Short Communication

A 96-Well Plate Assay for CYP4503A Induction Using Cryopreserved Human Hepatocytes

ABSTRACT:

A reliable and practical CYP3A induction assay with cryopreserved human hepatocytes in a 96-well format was developed. Various 96-well plates with different basement membrane were evaluated using prototypical inducers, rifampicin, phenytoin, and carbamazepine. Thin-layer (TL) Matrigel was found to yield the highest basal and induced levels of CYP3A activity as determined by testosterone 6β-hydroxylation. Concentration-dependent CYP3A induction of rifampicin was reproducible with the EC$_{50}$ values of 0.36 ± 0.28 μM from four batches of human hepatocytes using the 96-well plate with TL Matrigel. The rank order of induction potency for nine inducers or noninducers at a concentration of 10 μM were well comparable among the multiple donors, by expressing the results as percentage of change compared with the positive control, 10 μM rifampicin. Cotreatment of avasimibe or efavirenz with 10 μM rifampicin was found to reduce CYP3A activities induced by rifampicin at a lower rate than treatment with rifampicin alone, whereas treatment with phenobarbital and carbamazepine had no effect. From a comparison of induced CYP3A activities and gene expression levels, there were compounds that would cause induction of CYP3A4 mRNA but not activity, presumably due to their inhibitory effect on CYP3A activity. The cotreatment assay of test compound with rifampicin allows us to exclude the false-negative results caused by the cytotoxicity and/or the mechanism-based inactivation, when the drug candidate's ability for CYP3A induction is evaluating the enzyme activity. This 96-well plate assay, which is robust, reproducible, and convenient, has demonstrated the paramount applicability to the early drug discovery stage.

Introduction

CYP3A is regarded as one of the predominant cytochrome P450 (P450) isoforms among drug-metabolizing enzymes expressed in the human liver. The induction of CYP3A can result in clinically significant drug-drug interactions (DDIs) or autoinductions. The CYP3A inducers may cause reduction in therapeutic efficacy of comedication or an increase in risk of metabolite-induced toxicity. Therefore, it is important to evaluate the induction potential of new chemical entities in the early discovery stage (Dickins, 2004; Luo et al., 2004; Lin, 2006).

Several in vitro models have been developed to assess the potential of CYP3A induction, including nuclear receptor-based assays (Moore et al., 2000; El Sankary et al., 2001; Luo et al., 2002; Sinz et al., 2006), immortalized cell lines (Mills et al., 2004; Hariparsad et al., 2008), and primary hepatocytes. Among these models, the primary human hepatocytes have been considered as the most predictive model for assessing in vitro induction of P450 enzymes. Although fresh human hepatocytes are the standard for evaluating induction potential, attachable cryopreserved hepatocytes have been used more often due to increased availability. The enzymes in cryopreserved hepatocytes were shown to be inducible by standard P450 inducers, with the increased levels of mRNA, protein, and/or functional enzyme activity as the endpoint (Silva et al., 1999; Roymans et al., 2004, 2005).

A high-speed screening method for the evaluation of CYP1A2 and CYP3A4 induction potency has been established using real-time one-step reverse transcription-polymerase chain reaction (RT-PCR) in cryopreserved human hepatocytes (Nishimura et al., 2009). However, there have been few reports on the P450 induction assay procedures using cryopreserved hepatocytes with catalytic activity as the endpoint in microplates with 96 or more wells per plates. Although mRNA expression levels are also used for evaluating P450 induction, measurement of the enzyme activities has been recommended by the U.S. Food and Drug Administration (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf) as the most reliable method for quantifying a drug's induction potential. Moreover, extraction of total RNA from hepatocytes and the measurement of the mRNA levels in addition to the determination of P450 activities are time- and cost-consuming processes of P450 induction screening in the early discovery stage.

Fahmi et al. (2009) have recently demonstrated the applicability of a relative induction score (RIS) to predict clinical DDI using the human cryopreserved hepatocytes model. The RIS approach was based on combining in vitro induction parameters (EC$_{50}$ and E_{max}) from CYP3A4 mRNA induction data with the efficacious free plasma concentrations, and the RIS values were well correlated to the magnitude of clinical DDIs. However, donor-to-donor variability in the human hepatocytes has remained unresolved, in which in vitro induction parameters varied considerably among different batches of hepatocytes from different donors.

ABBREVIATIONS: P450, cytochrome P450; DDI, drug-drug interaction; RT-PCR, reverse transcription-polymerase chain reaction; RIS, relative induction score; TL, thin-layer; PLO/LM, poly-L-ornithine/laminin; PDL, poly-D-lysine; Ct, threshold cycle; RIF, rifampicin; %RIF, percentage of RIF response.

The aim of this study was to develop a reliable CYP3A induction assay method for drug discovery using cryopreserved human hepatocytes with CYP3A activity as the endpoint. We show that thin-layer (TL) Matrigel as the basement membrane on the bottom of 96-well plates is useful for maintaining the steady expression of CYP3A activity in cryopreserved hepatocytes. This CYP3A induction method with TL Matrigel was characterized using four batches of cryopreserved human hepatocytes.

Materials and Methods

Materials. Cryopreserved human hepatocytes from four donors were obtained from Celsis In Vitro Technologies (lots LOF, UFN, and LMP; Baltimore, MD) and CellzDirect, Inc. (lot 4201; Pittsboro, NC). Hepatocyte plating medium (In VitroGRO CP Medium), incubation medium (In VitroGRO HI Medium), and Torpedo Antibiotic Mix were obtained from Celsis In Vitro Technologies. Multiwell plates coated with TL Matrigel, collagen type I, collagen type IV, laminin, fibronectin, poly-L-ornithine/laminin (PLO/LM), and poly-D-lysine (PDL) were obtained from BD Biosciences (San Jose, CA). Rifampicin (RIF), phenytoin, carbamazepine, nifedipine, sulfinpyrazone, cimetidine, furosemide, and phenobarbital sodium were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Modafinil was purchased from Sigma-Aldrich (St. Louis, MO). Efavirenz, avasimibe, and ritonavir were produced at Takeda Pharmaceutical Company Limited (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Cell Culturing and Induction. Cryopreserved human hepatocytes were thawed in plating medium and transferred to TL Matrigel 96-well plates at a density of 0.4 to 0.8×10^5 viable cells/well. After 24-h incubation, the plating medium was removed and replaced with incubation medium supplemented with Torpedo Antibiotic Mix. The cells were then incubated for three additional days to establish the cultures. After the initial 4-day conditioning culture, the medium was removed and the hepatocytes were treated daily for 3 days with incubation medium, including test compounds in the absence or presence of 10 μM rifampicin. The wells of test compounds with 10 μM rifampicin were used to estimate the inhibitory effect of test compound on RIF-induced CYP3A activity. All incubations were conducted in a humidified atmosphere at 37°C in 5% CO_2.

CYP3A Measurement Activity. CYP3A activity was quantified by measuring the extent of 6β-hydroxytestosterone formation from testosterone using ultraperformance liquid chromatography systems (Waters, Milford, MA). The cultured hepatocytes were washed two times with Hanks' balanced salt solution containing 25 mM HEPES and incubated with 150 μl of the same buffer containing 100 μM testosterone at 37°C for 2 h. At the end of the incubation period, 120-μl aliquots of the media was transferred to a 96-well plate containing 10 μl of $HClO_4$ (5%), and 10 μl of the mixture was injected onto the column. Testosterone and 6β-hydroxytestosterone were chromatographed using an Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters) and UV detection at 250 nm. The mobile phase consist of solvent A (10 mM ammonium acetate/water/acetonitrile, 1:8:1) and solvent B (10 mM ammonium acetate/acetonitrile, 1:9), with a gradient profile starting at 25% B and then increasing to 95% B from 1 to 1.01 min, and remaining at 95% B until 2.4 min, then returning back to 25% B at 2.41 min, followed by re-equilibration until 2.5 min, all at flow rate of 0.5 ml/min.

Real-Time Quantitative RT-PCR. Total RNA was isolated using the SV 96 Total RNA Isolation Kit (Promega, Madison, WI). Quantification of cytochrome CYP3A4 mRNA was performed in a 384-well format using the TaqMan One-Step RT-PCR method by using the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The relative quantity of the target CYP3A4 gene compared with the endogenous control (hypoxanthine phosphoribosyltransferase I) was determined by the ΔΔCt method.

Data Analysis. The values of percentage of RIF response (%RIF) and fold of control (Fold) were calculated as shown below:

$$\% \text{ RIF} = \frac{\text{activity of test compound treated cells} - \text{activity of control}}{\text{activity of 10 μM rifampicin} - \text{activity of control}} \times 100$$

$$\text{Fold} = \frac{\text{activity of test compound treated cells}}{\text{activity of control}}$$

S.D.s of Fold and %RIF were calculated using the "propagation of error" formula. To determine the EC_{50} and E_{max} values, the data from concentration-response curves were fitted to a three-parameter sigmoid model. All curve fittings were carried out with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

Results and Discussion

Assessment of Various Plates with Different Attachment Substratum on CYP3A Induction Response. Because long-term culture (~8 days) is required for P450 induction assays, the improvement of cell attachment and prevention of gradual decline of CYP3A activity play an important role in the assay construction. We first assessed the 96-well plates with various types of basement membrane on the bottom of the plates. CYP3A inducibilities of human hepatocytes cultured in 96-well plates were compared for the seven types of basement membrane, including extracellular matrix components or synthetic compounds to enhance cell attachment, using prototypical inducers, rifampicin, phenytoin, and carbamazepine. Hepatocytes plated in 96-well plates with each basement membrane were allowed to recovery the culture for 3 days in incubation medium, and then

TABLE 1

Comparison of 96-well plates with various types of basement membrane on CYP3A induction. Hepatocytes (lot LOF) were cultured on a 96-well plate coated with two lots of TL Matrigel, collagen type I, collagen type IV, laminin, fibronectin, PLO/LM, or PDL. Hepatocytes cultures were treated with 0.1% dimethyl sulfoxide (control), 10 μM RIF, 30 μM phenytoin, or 30 μM carbamazepine, and determined the induced CYP3A activity. CYP3A induction responses, determined by 6β-hydroxytestosterone formation, are represented as CYP3A activity [pmol/(h · million cells)], fold induction, or %RIF. Data represent mean ± S.D. calculated from four separate wells.

|	TL Matrigel lot 14340	TL Matrigel lot 92069	Collagen Type I	Collagen Type IV	Laminin	Fibronectin	PLO/LM	PDL
Activity [pmol/(h · million cells)]								
Control	994 ± 152	1557 ± 450	250 ± 12	307 ± 35	336 ± 60	235 ± 9	193 ± 18	351 ± 21
10 μM Rifampicin	15699 ± 1856	25412 ± 2333	3940 ± 628	5644 ± 198	5522 ± 1038	3286 ± 523	1962 ± 599	9319 ± 908
30 μM Phenytoin	6507 ± 873	9396 ± 266	637 ± 74	897 ± 73	936 ± 212	423 ± 102	336 ± 74	1427 ± 250
30 μM Carbamazepine	4260 ± 535	7070 ± 487	532 ± 93	732 ± 83	1234 ± 106	442 ± 113	420 ± 76	1315 ± 80
Fold								
Control	1	1	1	1	1	1	1	1
10 μM Rifampicin	15.8 ± 3.1	16.3 ± 5	15.8 ± 2.6	18.4 ± 2.2	16.4 ± 4.3	14 ± 2.3	10.2 ± 3.3	26.6 ± 3
30 μM Phenytoin	6.5 ± 1.3	6 ± 1.8	2.5 ± 0.3	2.9 ± 0.4	2.8 ± 0.8	1.8 ± 0.4	1.7 ± 0.4	4.1 ± 0.8
30 μM Carbamazepine	4.3 ± 0.8	4.5 ± 1.4	2.1 ± 0.4	2.4 ± 0.4	3.7 ± 0.7	1.9 ± 0.5	2.2 ± 0.4	3.7 ± 0.3
%RIF								
Control	0	0	0	0	0	0	0	0
10 μM Rifampicin	100	100	100	100	100	100	100	100
30 μM Phenytoin	37.5 ± 7.7	32.9 ± 3.9	10.5 ± 2.7	11.1 ± 1.6	11.6 ± 4.8	6.2 ± 3.5	8.1 ± 5.1	12.0 ± 3.0
30 μM Carbamazepine	22.2 ± 4.7	23.1 ± 3.6	7.7 ± 2.9	8 ± 1.7	17.3 ± 4.2	6.8 ± 3.9	12.9 ± 6.2	10.8 ± 1.4
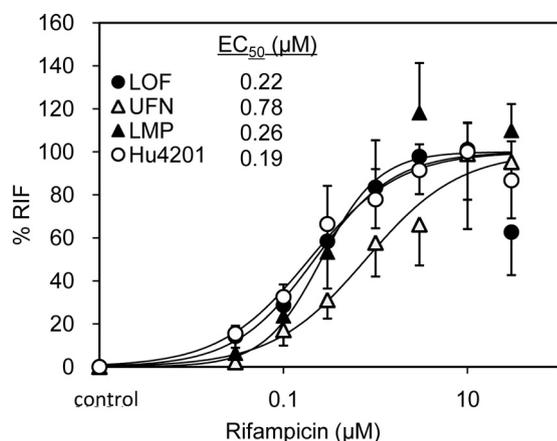
FIG. 1. Concentration-response curve for CYP3A induction by rifampicin in cryopreserved human hepatocytes from four different donors. Data represent mean ± S.D. calculated from four separate wells. Data points were fitted to a three-parameter sigmoid model.

incubated for an additional 3 days with inducers as described under *Materials and Methods*. TL Matrigel plates yielded the higher basal and induced CYP3A activity among these tested plates (Table 1). When the CYP3A induction potential was expressed as change in percentage compared with 10 μM rifampicin (%RIF), the positive control, CYP3A induction by phenytoin, and carbamazepine in the TL Matrigel plates were significantly higher than those in the other plates. Although the most common culture format is collagen type I and collagen-Matrigel sandwich culture (Chu et al., 2009), there are reports that Matrigel overlay to cultured primary rat hepatocytes had enhanced phenobarbital responsiveness on CYP2B and CYP3A gene induction (Sidhu et al., 1993, 2004). We observed the higher %RIF values of the CYP3A induction by carbamazepine and phenytoin in the TL Matrigel culture, and this finding suggests that Matrigel may enhance the CYP3A inducibility by unclear mechanism, in addition to improvement of cell attachment onto the 96-well plate. Furthermore, the TL Matrigel plate coated on the bottom is easier to handle than a gelled Matrigel plate commonly used as three-dimensional culture. From the results of this study, we have used the TL Matrigel plate for our CYP3A induction assay system in a 96-well format.

Assay Characterization with TL Matrigel 96-Well Plates. To validate TL Matrigel plate for CYP3A induction assay, we evaluated prototypical inducers using the TL Matrigel 96-well plate in different batches of human hepatocytes. Concentration-response studies with rifampicin were first conducted in four batches of human hepatocytes. As shown in Fig. 1, rifampicin-mediated CYP3A induction was reproducible in individual donors of hepatocytes, with the EC$_{50}$ values

of 0.36 ± 0.28 μM. Although the magnitude of CYP3A induction response expressed as fold increase varied according to the batches (data not shown), the variation among batches was reduced by using %RIF as an indicator of CYP3A induction response. To assess the ability and reproducibility of the assay, six inducers and two noninducers were evaluated with this established method in four batches of human hepatocytes. The compounds used as inducers were rifampicin, efavirenz, nifedipine, phenytoin, carbamazepine, sulfinpyrazone, and modafinil. The compounds used as noninducers were cimetidine and furosemide. All compounds were tested at 10 μM. As a result, CYP3A induction responses of the tested compounds expressed as %RIF were well comparable in different donors of hepatocytes, and the rank order of inducers and noninducers was reproducible in this assay (Fig. 2). These results indicate that this established CYP3A induction assay method with TL Matrigel plate is robust and reliable for evaluating the ranking of CYP3A induction potency for candidate compounds.

Cotreatment Assay of Prototypical Inducers with Rifampicin. The bell-shaped response of CYP3A induction is commonly encountered, in which the induction response increased at low drug concentrations with decreases in the response at higher concentrations. For a screening assay using only one or two test concentration points, the phenomenon of bell-shaped response is considered to be a risk for false-negative results in evaluating the CYP3A induction potency. Cytotoxicity, mechanism-based CYP3A inactivity, and/or solubility are often used to explain the bell-shaped response. Although common assessment of the cytotoxicity during the course of an experiment is recommended (Chu et al., 2009), it is insufficient to explain the other causes of the bell-shaped response. Therefore, we constructed the cotreatment assay of rifampicin with a test compound to detect the compound property, to exhibit the bell-shaped CYP3A induction response. Figure 3 shows the CYP3A induction by prototypical inducers, phenobarbital, carbamazepine, avasimibe, and efavirenz, in which hepatocytes were treated with the inducer alone or with the inducer and rifampicin. In this assay, hepatocytes were incubated with the inducer and 10 μM rifampicin for 3 days, and the induced CYP3A activities were determined. Phenobarbital and carbamazepine increased the CYP3A activity in a concentration-dependent manner. The RIF-induced CYP3A activities were constant in the cotreatment with phenobarbital or carbamazepine, even at the higher concentration of these inducers (Fig. 3). On the other hand, avasimibe and efavirenz showed the bell-shaped response with decrease in the induced CYP3A activity at higher concentrations of the inducers (Fig. 3). In the cotreatment of avasimibe or efavirenz, the RIF-induced CYP3A activity decreased with increasing the concentrations of avasimibe or efavirenz. These results indicate that avasimibe and efavirenz have inhibitory effects on the RIF-induced CYP3A activity for some rea-
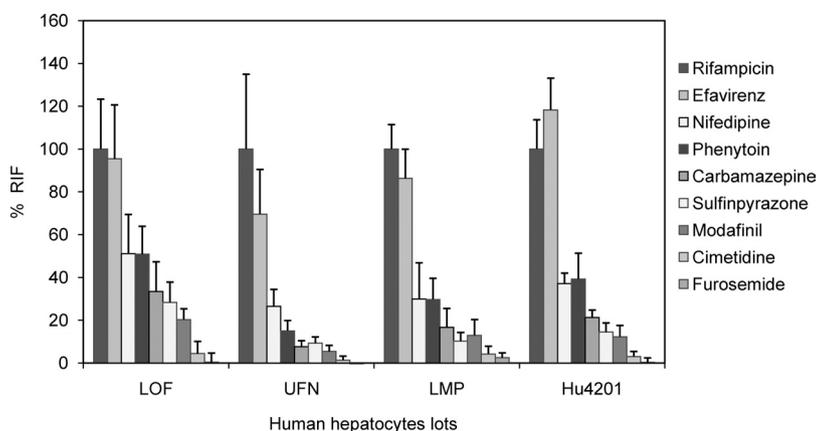
FIG. 2. CYP3A induction in cryopreserved human hepatocytes from four different donors treated with various inducers (rifampicin, efavirenz, nifedipine, phenytoin, carbamazepine, sulfinpyrazone, or modafinil) and noninducers (cimetidine or furosemide). The CYP3A induction response was expressed as a percentage of the 10 μM rifampicin response calculated from four separate wells. Error bars indicate S.D.
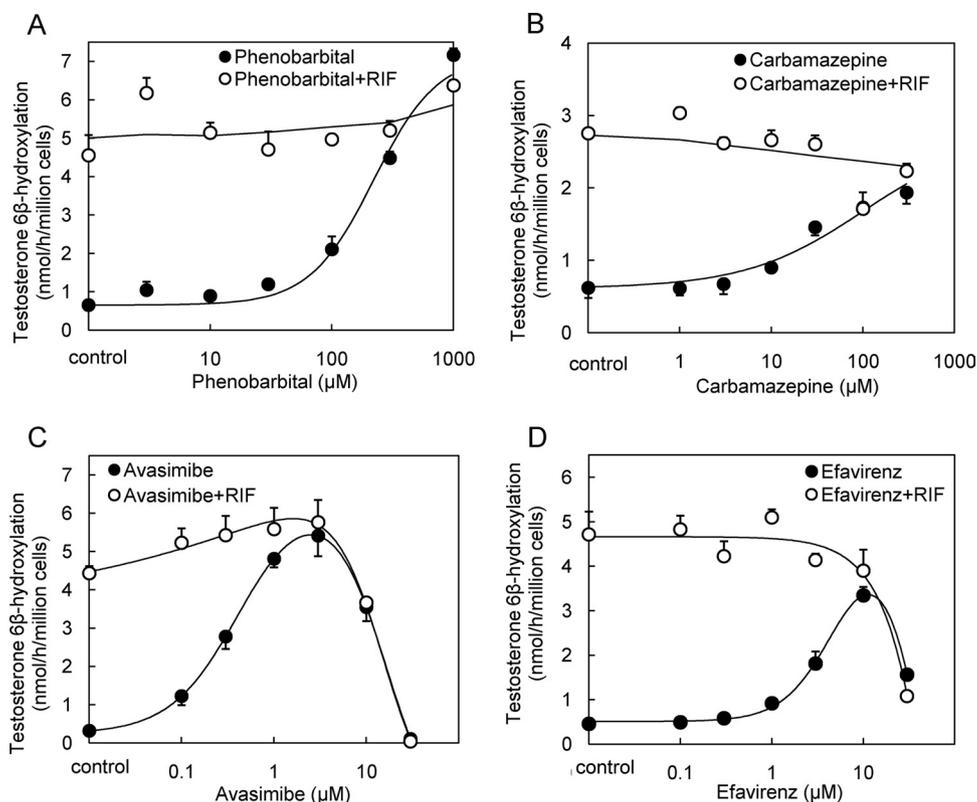
FIG. 3. Concentration-response curves for CYP3A induction by phenobarbital (A), carbamazepine (B), avasimibe (C), and efavirenz (D). Hepatocytes (lot LOF) were treated for 3 days with the inducer alone (●) or in combination with 10 μM rifampicin (○). Data are expressed as the mean ± S.D. of CYP3A activity (n = 3 or 4).

sons, i.e., cytotoxicity or mechanism-based CYP3A inactivation. By implementing the CYP3A induction assay together with the cotreatment assay, we can exclude the false-negative results in selecting drug candidates with no CYP3A induction potency, although the causes of the inhibitory effects of the compounds are unidentified in this cotreatment assay.

Comparison of Induced CYP3A Activities and mRNA Levels. Ritonavir, a mechanism-based inhibitor as well as inducer of CYP3A, indicated the inhibitory effect on the RIF-induced CYP3A activity in the cotreatment assay (Fig. 4). On the other hand, ritonavir was found not to inhibit the induction of CYP3A4 mRNA of rifampicin in identical hepatocytes. In cases where mechanism-based inactivation is a cause of inhibitory effect on CYP3A induction, measuring CYP3A4 mRNA expression could provide the useful information to evaluate whether the compounds have the induction potential. Figure 5 shows a correlation between CYP3A activities and CYP3A4 mRNA levels with 44 compounds at various concentrations. The induced CYP3A activity and mRNA level by tests compounds alone were used for the

plots on the graph, in which the compounds were categorized by the results of the cotreatment assay according to the potency of inhibitory effect on CYP3A induction by rifampicin. The CYP3A activity and CYP3A4 mRNA levels induced by the test compounds were well correlated, when the residual percentage of the RIF-induced CYP3A activity was more than 80%. The compounds that caused induction of CYP3A4 mRNA expression but not CYP3A activity were found to have a potent inhibitory effect on the RIF-induced CYP3A activity. These compounds suppressed the RIF-induced CYP3A activity by less
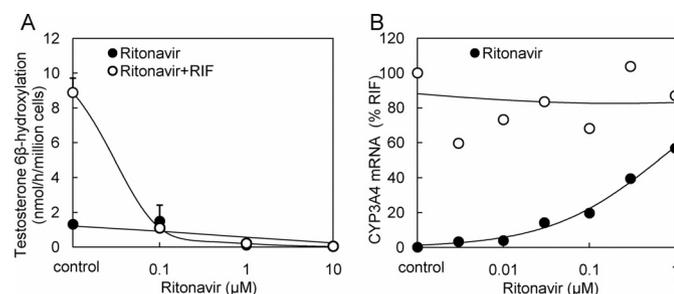

FIG. 4. Concentration-response curves for induction of CYP3A activity (A) and CYP3A4 mRNA expression (B) by ritonavir. Hepatocytes (lot LOF) were treated with ritonavir alone (●) or in combination with 10 μM rifampicin (○). Data are expressed as the mean ± S.D. of CYP3A activity or the mean of CYP3A4 mRNA level (n = 3 or 4).
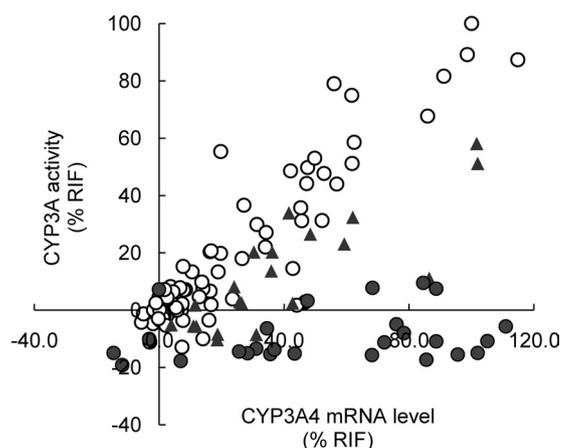

FIG. 5. Correlation between induced CYP3A activity and CYP3A4 mRNA level. Cryopreserved hepatocytes (lot LOF, UFN) were treated with 44 compounds at several concentrations (n = 128). The induced CYP3A activity and mRNA level by tests compounds alone were used for the plots. The data points were categorized by magnitude of the inhibitory effect of test compounds on RIF-induced CYP3A induction into three classes, >80% (○, non/weak), 30 to 80% (▲, middle), and <30% (●, potent), according to the percentages of the remaining CYP3A activity treated with 10 μM rifampicin in the presence of test compound to the activity treated with rifampicin alone. Data represent the mean calculated from four separate wells.

than 30% compared with those induced by rifampicin alone. The results suggest that measuring CYP3A4 mRNA is effective in assessing intrinsic CYP3A induction potential, especially in cases where test compounds have an inhibitory effect on the RIF-induced CYP3A activity.

In conclusion, we established a new CYP3A induction assay system using cryopreserved hepatocytes in a 96-well format with TL Matrigel plate. Our assay system offers several advantages in terms of higher capacity, robustness, and reproducibility. This assay system is applicable to the early drug discovery stage to rank the CYP3A induction potential for a large number of new drug candidates.

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