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Measurement of Human Cytochrome P450 Enzyme Induction Based on Mesalazine and Mosapride Citrate Treatments Using a Luminescent Assay

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Abstract

Drug metabolism mostly occurs in the liver. Cytochrome P450 (CYP) is a drug-metabolizing enzyme that is responsible for many important drug metabolism reactions. Recently, the US FDA and EU EMA have suggested that CYP enzyme induction can be measured by both enzymatic activity and mRNA expression. However, these experiments are time-consuming and their inter-assay variability can lead to misinterpretations of the results. To resolve these problems and establish a more powerful method to measure CYP induction, we determined CYP induction by using luminescent assay. Luminescent CYP assays link CYP enzyme activity to firefly luciferase luminescence technology. In this study, we measured the induction of CYP isozymes (1A2, 2B6, 2C9, and 3A4) in cryopreserved human hepatocytes (HMC424, 478, and 493) using a luminometer. We then examined the potential induction abilities (unknown so far) of mesalazine, a drug for colitis, and mosapride citrate, which is used as an antispasmodic drug. The results showed that mesalazine promotes CYP2B6 and 3A4 activities, while mosapride citrate promotes CYP1A2, 2B6, and 3A4 activities. Luminescent CYP assays offer rapid and safe advantages over LC-MS/MS and qRT-PCR methods. Furthermore, luminescent CYP assays decrease the interference between the optical properties of the test compound and the CYP substrates. Therefore, luminescent CYP assays are less labor intensive, rapid, and can be used as robust tools for high-throughput CYP screening during early drug discovery.

Key Words: CYP, Human hepatocytes, Luminescent assay, Mesalazine, Mosapride citrate

INTRODUCTION

Cytochrome P450 (CYP) enzymes, which are primarily located in the liver, account for more than 95% of the phase 1 metabolism of all drugs (Wrighton and Stevens, 1992; Ofotokun, 2005). More than 2,700 individual members of the CYP superfamily have been identified, and 57 CYP enzymes are recognized in humans (Mann, 2006). CYP enzymes are heme-containing membrane proteins and represent a family of isozymes responsible for the biotransformation of many drugs via oxidation (Ogu and Maxa, 2000).

Many adverse drug responses following multiple drug therapies have been associated with drug-drug interactions, involving inhibition and induction of drug-metabolizing enzymes (Garcia *et al*., 2008). Therefore, during drug discovery and lead optimization it is very important to screen for potential CYP inducibility of drug candidates, thereby enhancing the likelihood of developing drugs that are free of CYP-inducing properties (Garcia *et al*., 2008).

CYP induction ability is generally measured based on mRNA expression and enzymatic activity, as recommended by the FDA and EMA (Guideline on the investigation of drug interactions, 2013, European Medicines Agency, London). However, analyzing the results of these 2 measurement methods is time-consuming, and the results can be confounded by inter-assay variability. Additionally, enzymatic activity mea-

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surement requires expensive LC-MS/MS equipment that uses complex analytical processes. Thus, there is a need for more efficient methods of CYP induction, which could help to increase the pace of drug development.

Bioluminescent assays that monitor changes in luciferin concentration as the variable reactant rely on a series of luciferin derivatives; these are used as probe substrates for the enzymes of interest (Cali *et al*., 2008). Luminescent CYP assays utilize derivatives of beetle luciferin as the luminogenic probe for CYP substrates (Bosetti *et al*., 2005; Auld *et al*., 2013). These luciferin derivatives are metabolized by CYP to luciferins, which in turn react with luciferase and produce light (Bosetti *et al*., 2005). Assays are easily configured in multiwell plates where a homogenous luciferase mixture is added directly to CYP reactions that use luciferin derivatives as substrates (Bosetti *et al*., 2005). The amount of luciferin produced by a CYP is directly proportional to the light output, so light is used to measure the CYP activity (Bosetti *et al*., 2005). The energy input is chemical in nature, making the background minimal. In this case, a luminescent assay is more sensitive. Therefore, we performed CYP induction studies using a luminescent assay to compensate for the defects of mRNA expression and enzymatic activity measurements.

Routine assessment to identify the potential metabolism-mediated interactions of CYP1A2, CYP2B6 and CYP3A4 is recommended by the US FDA, because these CYP isozymes are involved in clinically important drug metabolisms FDA (Drug Interactions Studies-Study Design, Data Analysis, Implications for Dosing and Labeling Recommendations, 2012, Food and Drug Administration, Silver Spring). Therefore, we selected 4 CYP isozymes (CYP-1A2, -2B6, -2C9, and -3A4) and evaluated the effect of mesalazine and mosapride citrate on CYP. Mesalazine is a drug used to treat colitis, and mosapride citrate is an antispasmodic drug prescribed to patients with indigestion. Both drugs are frequently prescribed, but their CYP interactions are still not fully understood. Therefore, the investigation of mesalazine and mosapride citrate CYP interactions has clinical importance.

In this study, we established CYP luminescent assay using positive inducers and investigated the effects of mesalazine and mosapride citrate on CYP1A2, CYP2B6, CYP2C9, and CYP3A4 activities as detected by a luminometer. Furthermore, we used primary human hepatocytes for CYP induction, which a well-established model used to predict clinical results *in vitro*.

MATERIALS AND METHODS

Reagents and media

Omeprazole, rifampin, Krebs-Henseleit buffer, mesalazine, and mosapride citrate were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Phenobarbital was purchased from Jeil Pharmaceutical (Daegu, Korea). Cryopreserved human hepatocytes, the Gentest High Viability CryoHepatocyte Recovery Kit, hepatocyte culture medium, epidermal growth factor (EGF), L-glutamine, and matrigel matrix were purchased from BD Bioscience (San Jose, CA, USA). Penicillin/streptomycin, phosphate-buffered saline (PBS, pH 7.4), and gentamicin sulfate were purchased from Gibco® (Burlington, Canada). William's E medium (No Phenol Red), and GlutaMAX™ were purchased from Invitrogen (Seoul, Korea). The CYP-Glo™ CYP 1A2, 2C9, 3A4 assay and the CellTiter-Glo 2.0 assay were purchased from Promega (Madison, WI, USA). ITS+ culture supplement was purchased from Corning (Tewksbury, MA, USA).

Table 1. Characteristics of the human hepatocyte donors used in this study

Donor	Gender	Age	Race
HMC424	Male	64	Caucasian
HMC478	Male	64	Caucasian
HMC493	Male	75	Caucasian

Culture of human cryopreserved hepatocytes

Three different human cryopreserved hepatocyte cell lines (Table 1) were thawed using the Gentest High Viability Cryo-Hepatocyte Recovery Kit. After thawing, cells were seeded on 96-well collagen I-coated plates (1×10^5 cells/well) and incubated at 37°C with 5% CO_2 in a humidified atmosphere. Four hours after seeding, medium was replaced with 100 μL of matrigel buffer supplemented with William's E buffer without phenol red, 200 mM L-glutamine, 50 mg/mL gentamycin sulfate, 10 mM dexamethasone, ITS supplement, and 0.25 mg/mL matrigel matrix. Hepatocytes were then incubated for 24 h at 37°C with 5% CO_2 in a humidified atmosphere.

CYP induction

Hepatocytes were treated with positive inducers (1A2: 50 μM omeprazole; 2B6: 1000 μM phenobarbital; 2C9 and 3A4: 25 μM rifampin) and test substances (mesalazine: 0.32 μg/mL, 3.21 μg/mL, and 32.15 μg/mL; mosapride citrate: 8.69 ng/mL, 86.9 ng/mL, and 869 ng/mL) for 48 h, with media change every 24 h. Control hepatocytes were treated with 0.1% (v/v) DMSO. Hepatocytes were incubated at 37°C with 5% CO_2 in a humidified atmosphere.

Measuring CYP activity in cultured cells

Hepatocytes in the culture media containing positive inducers and test substances were removed from the plates and washed twice with Krebs-Henseleit buffer. Hepatocyte culture medium containing the CYP substrates (6 μM CYP-1A2, 3 μM CYP-2B6, 100 μM CYP-H, and 3 μM CYP-IPA) were added to the hepatocytes to measure the enzymatic activities of CYP1A2, CYP2B6, CYP2C9 and CYP3A4, respectively. Incubations were performed at 37°C with 5% CO_2 in a humidified atmosphere (CYP1A2 and CYP3A4: 60 min; CYP2B6: 120 min; CYP2C9: 240 min). After incubation, 100 μL aliquots of the hepatocyte culture media mixture from both wells (with cells, and control wells containing no cells) were transferred to a 96-well opaque white luminometer plate at room temperature. Next, a luciferin detection reagent (100 μL) was added to the samples to initiate a luminescent reaction. The plate was incubated at room temperature for 20 min, after which luminescence was measured using a luminometer. Net signals were calculated as below by subtracting the background luminescence values (no-cell control) from test substance-treated and vehicle control values. Finally, fold changes were calculated by dividing the net treated values by the net untreated values.

Table 2. Sequence of qRT-PCR primers used for the for the expression of genes

Gene	Sequence(5'-3')	Product size (bp)
1A2	F-ctgcagatccgcattggctc	399
	R-gtgctgtccgaagcacatgg	
2B6	F-ggagtagaggccatacggga	272
	R-aggaaggtggcgtccatgag	
2C9	F-ccctttggaagtggacccag	200
	R-acggtgccatcccttgactc	
3A4	F-ccactcctctcccagtgattgg	353
	R-gcgggcttcctcttgaacac	
GAPDH	F-ccaactgcttagcacccctg	272
	R-gtccaccactgacacgttgg	

Net signal calculation

CYP enzyme activity (ΔLuminescence/min)=Absorbance in induced hepatocyte-Absorbance in empty well

CYP mRNA expression analysis

The mRNA expression of CYP1A2, CYP2B6, 2C9 and CYP3A4 were analyzed by qRT-PCR. Total RNA was extracted using the easy-spin™ Total RNA Extraction Kit (iNtRON, Seongnam, Korea) and quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). cDNA was synthesized using SuperScript First-strand Synthesis system (Invitrogen, US) according to the manufacturer's instructions and quantified using a NanoDrop ND-1000 Spectrophotometer. One microgram of cDNA products was amplified on an IQ5 Real Time PCR detection system (Bio-Rad, Hemel Hempstead, United Kingdom) by the real-time PCR method. The PCR reactions were carried out in iQ® Realtime PCR plates (Bio-Rad) and the PCR mixtures consisted of 10 μl of 2X SYBR PCR premix (Bio-Rad), and 1 μl of 20 pmol forward and reverse primers (Table 2).

The PCR program consisted of the following steps: 50°C for 2 min followed by denaturation at 95°C for 10 min; 40 amplification cycles (95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec). Melting curve conditions were 55°C to 81°C at a ramp rate of 1°C per 30 sec. The relative quantification of gene transcription was calculated against the housekeeping gene, GAPDH. The amplified PCR products were qualified by melting curve analysis.

Statistical analysis

Statistical analyses were performed using 2-way ANOVA in Sigma Plot version 9.0 (Systat, San Jose, CA). Statistical significance was set at a p value of <0.05. Data were expressed as means ± SEM.

RESULTS

Verification of the CYP luminescent assay using positive inducers

To verify the luminescent assay, we evaluated the induction of CYP isozymes (1A2, 2B6, 2C9 and 3A4) using positive inducers in 3 different cryopreserved human hepatocyte cell lines. In accordance with FDA guidance, positive inducers (1A2: omeprazole, 2B6: phenobarbital, 2C9, 3A4: rifampin) were selected and used to treat hepatocytes from each of the 3 donors, to account for inter-individual variability (FDA, 2012). Although the fold-induction value varied between individuals, the induction of CYP isozymes (CYP1A2, 2B6, 2C9, and 3A4) was consistently observed, in a concentration-dependent manner, in each of the 3 hepatocyte cell lines (Fig. 1). These results indicated that the luminescent assay was reliable under the performed conditions.

The effects of mesalazine on CYP isozymes

Using the newly-developed luminescent assay, we investigated the potential CYP induction ability of mesalazine. According to previous studies, the maximum serum concentration (C_{max}) of mesalazine is 3.21 μg/mL (Wiersma *et al*., 2004). Hepatocytes were treated with 3 different concentrations as follows: 1/10-fold of C_{max}, C_{max}, and 10-fold of C_{max}. C_{max} is the average value of many patient populations, so it can occasionally cause false negative results. Thus, we used concentrations exceeding C_{max} to prevent false negative effects. FDA guidance stipulates that drugs may act as a potential inducer when CYP induction exceeds 2-fold after drug treatment (FDA, 2012). Therefore, we complied with FDA guidance and interpreted the CYP induction results accordingly.

Fig. 2 shows the effect of mesalazine on CYP induction. No significant differences in CYP1A2 and CYP2C9 induction were observed in each of the 3 hepatocyte cell lines in comparison to control. In other words, mesalazine cannot induce CYP1A2 and 2C9 activities. However, the activity of CYP2B6 was increased after mesalazine treatment (Fig. 2). The CYP2B6 induction values were 2.81 and 2.02-fold greater after 32.15 μg/mL mesalazine treatment in HMC478 and HMC493 cultures, respectively. In addition, the induction ability of CYP3A4 was also promoted by mesalazine (Fig. 2). The CYP3A4 induction values were 5.23 and 4.81-fold greater after 32.15 μg/mL mesalazine treatment in HMC424 and HMC478 cultures, respectively. In contrast to HMC424 and HMC478, the induction of HMC493 gradually increased by 4.28, 6.66, and, 23.76-fold at different concentrations of mesalazine.

To crosscheck the luminescent assay results, we performed mRNA expression measurements for CYP induction after mesalazine treatment. The mRNA expression of CYP2B6 and 3A4 were significantly increased (Fig. 3). The CYP2B6 induction values increased 2.06-fold in HMC424 and 3.26-fold in HMC493 at a mesalazine concentration of 32.15 μg/mL. In the case of HMC478, the expression of CYP2B6 increased at all concentrations (2.50, 2.95, and 7.95-fold). The mRNA expression of CYP3A4 was also induced at 32.15 μg/mL mesalazine (by 4.84-fold in HMC424, 2.75-fold in HMC478, and 3.46-fold in HMC493).

The effects of mosapride citrate on CYP isozymes

We also performed CYP interaction studies using mosapride citrate, based on its C_{max} value. The C_{max} of mosapride citrate is 86.9 ng/mL (Kim *et al*., 2012). The results showed that CYP1A2 and CYP3A4 activities were induced by 869 ng/mL mosapride citrate (Fig. 4). The activity of CYP1A2 increased 3.66 and 2.81-fold in HMC478 and HMC493 cultures, respectively, while the activity of CYP3A4 increased 2.10, 4.64, and 4.28-fold in each of the 3 donor cell lines. In addition, CYP2B6 activity was also induced by mosapride citrate (Fig. 4). The fold-inductions doubled at C_{max} and 10-fold of

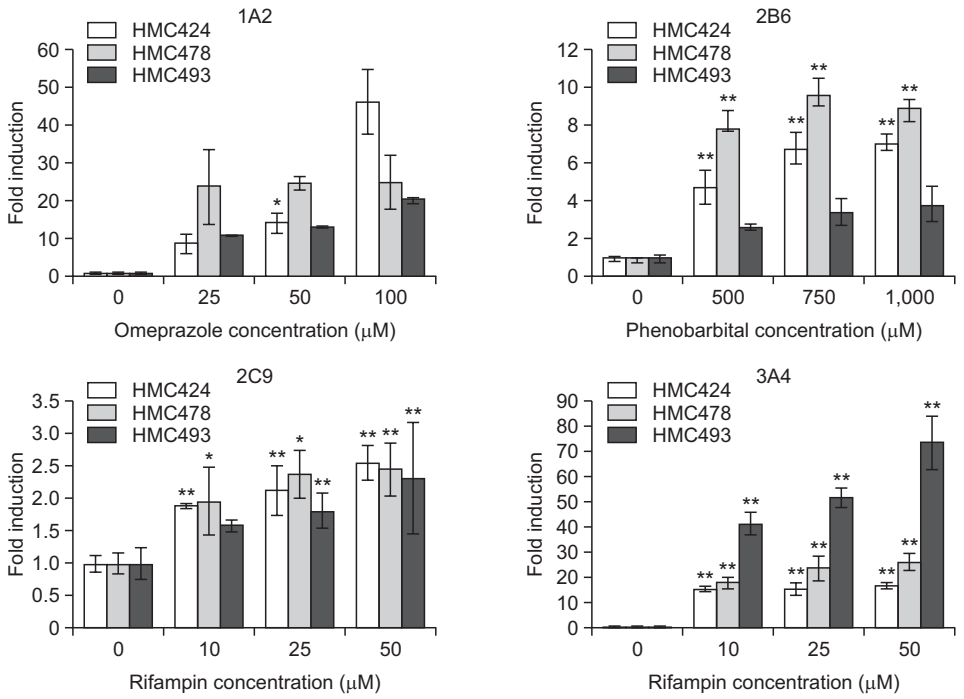

Fig. 1. The fold-induction of CYP isozymes after 48 hours treatment with positive inducers (1A2: 25, 50, and 100 μM omeprazole; 2B6: 500, 750, and 1000 μM Phenobarbital; 2C9, 3A4: 10, 25, and 50 μM rifampin) in 3 hepatocyte cell lines. **p*<0.05 and ***p*<0.01 *versus* each corresponding control.

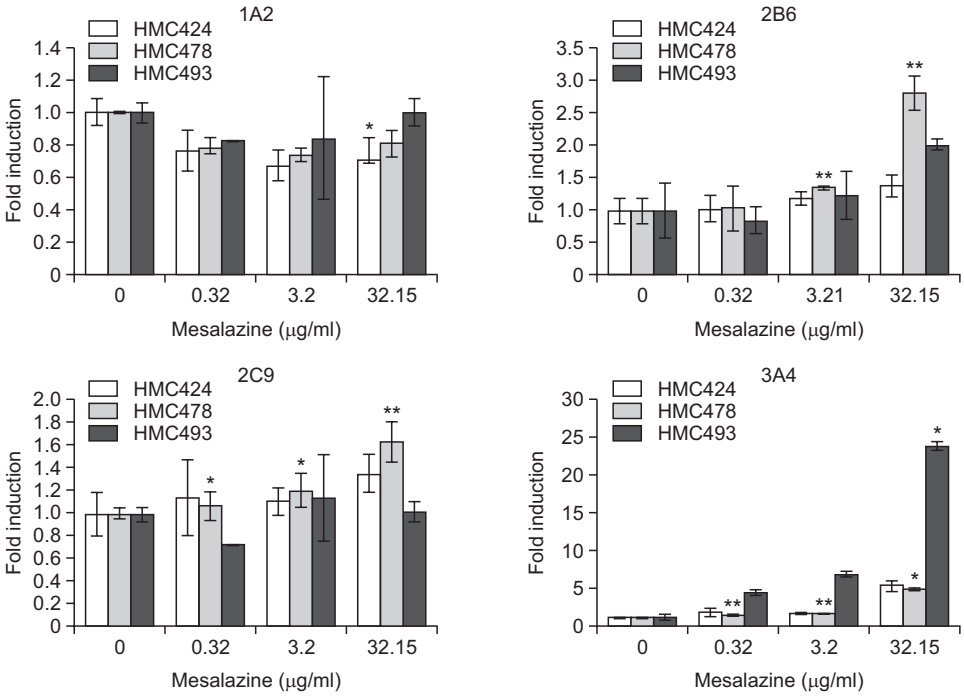

Fig. 2. The potential CYP induction ability of mesalazine (0.32, 3.2, and 32.15 μg/ml) in 3 hepatocyte cell lines, measured using a luminometer. **p*<0.05 and ***p*<0.01 *versus* each corresponding control.

C_{max}, with the exception of HMC424 hepatocytes. HMC424 treated with 869 ng/mL mosapride citrate showed a 5.46-fold increase, while HMC478 treated with 86.9 and 869 ng/mL mosapride citrate showed 3.14 and, 10.05-fold increases, respectively. Furthermore, the CYP2B6 activities of HMC493 were 2.63 and 8.12-fold increased after 8.69 and 869 ng/mL

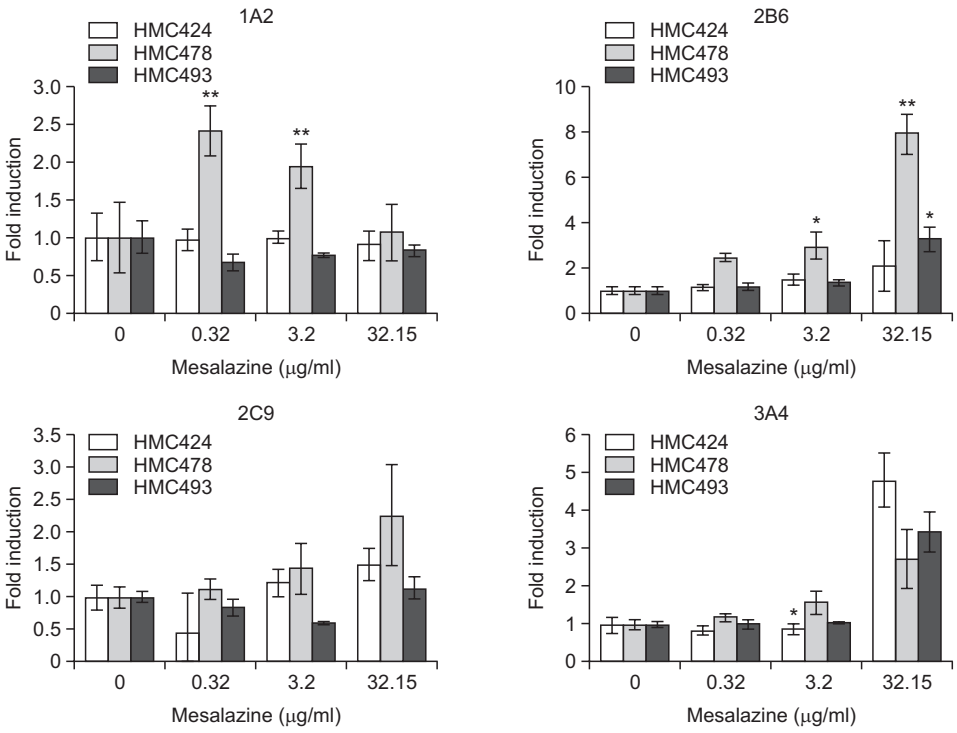

Fig. 3. The effects of mesalazine (0.32, 3.2, and 32.15 μg/ml) on CYP1A2, 2B6, 2C9, and 3A4 mRNA expression in 3 hepatocyte cell lines. **p*<0.05 and ***p*<0.01 *versus* each corresponding control.

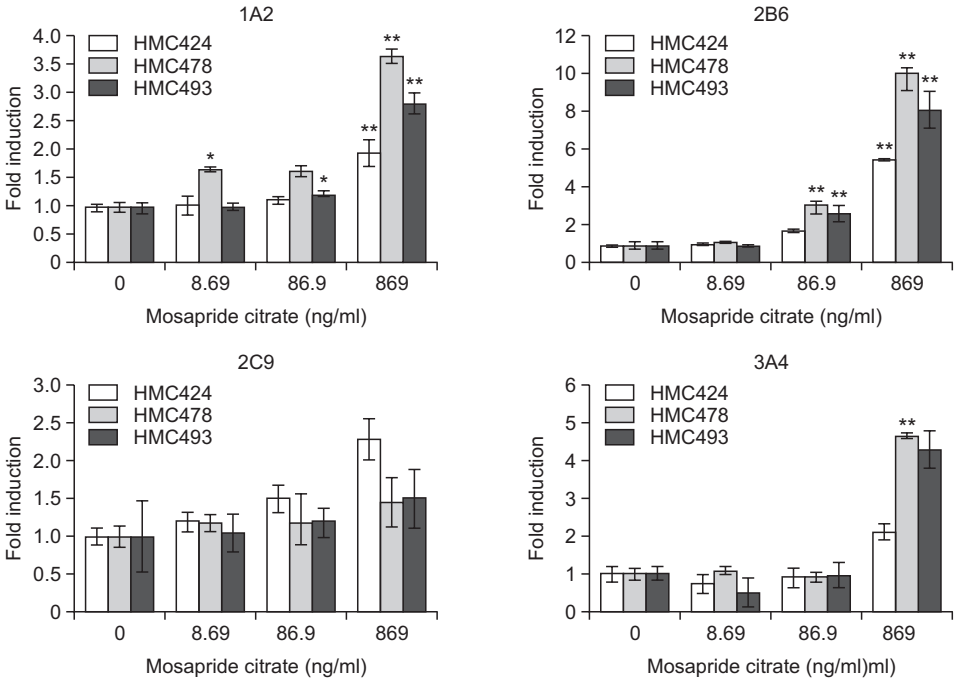

Fig. 4. The potential CYP induction ability of mosapride citrate (8.69, 86.9, and 869 ng/ml) in 3 hepatocyte cell lines measured using a luminometer. **p*<0.05 and ***p*<0.01 *versus* each corresponding control.

mosapride citrate treatments, respectively. On the other hand, CYP2C9 activity was not significantly changed by mosapride citrate treatment (Fig. 4).

The same as before, we investigated mosapride citrate induction ability using qRT-PCR. Mosapride citrate induced CYP1A2, 2B6, 2C9, and 3A4, but mRNA expression of each

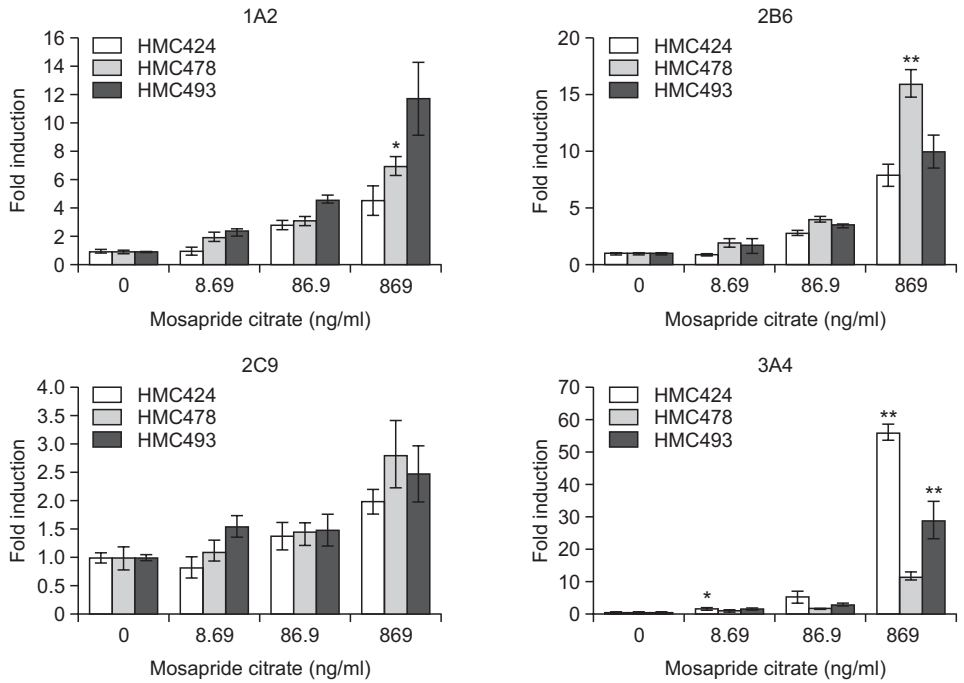

Fig. 5. The effects of mosapride citrate (8.69, 86.9, 869 ng/ml) on CYP1A2, 2B6, 2C9, 3A4 mRNA expression in 3 hepatocyte cell lines. **p*<0.05 and ***p*<0.01 *versus* each corresponding control.

of the 4 CYP isozymes differed depending on the donor cell line (Fig. 5). The fold-induction of CYP1A2 was 2.87 and 4.58-fold in HMC424 at 86.9 and 869 ng/mL mosapride citrate concentrations, respectively. In addition, the expression of 1A2 increased 2.00, 3.12, and 7.02-fold in HMC478, and 2.38, 4.64, 11.80-fold in HMC493 respectively. The mRNA level of CYP2B6 increased 2.85 and 8.05-fold in HMC424, 4.05 and 16.13-fold in HMC478, and 3.54 and 10.08-fold in HMC493 after 86.9 and 869 ng/mL mosapride citrate treatments, respectively. In the case of CYP2C9, HMC478 and HMC493 treated with 869 ng/mL mosapride citrate showed 2.81 and 2.47-fold increases, respectively. Lastly, the mRNA level of CYP3A4 increased 2.07, 5.54, and 56.14-fold in HMC424, and increased 2.45, 3.50, and 29.49-fold in HMC493 at increasing concentrations of mosapride citrate (8.69, 86.9, and 869 ng/mL, respectively). The induction of CYP3A4 in HMC478 was 12.09-fold at 869 ng/mL.

DISCUSSION

CYP is a major source of variability in drug pharmacokinetics and response (Zanger and Schwab, 2013) because the expression of each CYP is influenced by both endogenous (such as genetic polymorphisms and hormone levels) and exogenous factors (such as diet, nutraceutical chemical use, drug exposure, alcohol consumption, and cigarette smoking) (Parkinson *et al*., 2004). Therefore, an understanding of CYP isozyme interactions in response to drug treatment is important at the early phase of drug discovery.

mRNA expression measurement and enzymatic activity measurement are generally used in the study of CYP induction, as recommended by the FDA. However, there are limiting factors in these methods; for example, mRNA expression measurement can only detect induction at the level of gene transcription, while enzymatic activity measurement requires a substrate treatment process and use of expensive equipment. Accordingly, regulatory agencies are making efforts to establish more efficient assessment tools by introducing novel technologies for CYP induction measurement, thereby reducing the length of the drug development process. Thus, in this study we applied luminogenic technology as a new and efficient measurement tool for CYP induction. Moreover, we uncovered the poorly-understood CYP induction abilities of 2 drugs: mesalazine and mosapride citrate.

In this study, CYP2B6 and CYP3A4 were induced by mesalazine in a concentration-dependent manner (Fig. 2). In addition, the induction abilities of CYP1A2, 2B6, and 3A4 were promoted when mosapride citrate concentrations were increased (Fig. 4). Accordingly, mesalazine may act as a CYP2B6 and, CYP3A4 inducer, while mosapride citrate may influence CYP1A2, 2B6, and 3A4 induction *in vitro*.

Luminogenic CYP assays offer advantages of speed and safety over qRT-PCR and LC-MS/MS (Cali *et al*., 2006). Moreover, this luminogenic approach offers improved sensitivity and decreased interference between optical properties of the test compound and CYP substrate (Cali *et al*., 2006). Furthermore, we suggest that mRNA expression or enzymatic activity results for CYP induction could be validated by cross checking with luminescent assay results. Therefore, luminescent assays provide a simple, homogenous and robust assay format for rapid screening of compounds against CYP activities (Auld *et al*., 2013). Finally, luminescent assays can offer significant advantages over other alternative methods (Cali *et al*., 2008).

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