materials and Methods section, and then CYP3A4 mRNA was normalized to β-actin and compared with that of a vehicle control. The mean absolute ratio for the control hepatocytes was 0.051 ± 0.02. (A). For measurement of CYP3A4 activity, testosterone was incubated with intact hepatocytes and metabolite was analyzed as described in the Materials and Methods section, and then CYP3A4 activities were compared with those of a vehicle control. The mean activity of control hepatocytes was 2.3 ± 0.7 pmol/min/10^5cells (B). Results are expressed as means ± SD of three experiments.

Fig. 6. Metabolic clearance of MKC-963 in microsomes from insect cells expressing CYP enzymes (50 pmol/mL) (A) and the contributions (%) of each CYP enzyme to the total clearance of MKC-963 in five CYP enzymes corrected by the CYP contents in native human liver microsomes as described in the Materials and Methods section (B). MKC-963 (2 μM) was incubated in the presence of CYP1A2, CYP2C9, CYP2C19, CYP2D6 or CYP3A4 for 0, 5, 15 and 30 min, at 37 °C. Each value is the mean ± SD of triplicate assays.

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Table 1. Pharmacokinetic parameters of MKC-963 on day 1 and day 14 after repeated oral administration of 120 mg to six healthy subjects

	Day 1	Day 14
C_{max} (ng/mL)	96.2 ± 46.7	22.6 ± 14.8 **
t_{max} (h)	1 (1 – 1.5)	1 (0.5 –1)
AUC (h×ng/mL)	206.0 ± 76.5	64.8 ± 31.8 **
$t_{1/2}$ (h)	7.2 ± 2.1	10.9 ± 5.0

Data are expressed as means ± SD except for t_{max} data, which are given as median with range.

**$p < 0.01$.

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Figure 1

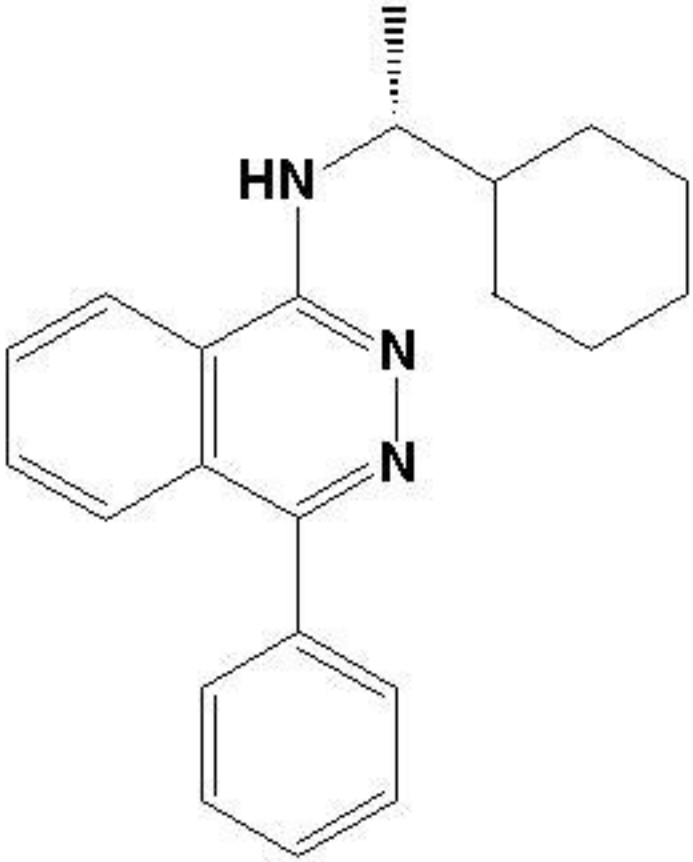


Figure 2

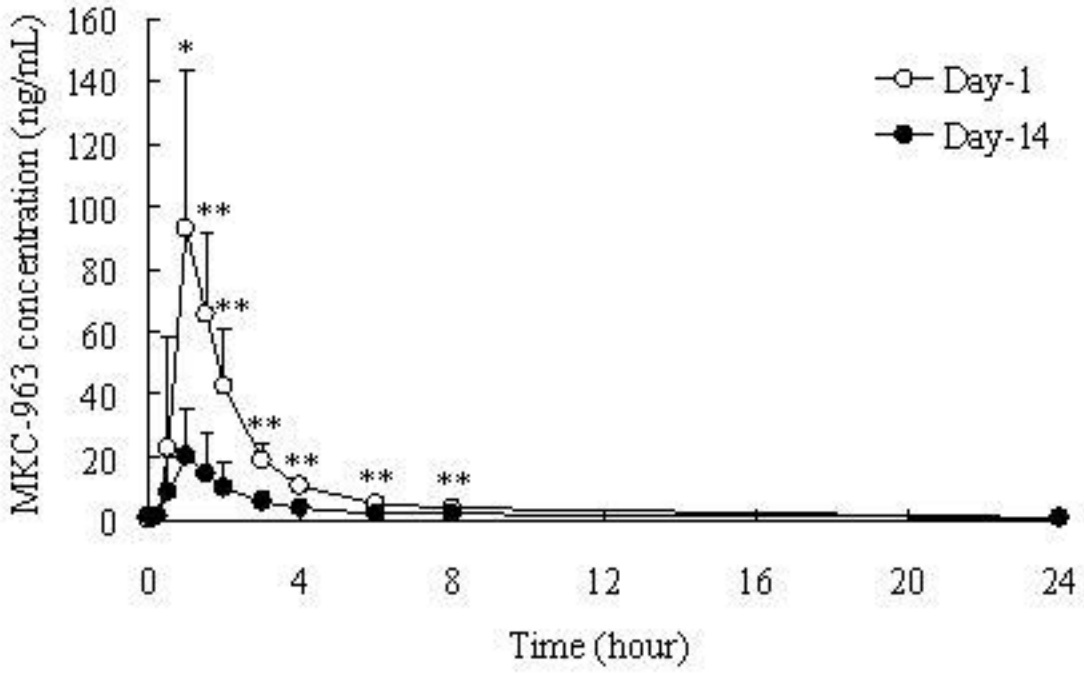

MKC-963 concentration (ng/mL)
160
140
120
100
80
60
40
20
0
*
**
**
**
**
**
**
Day-1
Day-14
0
4
8
12
16
20
24
Time (hour)

Figure 3

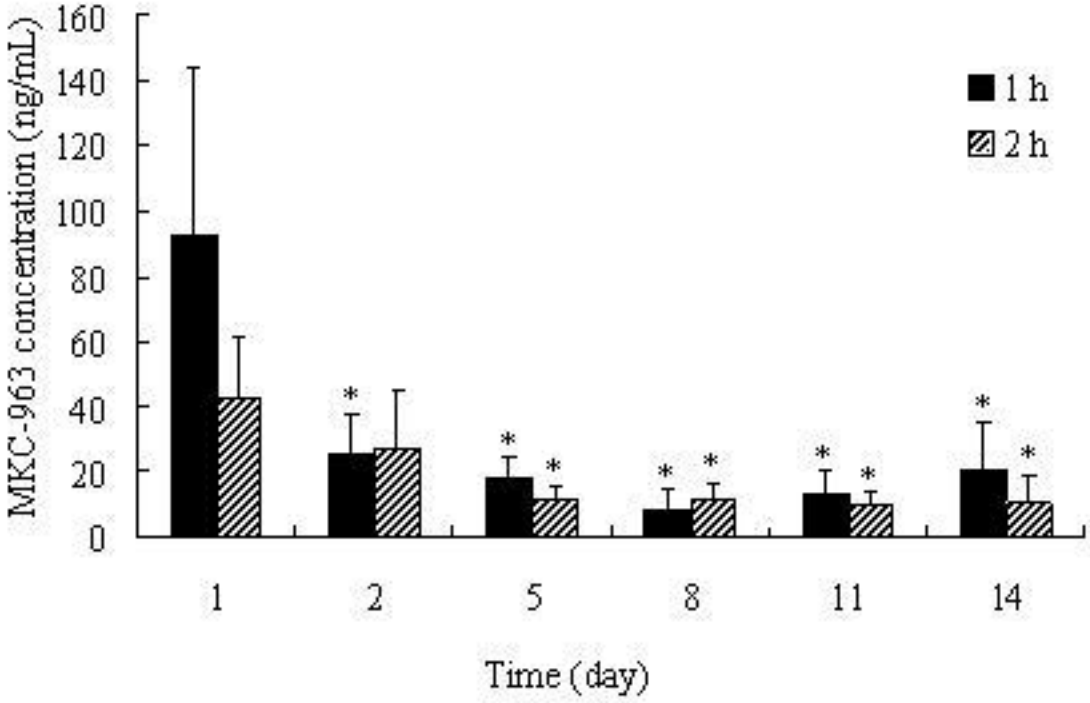

MKC-963 concentration (ng/mL)
160
140
120
100
80
60
40
20
0
1 h
2 h
*
1
2
5
8
11
14
Time (day)

Figure 4

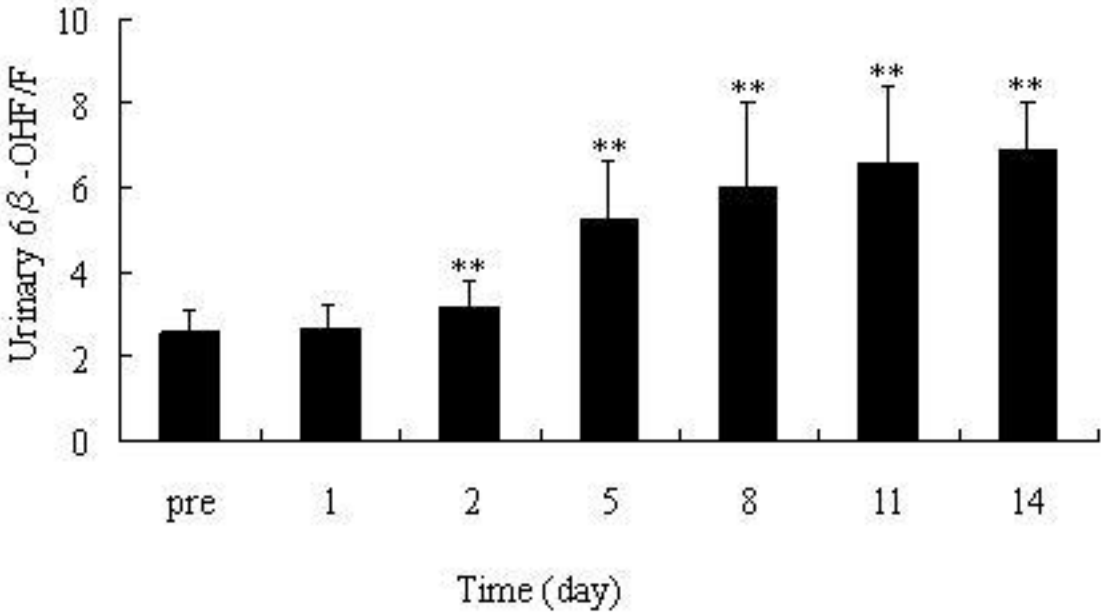


Figure 5

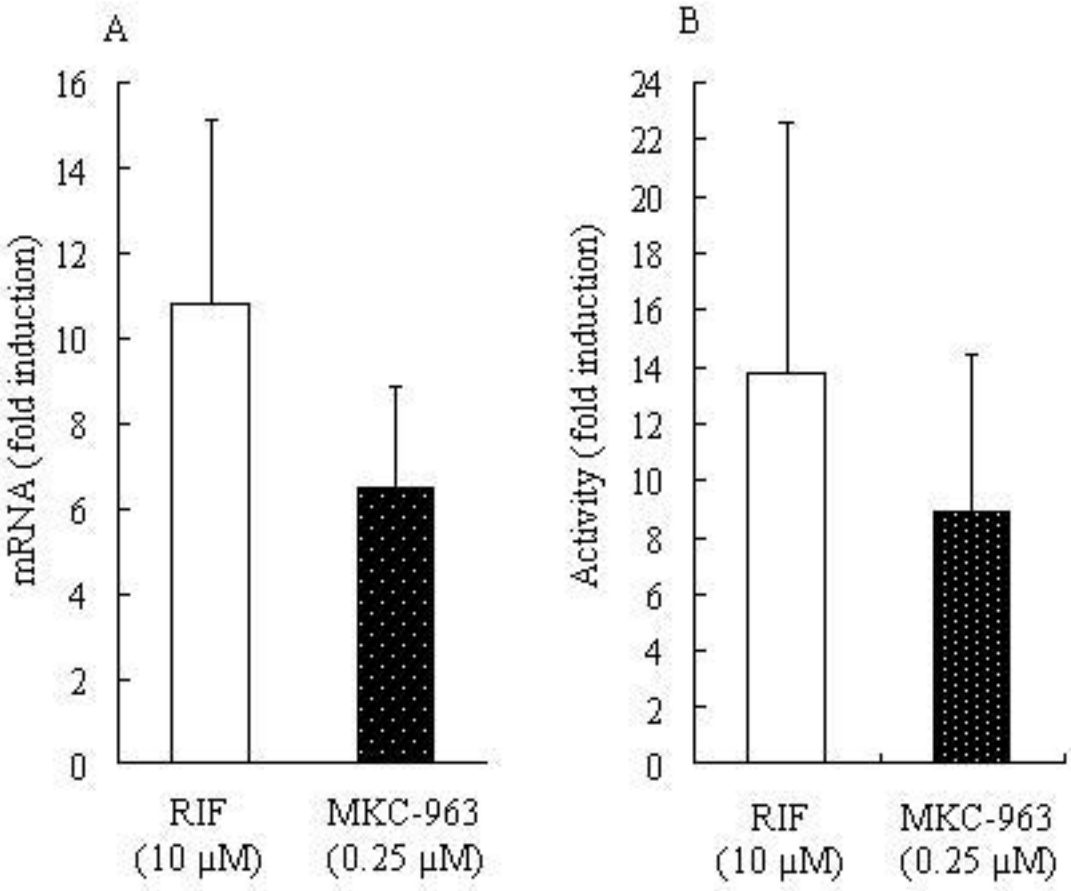
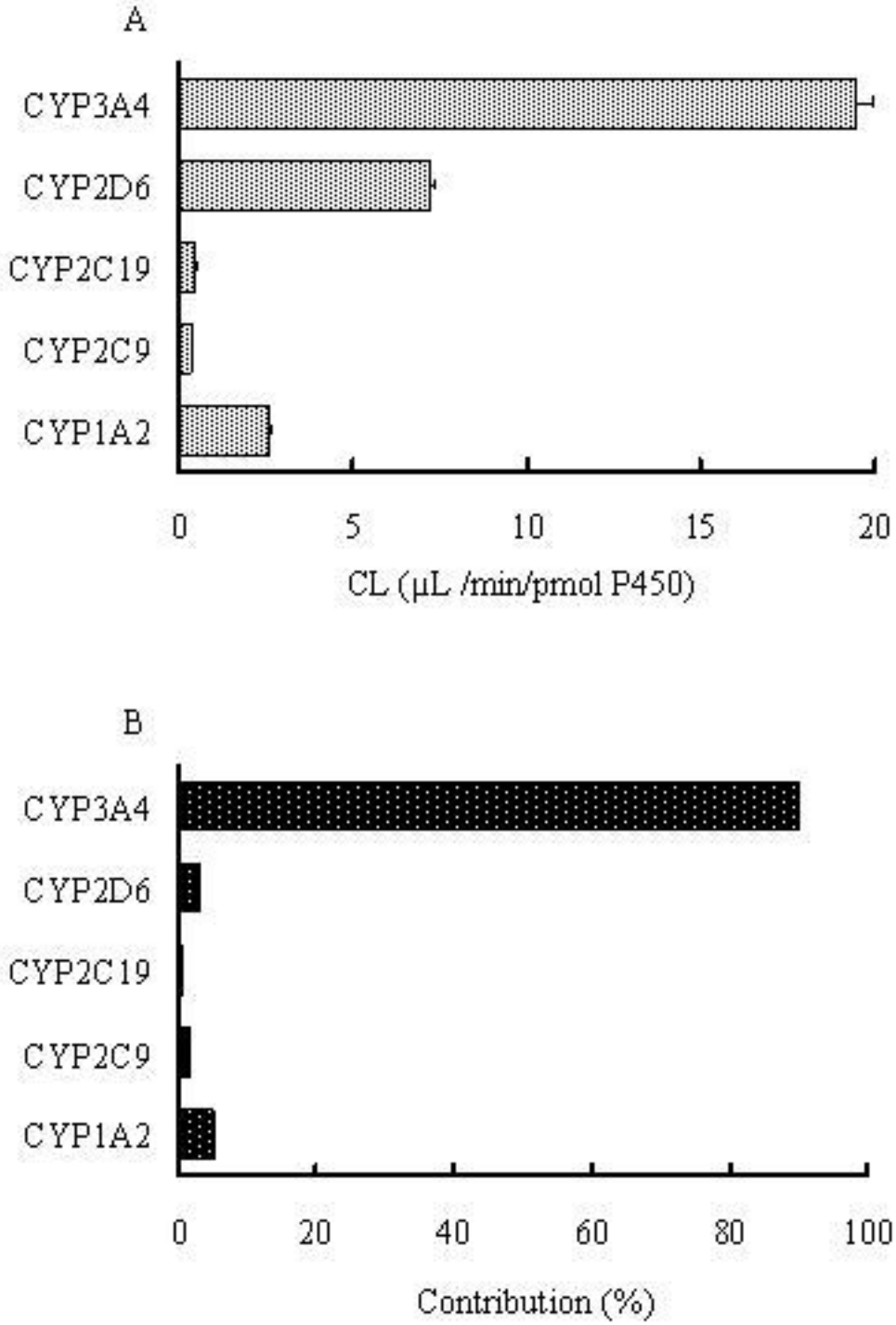

A
mRNA (fold induction)
16
14
12
10
8
6
4
2
0
RIF
(10 μM)
MKC-963
(0.25 μM)
B
Activity (fold induction)
24
22
20
18
16
14
12
10
8
6
4
2
0
RIF
(10 μM)
MKC-963
(0.25 μM)

Figure 6


A
CYP3A4
CYP2D6
CYP2C19
CYP2C9
CYP1A2
0
5
10
15
20
CL (μL /min/pmol P450)
B
CYP3A4
CYP2D6
CYP2C19
CYP2C9
CYP1A2
0
20
40
60
80
100
Contribution (%)