Induction of CYP3As in HepG2 Cells by Several Drugs.—Association between Induction of CYP3A4 and Expression of Glucocorticoid Receptor—

Tatsuhiro USUI,[1)] Yukiya SAITOH, and Fusao KOMADA*

Department of Drug Informatics, Faculty of Pharmaceutical Sciences, Josai University; Keyakidai, Sakado, Saitama 350–0295, Japan. Received November 5, 2002; accepted January 15, 2003

The cytochrome P-450 3A (CYP3A) enzyme family is responsible for most of the drug metabolism in the human liver. In this study, we demonstrated the inductive effects of phenobarbital, rifampicin, carbamazepine, phenytoin, prednisolone, ciclosporin and clotrimazole on CYP3A4, CYP3A5 and CYP3A7 mRNA expression, and established the relationship between the expression of human glucocorticoid receptor α (hGR) mRNA and the induction of CYP3A4 mRNA in cultured HepG2 cells by reverse transcription polymerase chain reaction (RT-PCR). Treatment with prednisolone, rifampicin and carbamazepine rapidly induced the level of CYP3A4 mRNA expression by 3- to 6-fold. However, phenytoin and phenobarbital gradually induced CYP3A4 mRNA level by 3 to 4-fold. The induction of CYP3A4 mRNA expression by clotrimazole and ciclosporin was negligible. Treatment with phenytoin, rifampicin, carbamazepine and ciclosporin induced approximately 2-fold increases in the expression of CYP3A5 mRNA, although prednisolone, phenytoin and clotrimazole had no effect. Treatment with rifampicin, phenytoin, clotrimazole and ciclosporin resulted in approximately a 2-fold induction of the CYP3A7 mRNA level. Treatment with rifampicin and ciclosporin induced the expression of hGRα mRNA significantly in comparison with controls, although the induction of hGRα mRNA following treatment with other drugs was negligible. In cluster analysis, the induced level of CYP3A4, CYP3A5, CYP3A7 and hGRα mRNA by these drugs could be classified into four major clusters. This suggested that each cluster might be associated with different mechanism(s) of induction by these drugs. Furthermore, we studied the associations between the expression of hGRα mRNA and the induced level of CYP3A4 mRNA by prednisolone and ciclosporin. Treatment with both prednisolone and ciclosporin showed synergistic effects on induction of CYP3A4 mRNA and, following treatment with both drugs, the expression level of CYP3A4 mRNA was 2-fold greater compared with prednisolone alone after the fifth day. Positive correlations were observed between the levels of hGRα mRNA expression and those of CYP3A4 mRNA. This observation shows that the regulation of CYP3A4 gene expression was hGRα-dependent and that ciclosporin may function as a regulator of expression *via* hGRα.

Key words induction; CYP3A4; CYP3A5; CYP3A7; glucocorticoid receptor; synergistic effect

Many drugs are mainly metabolized by the cytochrome P-450 3A (CYP3A) subfamily, which is the most abundant group of CYP enzymes in the liver, consisting of at least 3 isoforms: CYP3A4, CYP3A5 and CYP3A7. CYP3A4 is the most abundantly expressed CYP and accounts for approximately 40% of all CYP in the adult human liver, and is the major phase I xenobiotic metabolizing enzyme for several drugs.[2,3)] CYP3A5, 83% homologous with CYP3A4, is expressed at a much lower level than CYP3A4 in the liver, but is the main CYP3A isoform in the kidney.[4)] CYP3A7 is the major CYP isoform detected in human embryonic, fetal and newborn liver, although its levels are much lower than those of CYP3A4 in the adult liver.[5)]

The induction of CYP3As promotes the metabolism of other drugs, often with adverse consequences. Previous studies have indicated that these enzymes are induced by several drugs *in vivo* and *in vitro*. Sumida *et al.* have reported that the combination of HepG2 cells and reverse transcription polymerase chain reaction (RT-PCR) allows evaluation of the degree of CYP3A mRNA induction both easily and rapidly, and also that many drugs increase CYP3A mRNA expression by 2- to 5-fold in comparison with untreated controls.[6)] CYP3A4 has been shown to be induced strongly by rifampicin, phenobarbital, phenytoin, dexamethasone, prednisolone and taxol.[7,8)] In particular, prednisolone strongly induces CYP3A4 and this affects the pharmacokinetics of other drugs administered simultaneously. CYP3A5 is induced by dexamethasone and phenobarbital.[9,10)] CYP3A7 has been shown to be induced by environmental pollutants.[11)] The pharmacokinetics of these drugs alone and/or co-administered with other drugs may change over long-term administration, and many problems have been reported.[12,13)] Therefore, it is very important to predict the induction mechanisms of these drugs.

Members of the orphan nuclear receptor superfamily (constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptors) mediate the induction of hepatic P450s belonging to the CYP2, CYP3 and CYP4 families in response to the prototypical inducers phenobarbital, prednisolone 16 α-carbonitrile, rifampicin, and clofibric acid.[14)] Dexamethasone, lovastatin and rifampicin enhance PXR activator-mediated CYP3A4 gene expression in cultured human hepatocytes.[15,16)] El-Sankary *et al.* have reported that regulation of the CYP3A4 gene is receptor-dependent and that hydrocortisone may function as a regulator of basal expression *via* human PXR and the human glucocorticoid receptor (hGR).[17)] Furthermore, a member of the human CYP3A gene subfamily contains an enhancer that binds hGR and this binding is critical for transcriptional activation by dexamethasone.[9,15)] Alternative splicing of hGR pre-mRNA generates two highly homologous isoforms, hGRα and hGRβ,[18—20)] hGRα is a ligand-activated transcription factor that modulates the expression of glucocorticoid-responsive genes by binding to specific

* To whom correspondence should be addressed. e-mail: komada@josai.ac.jp

glucocorticoid response element DNA sequences. In contrast, hGRβ does not bind glucocorticoids and is transcriptionally inactive. In this study, we studied the effects of prednisolone, phenobarbital, rifampicin, carbamazepine, phenytoin, ciclosporin and clotrimazole on induction of CYP3A4, CYP3A5, CYP3A7, hGRα and hGRβ mRNA expression in Hep G2 cells. The pattern of induction by these drugs was analyzed by means of the tree clustering method.

Furthermore, we examined the synergistic effects of dual drug treatment on induction of CYP3A4 mRNA, *i.e.* the effect of ciclosporin on prednisolone-induced CYP3A4 mRNA levels. Prednisolone and ciclosporin are administered simultaneously to prevent rejection after tissue transplantation. Ciclosporin and its derivatives are used for P-glycoprotein-mediated exclusion of anticancer drugs from tumors with multidrug resistance (MDR).[21] Thus, prednisolone and/or other anticancer drugs are used with ciclosporin in the treatment of chronic lymphocytic leukemia, acute myeloid leukemia and acute lymphoblastic leukemia with MDR.[22] The association between the expression of hGRα mRNA and the induction of CYP3A4 mRNA by prednisolone and/or ciclosporin was also studied.

MATERIALS AND METHODS

Materials Human hepatoma cells (Hep G2 cells) were purchased from Riken Cell Bank (Tsukuba, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin–streptomycin mixture and RT-PCR kit (Ready To Go RT-PCR Beads) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), ICN Biomedicals Inc. (OH, U.S.A.), Bio Whittaker (ND, U.S.A.) and Amersham Pharmacia Biotech Inc. (NJ, U.S.A.), respectively. Mineral oil, agarose, ethidium bromide and molecular weight marker (Ladder Mix) were purchased from Sigma Chemical Co. (NJ, U.S.A.), Takara Co. (Tokyo, Japan), Gibco Co. (NY, U.S.A.) and Fermentas Inc. (MD, U.S.A.), respectively. The primers for CYP3A4, CYP3A5, CYP3A7, glyceraldehyde 3 phosphate dehydrogenase (GAP), hGRα, and hGRβ mRNAs were purchased from Sawady Technology Co., Ltd. (Tokyo, Japan) (Table 1).[6,23—25] Phenobarbital, rifampicin, phenytoin, prednisolone, clotrimazole, carbamazepine and verapamil were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ciclosporin was a gift from Novartis Pharma K. K. (Tokyo, Japan). All other chemicals were of reagent grade.

Quantification of the Levels of CYP3A4, CYP3A5, CYP3A7, hGR and GAP mRNA Aliquots of 6×10^6 Hep G2 cells were seeded in 100 mm tissue culture dishes containing 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin (penicillin–streptomycin mixture) at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. The test drugs were dissolved in dimethyl sulfoxide (DMSO). The incubation medium was replaced with each test medium containing the final concentration as a clinical plasma concentration of phenobarbital (86.11 μmol/l), rifampicin (12.15 μmol/l), carbamazepine (25.39 μmol/l), phenytoin (59.46 μmol/l), clotrimazole (100.0 nmol/l), prednisolone (1.83 and 9.15 μmol/l), ciclosporin (250.0 nmol/l), verapamil (100.0 μmol/l) and no drug in 10 μl DMSO on day 0.[6] The test medium was replaced with new test medium every day from day 1 to day 4. All experiments involving control and drug treatments were performed separately at least 3 times. Samples of mRNA were extracted from each culture on days 1 to 5 using a total RNA extraction kit (Rneasy Mini Kit, Qiagen, Hilden, Germany). RT-PCR was performed for CYP3A4, CYP3A5, CYP3A7, hGRα, hGRβ and GAP mRNAs under the conditions listed in Table 1. The levels of these mRNAs were quantified by the band density on agarose gels using NIH Image (National Institutes of Health, NJ, U.S.A.), and

Table 1. RT-PCR Conditions for CYP3A4, CYP3A5, CYP3A7, hGRα, hGRβ and GAP mRNA

A) Primers

CYP3A4 sense	5′-CAA GGA CAA CAT AGA TCC TTA CAT ATA CAC ACC CTT TGG AAG-3′	
CYP3A4 antisense	5′-AGC TCA ATG CAT GTA CAG AAT CCC CGG TTA-3′	
CYP3A5 sense	5′-CCC AGT TGC TAT TAG ACT TGA-3′	
CYP3A5 antisense	5′-GGG GCA CAG CTT TCT TGA AGA CCA-3′	
CYP3A7 sense	5′-AGT ATA GAA AAG TCT GGG GTA TTT ATG ACT-3′	
CYP3A7 antisense	5′-TAT TGA GAG AAC GAA TGG ATC TAA TGG-3′	
		(refs. 24, 25)
hGRα sense	5′-ACC AAT CAG ATA CCA AAA TA-3′	
hGRα antisense	5′-ATA CAC CAA CAG AAA GTC TA-3′	
hGRβ sense	5′-AAA GCA CAT CTC ACA CAT TA-3′	
hGRβ antisense	5′-AAA ACA CAT TCA CCT ACA GC-3′	
		(ref. 26)
GAP sense	5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′	
GAP antisense	5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′	
		(ref. 6)

B) RT-PCR conditions

	CYP3A4	CYP3A5	CYP3A7	hGRα	hGRβ	GAP
RT	40 °C 30 min	40 °C 30 min	40 °C 30 min	40 °C 30 min	40 °C 30 min	40 °C 30 min
	95 °C 5 min	95 °C 5 min	95 °C 5 min	95 °C 5 min	95 °C 5 min	95 °C 5 min
PCR	94 °C 30 s	94 °C 30 s	94 °C 30 s	94 °C 30 s	94 °C 30 s	94 °C 30 s
	60 °C 40 s	62 °C 40 s	62 °C 80 s	54 °C 45 s	54 °C 45 s	58 °C 60 s
	72 °C 30 s	72 °C 30 s	72 °C 40 s	72 °C 90 s	72 °C 90 s	72 °C 60 s
Products	398 bp	455 bp	473 bp	464 bp	797 bp	983 bp

the ratio of the expression levels of CYP3A and hGR mRNAs was normalized relative to that of GAP mRNA. The RT-PCR kinetic curves of these ratios of mRNAs did not reach a plateau. This indicated that the ratios of the levels of CYP3A mRNA/GAP mRNA and hGR mRNA/GAP mRNA in this study could be determined quantitatively at least up to a ratio of 3. Therefore, we could estimate the levels of CYP3A and hGR mRNA expression quantitatively. Each experiment was performed in 1 to 3 culture dishes, and means±S.D. were calculated using data (total $n=3$ to 9 dishes) from at least 3 separate experiments.

Data Analysis Statistical analysis was performed by analysis of variance (ANOVA). A level of $p<0.05$ was considered statistically significant. The data were analyzed by the tree clustering method with the Ward's method for linkage rules and the Euclidean distance for distance estimation using Statistica™ (StatSoft Japan Inc., Tokyo, Japan).

RESULTS

Induction of CYP3A4, CYP3A5 and CYP3A7 mRNA by Several Drugs Figure 1 and Table 2 show the level of induction of CYP3A4, CYP3A5 and CYP3A7 mRNA expression by several drugs. Treatments with prednisolone, phenytoin, carbamazepine and rifampicin induced the level of CYP3A4 mRNA expression by 4- to 6-fold, in comparison with control conditions (DMSO treatment alone) (Fig. 1A). The level of CYP3A4 mRNA expression by phenobarbital was more than 3-fold that under control conditions. The level of CYP3A4 mRNA expression in culture treated with prednisolone was more than 2.5-fold that in controls on the first day, although phenytoin and phenobarbital gradually induced CYP3A4 mRNA expression. The levels of CYP3A4 mRNA expression induced by prednisolone and rifampicin treatment reached almost steady-state conditions after the second day. The induced level of CYP3A4 mRNA expression by clotrimazole and ciclosporin was negligible. Phenytoin treatment induced an increase of approximately 2-fold in CYP3A5 mRNA expression after the fourth day. The expression of CYP3A5 mRNA was induced significantly by rifampicin, carbamazepine and ciclosporin treatments, although other drugs had no such effect (Fig. 1B). The CYP3A7 mRNA expression was weakly induced by treatment with all the test drugs, and rifampicin, phenytoin, clotrimazole and ciclosporin also increased the expression by approximately 2-fold (Fig. 1C). The rate of induction by rifampicin was more rapid than that by phenytoin.

These observations showed that both prednisolone and phenobarbital markedly induced CYP3A4 mRNA and weakly induced CYP3A7 mRNA, but the former showed a greater and more rapid induction of CYP3A4 mRNA expression than the latter. Rifampicin, carbamazepine and phenytoin induced the expression of all CYP3A mRNAs examined. The levels of CYP3A5 and CYP3A7 mRNA expression were induced by treatment with ciclosporin. However, clotrimazole

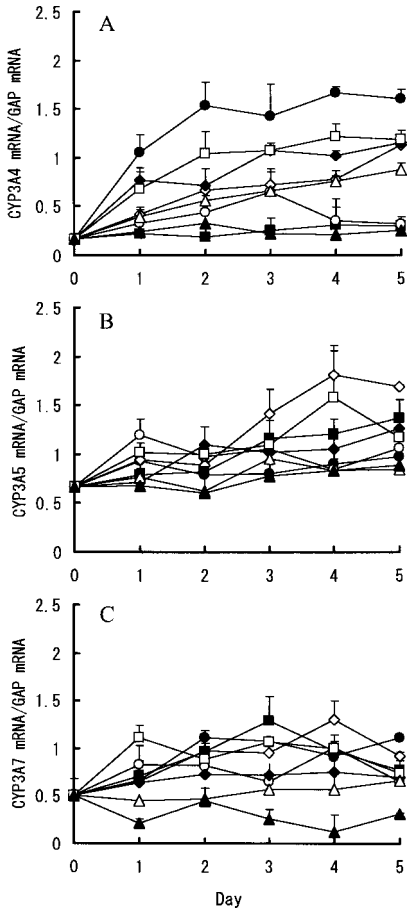

Fig. 1. Levels of CYP3A4, CYP3A5 and CYP3A7 mRNA Expression in Cultures Treated with Several Drugs

A: Levels of CYP3A4 mRNA expression. B: Levels of CYP3A5 mRNA expression. C: Levels of CYP3A7 mRNA expression. ▲, DMSO treatment; △, phenobarbital treatment (86.11 μmol/l); ●, prednisolone treatment (1.83 μmol/l); ○, clotrimazole treatment (100.0 nmol/l); ■, ciclosporin treatment (250 nmol/l); □, rifampicin treatment (12.15 μmol/l); ◆, carbamazepine treatment (25.39 μmol/l); ◇, phenytoin treatment (59.46 μmol/l). Data represent means±S.D. ($n=3$—9 dishes).

Table 2. Maximal Induction of CYP3A4, CYP3A5 and CYP3A7 mRNA by Several Drugs

Drug	CYP3A4 mRNA		CYP3A5 mRNA		CYP3A7 mRNA	
	x-fold	day	x-fold	day	x-fold	day
Prednisolone	6.26±0.37***	5	1.30±0.16	2	2.49±0.03*	2
Rifampicin	4.47±0.50***	4	1.90±0.58*	4	2.40±0.11***	5
Carbamazepine	4.51±0.35***	5	1.43±0.33*	5	2.25±0.37***	5
Phenytoin	4.40±1.84**	5	2.18±0.64**	4	2.96±0.16***	5
Phenobarbital	3.06±0.19**	3	1.22±0.14	3	2.14±0.20**	5
Clotrimazole	3.50±0.81	3	1.20±0.19	5	2.11±0.31***	5
Ciclosporin	1.17±0.58	2	1.54±0.05***	5	2.50±0.33***	5

x-Fold (mean±S.D.) represents the maximal expression level divided by the expression level in the DMSO-treated control on the same day. Day x represents the day on which the maximal expression level was observed. *; $p<0.05$, **; $p<0.01$ and ***; $p<0.001$: significant differences in comparison with the expression levels in the DMSO-treated control on the same day.

induced only the expression of CYP3A7 mRNA. This finding indicated that induction of CYP3A4, CYP3A5 and CYP3A7 mRNA by these drugs may be mediated by a different mechanism(s).

Induction of hGRα mRNA by Several Drugs Figure 2 shows the level of induction of the expression of hGRα mRNA by several drugs. Cultures treated with DMSO alone (control conditions) showed a gradual increase in the expression of hGRα mRNA by approximately 2-fold after the fifth day. The levels of hGRα mRNA expression in cultures treated with rifampicin and ciclosporin were increased in comparison with controls, although other drugs produced no induction of hGRα mRNA. On the other hand, the level of hGRα mRNA expression was decreased by treatment with phenytoin in comparison with controls. None of the examined drugs induced expression of hGRβ mRNA (data not shown).

Cluster Analysis of Induction of CYP3As and hGRα mRNA by Several Drugs The levels of CYP3A4, CYP3A5, CYP3A7 and hGRα mRNA induced by several drugs were analyzed by the tree clustering method (Fig. 3). The linkage distance in the horizontal hierarchical tree plot (dendrogram) between clusters of carbamazepine and that of rifampicin was shorter than that of phenobarbital and phenytoin, and thus, clusters of carbamazepine–rifampicin and that of phenobarbital–phenytoin were linked after about 35% of the linkage distance/maximal distance. Furthermore, the induction patterns of these drugs were classified into four major clusters at about 30% of the linkage distance/maximal distance: a cluster of phenobarbital–phenytoin, that of ciclosporin–clotrimazole, that of prednisolone alone and that of carbamazepine–rifampicin. In addition, the relationship between the cluster of phenobarbital–phenytoin and that of carbamazepine–rifampicin was closer than that of prednisolone, and the cluster of ciclosporin–clotrimazole had the longest linkage distance compared with the other clusters.

Synergistic Effect of Dual Treatment with Prednisolone and Ciclosporin on Induction of CYP3A4 mRNA We studied the effects of ciclosporin on the induction of CYP3A4 and hGRα mRNA by prednisolone. The prednisolone-induced expression of CYP3A4 mRNA increased rapidly, with the increase on the first day being more than 3-fold, and reached a steady-state after the second day of approximately 6-fold in comparison with controls (Fig. 1A). In contrast, the induced level of CYP3A4 mRNA expression by ciclosporin was negligible. Figure 2 shows the induction of hGRα mRNA expression by prednisolone and ciclosporin. Treatment with ciclosporin significantly induced the expression of hGRα mRNA in comparison with controls from the third to the fifth day, although prednisolone affected hGRα mRNA level only on the fifth day.

Figure 4 shows the effects of ciclosporin on prednisolone-induced CYP3A4 mRNA expression. Dual treatment with prednisolone and ciclosporin had synergistic effects on the induction of CYP3A4 mRNA. The levels of CYP3A4 mRNA expression in cultures treated with both prednisolone and ciclosporin together from the first to the third day were the same as those in cultures treated with prednisolone alone. However, the expression level following dual treatment on the fourth day was significantly increased in comparison with that following treatment with prednisolone alone. Furthermore, the level of CYP3A4 mRNA expression in cultures treated with both prednisolone and ciclosporin on the fifth day was 2-fold that induced by prednisolone alone (Fig. 4A). Treatment with ciclosporin followed by prednisolone from the second day resulted in a significant increase in the level of CYP3A4 mRNA expression on the fifth day only (Fig. 4B). In contrast, treatment with prednisolone followed by ciclosporin from the second day resulted in a marked and rapid increase in the level of CYP3A4 mRNA in comparison with ciclosporin treatment only on the first to third day, and levels of CYP3A4 mRNA expression on the third and fifth days were significantly higher than those after prednisolone treatment only on the first to third day (Fig. 4C). Furthermore, cessation of ciclosporin treatment affected the synergistic effect of dual treatment with prednisolone and ciclosporin on the expression of CYP3A4 mRNA, and only that on the fifth day was slightly increased (Fig. 4D). These findings indicate that ciclosporin affects the expression of CYP3A4 mRNA by prednisolone, and this effect of ciclosporin requires approximately 3 d.

A synergistic effect of dual treatment with prednisolone and ciclosporin on the expression of hGRα mRNA was observed by the fourth day compared with prednisolone-only treatment (Fig. 5A). When treatment with ciclosporin was

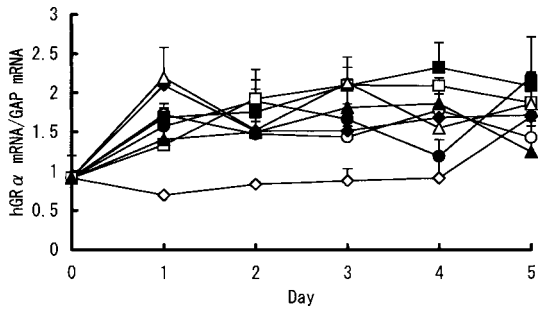

Fig. 2. Levels of hGRα mRNA Expression Following Treatment with Several Drugs

▲, DMSO treatment; △, phenobarbital treatment (86.11 μmol/l); ●, prednisolone treatment (1.83 μmol/l); ○, clotrimazole treatment (100.0 nmol/l); ■, ciclosporin treatment (250 nmol/l); □, rifampicin treatment (12.15 μmol/l); ◆, carbamazepine treatment (25.39 μmol/l); ◇, phenytoin treatment (59.46 μmol/l). Data represent means±S.D. (n=3—9 dishes).

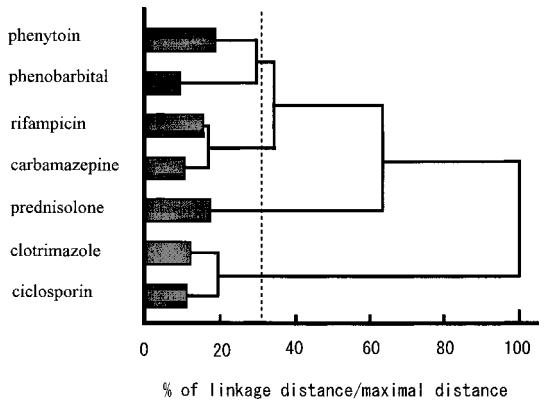

Fig. 3. Horizontal Hierarchical Tree Plot Showing Induced Levels of CYP3A4, CYP3A5, CYP3A7 and hGRα mRNA Expression by Several Drugs

Each rectangle indicates a cluster in which data were analyzed for each drug treatment in separate experiments at least 3 times, and the length of the rectangle indicates the maximum linkage distance within each drug treatment.

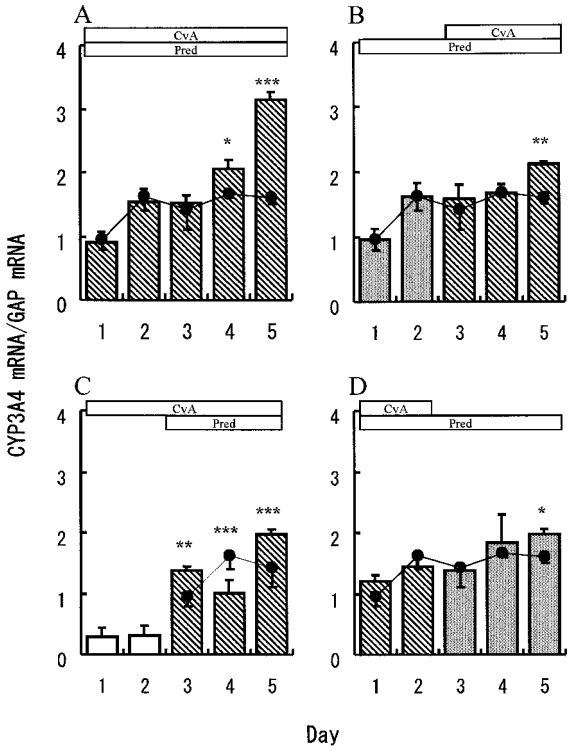

Fig. 4. Effects of Ciclosporin on Induction of CYP3A4 mRNA by Prednisolone

●, Levels of CYP3A4 mRNA expression in cultures treated with prednisolone alone (1.83 μmol/l); ▨, prednisolone treatment (1.83 μmol/l); □, ciclosporin treatment (250 nmol/l); ▧, treatment with both prednisolone (1.83 μmol/l) and ciclosporin (250 nmol/l). Ciclosporin and prednisolone are abbreviated as CyA and Pred, respectively. Data represent the means±S.D. (n=3—9 dishes). *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$: significantly different from treatment with prednisolone alone (●).

followed by prednisolone treatment from the second day, synergistic effects were observed on the fourth day compared with prednisolone-only treatment (Fig. 5B). Treatment with prednisolone followed by ciclosporin from the second day produced synergistic effects on the expression of hGRα mRNA on the third day only compared with prednisolone-only treatment on the third day (Fig. 5C). Cessation of ciclosporin treatment affected the synergistic effect of the dual treatment from the third to fifth day, and levels of hGRα mRNA expression on the third to fourth day were the same as those of prednisolone-only treatment (Fig. 5D). These observations indicate that ciclosporin affects the expression of hGRα mRNA induced by prednisolone, but that this effect is a weak one.

As shown in Fig. 6, the induction of CYP3A4 mRNA expression by prednisolone is dose-dependent. The co-administration of verapamil, which is a modulator of p-glycoprotein, did not affect the prednisolone-induced CYP3A4 mRNA expression. This observation indicates that the increase in prednisolone-induced CYP3A4 mRNA expression by ciclosporin is not due to inhibition of P-glycoprotein.

Correlation between the Levels of CYP3A4 and hGRα mRNA Expression We examined whether the ciclosporin–induced hGRα mRNA expression affected the induction of CYP3A4 mRNA by prednisolone. Figure 7 shows the relationship between levels of CYP3A4 mRNA and hGRα mRNA expression. The data show levels of CYP3A4 mRNA and hGRα mRNA expression in cultures treated with prednisolone alone on the second day (Fig. 1), fifth day (Figs. 4B, 5B), and third to fifth day (Figs. 4C, 5C), because the effect of ciclosporin on the expression of CYP3A4 mRNA induced by prednisolone required approximately 3 d. CYP3A4 mRNA levels correlated significantly with hGRα mRNA levels ($p<0.001$). A positive correlation was observed between the levels of CYP3A4 mRNA and hGRα mRNA expression in cultures treated with both prednisolone and ciclosporin, suggesting that hGRα is partially involved in the regulation of CYP3A4 gene expression by prednisolone.

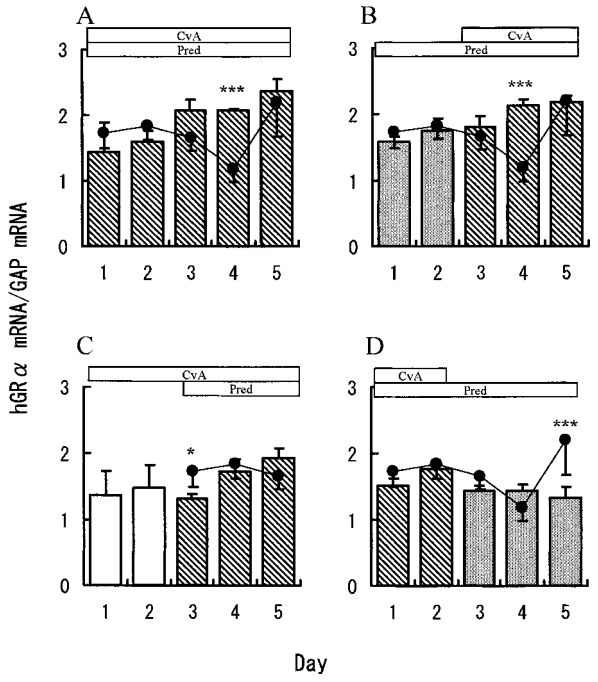

Fig. 5. Effects of Ciclosporin on Induction of hGRα mRNA by Prednisolone

●, Levels of hGRα mRNA expression in cultures treated with prednisolone alone (1.83 μmol/l); ▨, prednisolone treatment (1.83 μmol/l); □, ciclosporin treatment (250 nmol/l); ▧, treatment with both prednisolone (1.83 μmol/l) and ciclosporin (250 nmol/l). Ciclosporin and prednisolone are abbreviated as CyA and Pred, respectively. Data represent the means±S.D. (n=3—9 dishes). *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$: significantly different from treatment with prednisolone alone (●).

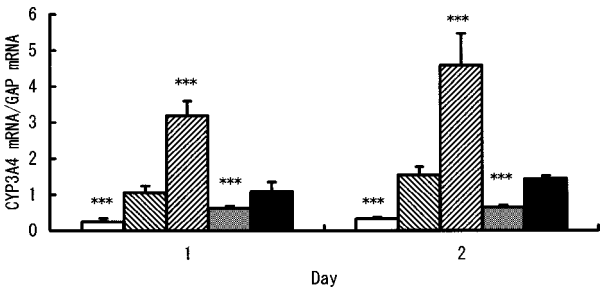

Fig. 6. Effects of Verapamil on Induction of CYP3A4 mRNA by Prednisolone

□, DMSO treatment; ▧, prednisolone treatment (1.83 μmol/l); ▨, prednisolone treatment (9.15 μmol/l); ▩, verapamil treatment (100 μmol/l); and ■, treatment with both prednisolone (1.83 μmol/l) and verapamil (100 μmol/l). Data represent the means±S.D. (n=3—6 dishes). ***, $p<0.001$: significantly different from prednisolone treatment (1.83 μM).

DISCUSSION

The levels of CYP3A4 mRNA expression were induced

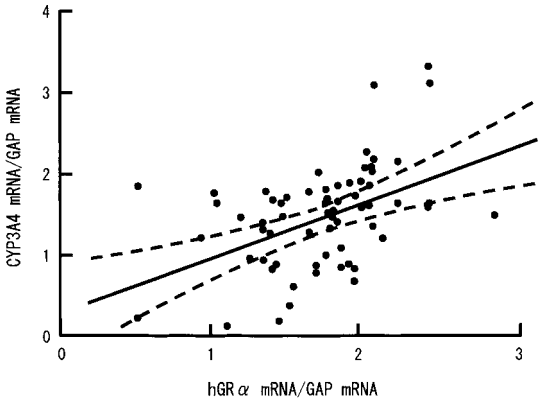

Fig. 7. Correlation between Expression of CYP3A4 and hGRα mRNA

——, CYP3A4=0.652* hGR+0.303, R=0.462; -----, 95% CI and $p<0.001$. Data points ($n=66$) show levels of CYP3A4 mRNA and hGRα mRNA expression in cultures treated with prednisolone alone on the second day (Fig. 1), on the fifth day (Figs. 4B, 5B), and on the third to fifth day (Figs. 4C, 5C).

more 3 to 6-fold by treatment with prednisolone, rifampicin, carbamazepine, phenytoin and phenobarbital in comparison with controls. Prednisolone, rifampicin and carbamazepine increased the expression of CYP3A4 mRNA rapidly, although the effect of phenytoin and phenobarbital was slow. Therefore, when prednisolone, rifampicin and/or carbamazepine were administered, the early detection of blood concentrations of drugs that are rapidly metabolized by CYP3A4 may be necessary.[26—28] For example, blood concentrations of indinavir, saquinavir and nerfinavir were decreased by 80 to 90% when taken together with these drugs. Phenytoin induced CYP3A5 mRNA expression. Anti-epileptics are administered to prevent convulsions and/or seizures in patients with brain tumors. When anti-tumor drugs that are rapidly metabolized by CYP3A5, such as docetaxel, paclitaxel, vincristine and vindesine, are administered to patients treated with phenytoin, the anti-tumor effects of these drugs may be markedly decreased.[27,29] All drugs investigated in the present study, weakly induced CYP3A7 mRNA expression. CYP3A7 is the major CYP isoform detected in the human embryonic, fetal and newborn liver, although its levels are much lower than those of CYP3A4 in the adult liver.[5] Nuclear receptors CAR and PXR play key roles in regulating CYP3A7 expression from early gestation until the perinatal period, when there is a switch in expression to CYP3A4.[30] Ogg *et al.* have reported that induction of CYP3A7 is only observed following co-transfection of plasmids containing components of the 5′-flanking region of CYP3A7 and encoding the hGR into the hepatoma cell line HuH-7.[31] Thus, CYP3A7 is induced by overexpression of hGR. Therefore, the induction of CYP3A7 mRNA following treatment with rifampicin and ciclosporin may have been activated by increased levels of hGRα mRNA expression by these drugs.

The induction patterns of CYP3As and hGRα mRNA by these drugs were analyzed by the tree clustering method. This statistical method interprets a large volume of information and organizes observed data into meaningful structures, that is, to develop taxonomies. This technique has successfully been applied to unravel latent similarities among gene expression profiles and microarray data.[32—34] The linkage distance between the carbamazepine and rifampicin clusters was shorter than that of the other drugs. This result suggests that carbamazepine and rifampicin may possess the same or a closely related induction mechanism(s). The cluster of carbamazepine–rifampicin and that of phenobarbital–phenytoin are linked. Furthermore, the induction patterns of CYP3As and hGRα mRNA by these drugs were classified into four major clusters: *i.e.* the cluster of phenobarbital–phenytoin, that of ciclosporin–clotrimazole, that of prednisolone alone and that of carbamazepine–rifampicin. The relationship between the cluster of phenobarbital–phenytoin and that of carbamazepine–rifampicin was closer than that of prednisolone, and the cluster of ciclosporin–clotrimazole had the longest linkage distance to other clusters. Indeed, two nuclear receptors, PXR and CAR, have recently been proposed to mediate CYP3As gene induction in response to xenobiotics, as the transactivated DNA nuclear response elements located in the promoter region of this gene.[14,35,36] PXR and CAR are activated by rifampicin and phenobarbital, respectively.[37,38] This result suggests that drugs in closely grouped clusters may possess similar induction mechanisms compared with drugs in other clusters with greater linkage distances. Therefore, reciprocal and complex interactions may occur when drugs belonging to different clusters are co-administered.

The level of hGRα mRNA expression was reduced by treatment with phenytoin in comparison with controls, although phenytoin did not induce the expression of hGRβ mRNA. When both hGRα and hGRβ isoforms are expressed in the same cell, hGRβ potentially functions as a dominant negative inhibitor of hGRα activity.[39—41] Therefore, we speculated that degradation of hGRβ may be prevented by phenytoin. However, the nuclear basis for these variations in hGRα mRNA expression is poorly understood.

We examined the relationship between ciclosporin-induced hGRα mRNA expression and prednisolone-induced CYP3A4 mRNA expression. Prednisolone and ciclosporin are administered simultaneously to prevent rejection after tissue transplantation. Also, prednisolone and/or other anticancer drugs are used with ciclosporin in the treatment of some kinds of leukemia associated with MDR1.[21,22] We demonstrated an association between the induction of expression of hGRα mRNA by ciclosporin and that of CYP3A4 mRNA by prednisolone. Treatment with prednisolone strongly and rapidly induced the level of CYP3A4 mRNA in a dose-dependent manner, although the effect of ciclosporin was negligible. On the other hand, treatment with ciclosporin significantly induced the expression of hGRα mRNA, while prednisolone did not. Many members of the orphan nuclear receptor superfamily mediate the expression of CYP3As.[14] hGR is a good candidate for a CYP3A-induced mediator.[17] Furthermore, a member of the human CYP3A gene subfamily contains an enhancer that binds hGR, and this binding is critical for transcriptional activation.[9,15]

No synergistic effects of dual treatment with prednisolone and ciclosporin on the induction of CYP3A4 mRNA were observed from the first to the third day. However, the expression level increased significantly on the fourth and fifth days in comparison with cultures treated with prednisolone alone. Treatment with ciclosporin followed by prednisolone from the second day resulted in a significant increase in the level of CYP3A4 mRNA expression on the fifth day only. In contrast, treatment with prednisolone followed by ciclosporin from the second day resulted in a marked and rapid increase

in the level of CYP3A4 mRNA expression in comparison with ciclosporin treatment only on the first to third day, and expression levels on the third and fifth days were significantly higher than those observed with prednisolone treatment only from the first to third day. However, the level of CYP3A4 mRNA expression on the fourth day was significantly lower than those observed with prednisolone-only treatment. We speculated that the reduction of CYP3A4 mRNA expression may occur as a result of the major consumption of hGRα following the addition of prednisolone.

The levels of CYP3A4 mRNA expression and hGRα mRNA, increased by dual treatment with prednisolone and ciclosporin, decreased after stopping ciclosporin treatment. A significant increase in the expression of hGRα mRNA induced by ciclosporin was observed with a lag-time of approximately 2 d. Therefore, ciclosporin affected the expression of CYP3A4 mRNA induced by prednisolone, and this effect required approximately 3 d. Moreover, hGRα mRNA expressed in the absence of hormone and xenobiotics is located in the cell cytoplasm as part of a large multiprotein complex. This complex has been identified as being either a chaperone (hsp70, hsp90), co-chaperone (hsp40, p60/hop), immunophilin (FKBP59, Cyp40) or another compound (p23).[42,43] Ciclosporin, an immunophilin binding exclusively to cyp40, was able to potentiate the dexamethasone effect approximately 3- to 4-fold.[44,45] This complex dissociated in response to hormonal and xenobiotic stimuli and translocates to the nucleus. Hence, our results suggest that potentiation of prednisolone action by ciclosporin may proceed through enhanced hGRα nuclear translocation.

A positive correlation ($p<0.001$) was observed between the levels of CYP3A4 mRNA and hGRα mRNA expression. These observations indicate that the ciclosporin-induced hGRα mRNA expression affects the expression of CYP3A4 mRNA induced by prednisolone. Ogg *et al.* have shown that hGR is involved in the induction of CYP3A4 gene expression by some CYP3A4 inducers using cells transfected with a plasmid encoding hGR, and RU-486, an antagonist of the glucocorticoid receptor, completely blocks the expression of the reporter gene for CYP3A4.[31] Furthermore, a study of cells transfected with expression plasmids encoding hGR and/or hPXR alone or in combination suggested that hydrocortisone functions as a regulator of basal expression *via* hGR and hPXR.[16] In contrast, a functional PXR element is present in the CYP3A7 gene promoter, and PXR may regulate the expression of CYP3A7 mRNA.[46]

Ciclosporin has been shown to be a substrate of CYP3A4 and a modulator of P-glycoprotein.[47] Ciclosporin and its derivatives are used for P-glycoprotein-mediated exclusion of anticancer drugs from tumors with MDR1.[21] It is possible that the concentration of prednisolone in HepG2 cells is increased by the effect of ciclosporin on P-glycoprotein. However, verapamil, which is a strong modulator of P-glycoprotein, had no effect on prednisolone-induced CYP3A4 mRNA expression in this study. Methylprednisolone and dexamethasone transport have been reported to be affected by both verapamil and ciclosporin.[48,49] However, a 6 α-methyl group in the steroid structure seems to be a prerequisite for substrates of intestinal P-glycoprotein.[50] Ciclosporin regulates expression of many cytokines to affect many immunocompetent cells *in vivo*. In this *in vitro* study, ciclosporin may not have been affected by cytokines because the hepatoma cell line HepG2 was used. Pascussi *et al.* have demonstrated the down-regulation of P450s CYP by cytokines.[51] Interleukin-6 (IL-6) rapidly and markedly decreases the expression of PXR and constitutively activated receptor mRNAs, but does not affect the levels of dioxin receptor or glucocorticoid receptor mRNA. IL-6 decreases both rifampicin- and phenobarbital-mediated induction of CYP2B6, CYP2C8, CYP2C9, and CYP3A4. These observations indicate that the synergistic effects of ciclosporin on the prednisolone-induced expression of CYP3A4 mRNA are not due to the inhibition of P-glycoprotein or cytokines. The present results indicate that the regulation of CYP3A4 gene expression may be hGRα-dependent and that ciclosporin may function as a regulator of the expression *via* hGRα. The present results indicating that hGRα regulates CYP3A4 gene expression provides a basis for the efficient identification and elimination of candidate drugs that may interact with other treatments.

REFERENCES AND NOTES

1) Present address: *Division of Immunobiochemistry, Department of Clinical Dietitics and Human Nutrition, Faculty of Pharmaceutical Sciences, Josai University, Keyakidai, Sakado, Saitama 350–0295, Japan.*
2) Li A. P., Kaminski D. L., Rasmussen A., *Toxicology*, **104**, 1—8 (1995).
3) Nelson D. R., Koymans L., Kamataki T., Stegeman J. J., Feyereisen R., Waxman D. J., Waterman M. R., Gotoh O., Coon M. J., Estabrook R. W., Gunsalus I. C., Nebert D. W., *Pharmacogenetics*, **6**, 1—42 (1996).
4) de Wildt S. N., Kearns G. L., Leeder J. S., Van den Anker J. N., *Clin. Pharmacokinet.*, **37**, 485—505 (1999).
5) Ring J. A., Ghabrial H., Ching M. S., Smallwood R. A., Morgan D. J., *Pharmacol. Ther.*, **84**, 429—445 (1999).
6) Sumida A., Yamamoto I., Zhou Q., Morisaki T., Azuma J., *Biol. Pharm. Bull.*, **22**, 61—65 (1999).
7) Sumida A., Fukuen S., Yamamoto I., Matsuda H., Naohara M., Azuma J., *Biochem. Biophys. Res. Commun.*, **267**, 756—760 (2000).
8) Rodriguez-Antona C., Jover R., Gomez-Lechon M. J., Castell J. V., *Arch. Biochem. Biophys.*, **376**, 109—116 (2000).
9) Schuetz J. D., Schuetz E. G., Thottassery J. V., Guzelian P. S., Strom S., Sun D., *Mol. Pharmacol.*, **49**, 63—72 (1996).
10) Hukkanen J., Lassila A., Paivarinta K., Valanne S., Sarpo S., Hakkola J., Pelkonen O., Raunio H., *Am. J. Respir. Cell Mol. Biol.*, **22**, 360—366 (2000).
11) Dubois M., Plaisance H., Thome J. P., Kremers P., *Ecotoxicol. Environ. Saf.*, **34**, 205—215 (1996).
12) Gillum J. G., Israel D. S., Polk R. E., *Clin. Pharmacokinet.*, **25**, 450—482 (1993).
13) Bertz R. J., Granneman G. R., *Clin. Pharmacokinet.*, **32**, 210—258 (1997).
14) Waxman D. J., *Arch. Biochem. Biophys.*, **369**, 11—23 (1999).
15) Pascussi J. M., Drocourt L., Fabre J. M., Maurel P., Vilarem M. J., *Mol. Pharmacol.*, **58**, 361—372 (2000).
16) Kliewer S. A., Lehmann J. M., Milburn M. V., Willson T. M., *Recent Prog. Horm. Res.*, **54**, 345—368 (1999).
17) El-Sankary W., Plant N. J., Gibson G. G., Moore D. J., *Drug Metab. Dispos.*, **28**, 493—496 (2000).
18) Bamberger C. M., Bamberger A. M., de Castro M., Chrousos G. P., *J. Clin. Invest.*, **95**, 2435—2441 (1995).
19) Encío I. J., Detera-Wadleigh S. D., *J. Biol. Chem.*, **266**, 7182—7188 (1991).
20) Dahia P. L., Honegger J., Reincke M., Jacobs R. A., Mirtella A., Fahlbusch R., Besser G. M., Chew S. L., Grossman A. B., *J. Clin. Endocrinol. Metab.*, **82**, 1088—1093 (1997).
21) Kusunoki N., Takara K., Tanigawara Y., Yamauchi A., Ueda K., Komada F., Ku Y., Kuroda Y., Saitoh Y., Okumura K., *Jpn. J. Cancer Res.*, **89**, 1120—1128 (1998).
22) Vilpo J., Koski T., Vilpo L., *Haematologica*, **85**, 806—813 (2000).
23) Kolars J. C., Lown K. S., Schmiedlin-Ren P., Ghosh M., Fang C., Wrighton S. A., Merion R. M., Watkins P. B., *Pharmacogenetics*, **4**,

247—259 (1994).
24) Schuetz J. D., Beach D. L., Guzelian P. S., *Pharmacogenetics*, **4**, 11—20 (1994).
25) Raddatz D., Henneken M., Armbrust T., Ramadori G., *Hepatology*, **24**, 928—933 (1996).
26) Villikka K., Kivistö K. T., Backman J. T., Olkkola K. T., Neuvonen P. J., *Clin. Pharmcol. Ther.*, **61**, 8—14 (1997).
27) Watanabe M., Tateishi T., Asoh M., Nakura H., Tanaka M., Kumai T., Kobayashi S., *Life Sci.*, **63**, 1685—1692 (1998).
28) Hsu A., Granneman G. R., Bertz R. J., *Clin. Pharmacokinet.*, **35**, 275—291 (1998).
29) Rendic S., Di Carlo F. J., *Drug Metab. Rev.*, **29**, 413—580 (1997).
30) Bertilsson G., Berkenstam A., Blomquist P., *Biochem. Biophys. Res. Commun.*, **280**, 139—144 (2001).
31) Ogg M. S., Williams J. M., Tarbit M., Goldfarb P. S., Gray T. J., Gibson G. G., *Xenobiotica*, **29**, 269—279 (1999).
32) Sultan M., Wigle D. A., Cumbaa C. A., Maziarz M., Glasgow J., Tsao M. S., Jurisica I., *Bioinformatics*, **18**, S111—S119 (2002).
33) Lukashin A. V., Fuchs R., *Bioinformatics*, **17**, 405—414 (2001).
34) Quackenbush J., *Nat. Rev. Genet.*, **2**, 418—427 (2001).
35) Xie W., Barwick J. L., Simon C. M., Pierce A. M., Safe S., Blumberg B., Guzelian P. S., Evans R. M., *Genes Dev.*, **14**, 3014—3023 (2000).
36) Quattrochi L. C., Guzelian P. S., *Drug Metab. Dispos.*, **29**, 615—622 (2001).
37) Lehmann J. M., McKee D. D., Watson M. A., Willson T. M., Moore J. T., Kliewer S. A., *J. Clin. Invest.*, **102**, 1016—1023 (1998).
38) Zelko I., Negishi M., *Biochem. Biophys. Res. Commun.*, **277**, 1—6 (2000).
39) Oakley R. H., Jewell C. M., Yudt M. R., Bofetiado D. M., Cidlowski J. A., *J. Biol. Chem.*, **274**, 27857—27866 (1999).
40) Oakley R. H., Sar M., Cidlowski J. A., *J. Biol. Chem.*, **271**, 9550—9559 (1996).
41) Strickland I., Kisich K., Hauk P. J., Vottero A., Chrousos G. P., Klemm D.J., Leung D. Y., *J. Exp. Med.*, **193**, 585—594 (2001).
42) Prima V., Depoix C., Masselot B., Formstecher P., Lefebvre P., *J. Steroid Biochem. Mol. Biol.*, **72**, 1—12 (2000).
43) Edinger R. S., Watkins S. C., Pearce D., Johnson J. P., *Am. J. Physiol. Renal. Physiol.*, **283**, F254—261 (2002).
44) Renoir J. M., Mercier-Bodard C., Hoffmann K., Le Bihan S., Ning Y. M., Sanchez E. R., Handschumacher R. E., Baulieu E. E., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 4977—4981 (1995).
45) Owens-Grillo J. K., Hoffmann K., Hutchison K. A., Yem A. W., Deibel M. R., Jr., Handschumacher R. E., Pratt W. B., *J. Biol. Chem.*, **270**, 20479—20484 (1995).
46) Pascussi J. M., Jounaidi Y., Drocourt L., Domergue J., Balabaud C., Maurel P., Vilarem M. J., *Biochem. Biophys. Res. Commun.*, **260**, 377—381 (1999).
47) Lown K. S., Mayo R. R., Leichtman A. B., Hsiao H. L., Turgeon D. K., Schmiedlin-Ren P., Brown M. B., Guo W., Rossi S. J., Benet L. Z., Watkins P. B., *Clin. Pharmacol. Ther.*, **62**, 248—260 (1997).
48) Koszdin K. L., Shen D. D., Bernards C. M., *Anesthesiology*, **92**, 156—163 (2000).
49) Nakayama A., Eguchi O., Hatakeyama M., Saitoh H., Takada M., *Biol. Pharm. Bull.*, **22**, 535—538 (1999).
50) Saitoh H., Hatakeyama M., Eguchi O., Oda M., Takada M., *J. Pharm. Sci.*, **87**, 73—75 (1998).
51) Pascussi J. M., Gerbal-Chaloin S., Pichard-Garcia L., Daujat M., Fabre J. M., Maurel P., Vilarem M. J., *Biochem. Biophys. Res. Commun.*, **274**, 707—713 (2000).