Progesterone receptor membrane component 1 modulates human cytochrome P450 activities in an isoform-dependent manner

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Running title: PGRMC1 modulates human drug-metabolizing P450 activities

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Number of text pages: 34
Number of tables: 5
Number of figures: 6
Number of supplemental table: 1
Number of supplemental figures: 2
Number of references: 30
Number of words in abstracts: 248 words
Number of words in introduction: 484 words
Number of words in discussion: 1308 words

ABBREVIATIONS: Ad, adenovirus; CPR, NADPH-cytochrome P450 reductase; GFP, green fluorescent protein; HLM, human liver microsomes; HPLC, high performance-liquid chromatography; MOI, multiplicity of infection; PGRMC1, progesterone receptor membrane component 1; P450 and CYP, cytochrome P450.
Abstract

Cytochrome P450s (CYP) catalyze the metabolism of a wide spectrum of compounds. Recently, progesterone receptor membrane component 1 (PGRMC1), which shares a key structural motif with cytochrome b₅, has been reported to bind to sterol- or steroid-synthesizing P450s, enhancing their activities. In this study, we investigated whether PGRMC1 affects human drug-metabolizing P450 activities. Using co-expression systems for PGRMC1 and P450s (CYP3A4, CYP2C9, or CYP2E1) in HepG2 cells, we found that PGRMC1 decreased the $V_{\text{max}}$ values and increased the $K_{\text{m}}$ values of the CYP3A4 activities, and it decreased the $V_{\text{max}}$ values but did not affect the $K_{\text{m}}$ values of the CYP2C9 activities. In contrast, PGRMC1 hardly affected the CYP2E1 activities. These results suggest that PGRMC1 negatively modulates the drug-metabolizing activities of P450, although it was isoform-, but not substrate-dependent.

Interestingly, co-immunoprecipitation analysis using co-expression systems for FLAG-PGRMC1 and Myc-P450s in HEK293 cells revealed that PGRMC1 interacts with all three P450s, although the affinity seemed to vary. In 29 human liver microsomes (HLM), there was 5-fold variability in the PGRMC1 protein levels. By the correlation analyses using the P450 activities and the PGRMC1 levels, we could neither observe the contribution of PGRMC1 to the P450 activities in HLM, nor that of the NADPH-cytochrome P450 reductase or cytochrome b₅. In conclusion, in contrast to sterol- or steroid-synthesizing P450s, we found that PGRMC1 negatively modulates the human drug-metabolizing activities of P450 through direct interaction. Further studies are needed to determine the clinical significance of PGRMC1 in the pharmacokinetics of drugs.
Introduction

Cytochrome P450 (P450, CYP) enzymes are heme-containing proteins that catalyze the metabolism of a wide variety of structurally diverse compounds (Nelson et al., 2004; Nebert and Russell, 2002). There are as many as 57 functional CYP genes and 58 pseudogenes in humans (http://drnelson.utmem.edu/CytochromeP450.html). Among them, three families, CYP1, CYP2, and CYP3, contribute to the oxidative metabolism of more than 70% of clinical drugs. Other CYP families (e.g., CYP4, CYP7, CYP11, CYP17, CYP19, CYP21, CYP51) are involved in the metabolism of endogenous molecules such as steroids, bile acids, leukotrienes, and eicosanoids. P450 enzymes can exert their function by receiving electrons from NADPH-cytochrome P450 reductase (CPR) or cytochrome b₅ (Guengerich, 2002). CPR is indispensable for the P450 activities, whereas cytochrome b₃ has a significant role in the activities of some P450s (Shimada et al., 1994; Locuson et al., 2006).

Progesterone receptor membrane component 1 (PGRMC1) was originally identified as a membrane-associated non-genomic receptor for progesterone (Cahill, 2007). Although it is predominantly localized in endoplasmic reticulum, it seems to be detected in the plasma membrane, nucleus, and cytoplasm (Lösel et al., 2008; Chahill, 2007). PGRMC1 is widespread in eukaryotes from yeast to human. Despite its name, it is controversial whether progesterone binds to PGRMC1 (Min et al., 2005; Peluso et al., 2008b), and its function has not been fully understood. PGRMC1 is expressed in many tissues including liver, kidney, brain, breast, and adrenals (Lösel et al., 2008; Chahill, 2007). PGRMC1, a 22-kDa protein, contains a transmembrane domain at the N-terminal and a cytochrome b₃-like domain to which heme binds in the middle (Mifsud and Bateman, 2002; Min et al., 2004 and 2005; Ghosh et al., 2005). As for the association of PGRMC1 with the P450 activities, there are several reports as follows. Laird et al. (1988) reported that monoclonal antibody to rat inner zone antigen (IZA), a synonym of PGRMC1, blocked the 21-hydroxylation of progesterone in rat adrenal tissue. Min et al. (2005) reported that co-expression of PGRMC1, but not that of a heme-deficient PGRMC1 mutant, enhanced the human CYP21A2 activity in COS-7 cells. Hughes et al. (2007) demonstrated that damage associated protein (Dap) 1, the yeast homolog of PGRMC1, binds
and positively regulates CYP51A1 and CYP61A1 which catalyze sterol biosynthesis, and knockdown of endogenous PGRMC1 in HEK293 cells resulted in the decreased cholesterol synthesis catalyzed by human CYP51A1. Thus, it has been demonstrated that PGRMC1 positively regulates P450-mediated sterol or steroid syntheses, making it “a helping hand for P450 proteins” (Debose-Boyd, 2007).

In contrast to endobiotic-metabolizing P450s, there is limited information on the effects of PGRMC1 on xenobiotic-metabolizing P450s. Although it was shown, by co-immunoprecipitation using a co-expression system in HEK293 cells, that PGRMC1 bound to CYP3A4 (Hughes et al., 2007), the functional significance remains to be clarified. In this study, we sought to investigate whether PGRMC1 might be a regulator of human drug-metabolizing P450 activities, focusing on CYP3A4, CYP2C9 and CYP2E1.
Materials and Methods

Chemicals and Reagents. Testosterone was purchased from Wako Pure Chemical Industries (Osaka, Japan). 7-Ethoxycoumarin, 7-hydroxycoumarin, chlorozoxzone, 6-hydroxychlorozoxzone, diclofenac, and S-warfarin were from Sigma-Aldrich (St. Louis, MO). 4’-Hydroxydiclofenac, 6β-hydroxytestosterone, and 7-hydroxywarfarin were purchased from BD Gentest (Woburn, MA), Steraloids (Wilton, NH), and Ultrafine Chemicals (Manchester, UK), respectively. Midazolam and 1’-hydroxymidazolam were kindly provided by Astellas Pharmaceutical (Tokyo, Japan). Clonazepam was kindly provided by Roche (Basel, Switzerland). NADP⁺, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Oriental Yeast (Tokyo, Japan). Adenovirus Expression Vector kit (Dual Version) and QuickTiter Adenovirus Titer Immunoassay kit were from Takara (Osaka, Japan) and Cell Biolabs (Tokyo, Japan), respectively. The pTARGET vector was purchased from Promega (Madison, WI). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). All primers were commercially synthesized at Hokkaido System Science (Sapporo, Japan). Rabbit anti-human CYP3A4 polyclonal antibody and rabbit anti-human CYP2C9 antibody were from BD Gentest. Goat anti-rat CYP2E1 polyclonal antibody was from Nosan (Yokohama, Japan). Rabbit anti-human PGRMC1 polyclonal antibody and mouse anti-FLAG monoclonal antibody (M2) were from Sigma-Aldrich. Rabbit anti-human/rat CPR antibody was from CHEMICON (Temecula, CA). Rabbit anti-cytochrome b₅ antibody and mouse anti-c-Myc monoclonal antibody (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 680 donkey anti-goat IgG was from Invitrogen. IRDye 680 goat anti-rabbit IgG and goat anti-mouse IgG were from LI-COR Biosciences (Lincoln, NE). All other chemicals and solvents were of the highest grade commercially available.

Construction of Recombinant Adenoviruses and Plasmids. Recombinant adenoviruses expressing CYP3A4 (AdCYP3A4), CYP2C9 (AdCYP2C9), and green fluorescence protein (AdGFP) were constructed as in our previous studies (Hosomi et al., 2010; Iwamura et al., 2011).
Recombinant adenoviruses expressing PGRMC1 (AdPGRMC1) and CYP2E1 (AdCYP2E1) were created as follows. A fragment containing the full-length coding region of the human PGRMC1 or CYP2E1 cDNA was amplified by PCR using the primer pairs shown in Table 1 with a human liver cDNA as a template. The fragments were subcloned into the pAxCAwtit vector at a *Swa* I site. These vectors and the adenovirus genome DNA-terminal protein complex were co-transfected into HEK293 cells by Lipofectoamine 2000. The recombinant adenovirus was isolated and propagated. Viral titers were determined using the QuickTiter Adenovirus Titer Immunoassay kit. The multiplicity of infection (MOI) was defined as the ratio of infectious units divided by the number of cells.

Expression vector for human PGRMC1 containing FLAG tag at the C-terminus (FLAG-PGRMC1) was constructed as follows. Human PGRMC1 cDNA was amplified by PCR using the primers S-PGRMC1 and AS-FLAG PGRMC1 (Table 1) with the human liver cDNA as a template. The AS-FLAG PGRMC1 primer contains complementary sequences of FLAG tag and a stop codon. The PCR product was digested with *BamH*I and *Xho*I and ligated into the pcDNA3.1 Hygro+ vector (Invitrogen).

Expression vectors for human P450s (CYP3A4, CYP2C9, and CYP2E1) containing 3×Myc tag at the C-terminus (Myc-P450) were constructed as follows. Once P450 cDNA lacking a stop codon was amplified by PCR with the primer pairs shown in Table 1, it was digested with *Xho*I and *Kpn*I and subcloned into the pTARGET vector digested with the same restriction enzymes (pTARGET/P450 stop-). A double strand DNA fragment containing three tandem copies of Myc tag sequence followed by a stop codon (Table 1) was subcloned into the pTARGET/P450 stop- plasmids digested with *Kpn*I and *Not*I. The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analyses.

**Cell Cultures.** Human embryonic kidney cell line HEK293 and human hepatocellular carcinoma cell line HepG2 were obtained from American Type Culture Collection (Rockville, MD) and Riken Gene Bank (Tsukuba, Japan), respectively. These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan).
supplemented with 0.1 mM non-essential amino acid (NEAA) (Invitrogen) and 10% fetal bovine serum (FBS) (Invitrogen). For the construction of the co-expression systems for FLAG-PGRMC1 and Myc-P450, the HEK293 cells were cultured in DMEM containing 4.5 g/liter glucose, 10 mM HEPES, and 10% FBS. Human cryopreserved hepatocytes, lot H704 (Caucasian, female, 49 years old), were purchased from XenoTech (Lenexa, KS). The hepatocytes were cultured in hepatocyte culture medium (HCM) (Cambrex, East Rutherford, NJ) on a plate coated with Cell Matrix Type I-C (Nitta Gelatin, Tokyo, Japan). These cells were maintained at 37°C under an atmosphere of 5% CO₂, 95% air.

**Infection of Recombinant Adenoviruses to HepG2 Cells or Cryopreserved Human Hepatocytes.** HepG2 cells were seeded at 7.5 × 10⁵ cells/well into a 6-well plate and were allowed to grow confluent. The cells were infected with a constant MOI of AdCYP (AdCYP3A4: 5, AdCYP2C9: 20, AdCYP2E1: 25, represented as ×1 in Fig. 1) and varied MOI of AdPGRMC1 (0, 2.5, 5, 10, represented as 0, ×1, ×2, ×4). To make the total MOI the same value in four different experimental conditions, AdGFP was infected. After 24 h, the cultured medium was replaced with fresh medium without adenovirus. After 48 h, total cell homogenates were prepared by homogenization with TGE buffer (10 mM Tris-HCl (pH 7.4), 20% glycerol, and 0.1 mM EDTA). The protein concentration was determined using Bradford protein assay reagent (Bio-Rad, Hercules, CA) with γ-globulin as a standard.

The human hepatocytes were seeded at 1.5 × 10⁶ cells/well into a 6-well plate. After 3 h, the medium was changed to HCM (albumin- and antibiotics-free) containing AdPGRMC1 or AdGFP at MOI 30. After 1 h, the medium was replaced with fresh HCM. After 48 h, the total cell homogenates were prepared as described above.

**Human Liver Microsomes.** Pooled human liver microsomes (HLM) (n = 50) and individual HLM (20 donors) were purchased from BD Gentest. Human liver samples from 9 donors were obtained from Human and Animal Bridging Research Organization (Chiba, Japan), which is in partnership with the National Disease Research Interchange (Philadelphia, PA). Microsomes
were prepared according to the method described previously (Tabata et al., 2004).

**SDS-PAGE and Western Blotting.** Total cell homogenates or HLM (20 µg) were separated with 10% SDS-polyacrylamide gel electrophoresis (PAGE) for the detection of P450s, and with 15% SDS-PAGE for the detection of PGRMC1. The separated proteins were electrotransferred onto the polyvinylidene difluoride membrane Immobilon-P (Millipore, Billerica, MA). The membranes were probed with rabbit anti-human CYP3A4, goat anti-rat CYP2E1, rabbit anti-human CYP2C9, rabbit anti-human PGRMC1, anti-human/rat CPR or anti-cytochrome b₅ antibodies and the corresponding fluorescent dye-conjugated second antibodies. The band densities were quantified with Odyssey Infrared Imaging system (LI-COR Biosciences). The expression levels of P450 proteins were defined on the basis of a standard curve using P450 Supersomes (BD Gentest).

**Enzyme Assays.** A typical incubation mixture (final volume of 0.2 mL) contained 0.4 mg/mL total cell homogenates or 0.2 mg/mL HLM, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 5 mM MgCl₂ and 1 unit/mL glucose 6-phosphate dehydrogenase) and each substrate. The reaction mixture was preincubated at 37°C for 2 min and the reaction was started by adding the NADPH-generating system.

The testosterone 6β-hydroxylase activity was determined as described previously (Nakajima et al., 1999) with a 20-min reaction time. The product formation was determined using HPLC with a LachromUltra C18 (4.6 × 100 mm; 3 µm) column (HITACHI, Tokyo, Japan) and monitored at 240 nm.

The midazolam 1'-hydroxylase activity was determined as described previously (Kronbach et al., 1989) with slight modifications. The reaction mixture was incubated at 37°C for 15 min and the reaction was terminated by adding 100 µL of ice-cold methanol. Clonazepam (20 ng) was added as an internal standard. After the removal of the protein by centrifugation at 10,000 g for 5 min, a 20 µL-portion of the sample was subjected to liquid chromatography-tandem mass
spectrometry (LC-MS/MS) system with an HP 1100 system including a binary pump, an automatic sampler, and a column oven (AB SCIEX, Tokyo, Japan), which was equipped with a ZORBAX SB-C18 (2.1 × 50 mm; 3.5 µm) column (Agilent Technologies). The column temperature was 20°C and the flow rate was 0.2 mL/min. The mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A linear gradient was used from 20% B to 90% B over 2 to 3 min followed by 90% B for 7 min and then the column was allowed to re-equilibrate at the initial conditions for 4 min. The LC was connected to a PE Sciex API 2000 tandem mass spectrometer (AB SCIEX) operated in the positive electrospray ionization mode. The turbo gas was maintained at 550°C. Nitrogen was used as the nebulizing gas, turbo gas, and curtain gas at 40, 90, and 40 psi, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using nitrogen as the collision gas. The collision energy was 37 V. The mass/charge (m/z) ion transitions were recorded in the multiple reaction monitoring mode: m/z 342.0 and 203.0 for 1'-hydroxymidazolam; m/z 315.9 and 270.1 for clonazepam.

The S-warfarin 7-hydroxylase activity was determined as described previously (Yamazaki et al., 1997) with a 20-min incubation time. The product formation was determined using HPLC with a Mightysil RP-18 (4.6 × 150 mm; 5 µm) column (Kanto Chemical, Tokyo, Japan) and monitored with the excitation wavelength set at 320 nm and emission at 415 nm.

The diclofenac 4'-hydroxylase activity was determined by the method by Katoh et al. (2004) with a 30-min incubation time. The product formation was determined using HPLC with a TSK-GEL ODS-80Tm (4.6 × 250 mm; 5 µm) column (TOSOH, Tokyo, Japan) and monitored at 280 nm.

The chlorzoxazone 6-hydroxylase activity was determined as described previously (Mohri et al., 2010) with a 30-min incubation time. The product formation was determined using HPLC with a LachromUltra C18 (4.6 × 100 mm; 3 µm) column and monitored at 295 nm.

The 7-ethoxycoumarin O-deethylase activity was determined as described previously (Yamazaki et al., 1999) with a 30-min incubation time. The product formation was determined
using HPLC with a Mightysil RP-18 (4.6 × 150 mm; 5 µm) column and monitored with the excitation wavelength set at 338 nm and emission at 458 nm.

Kinetic parameters were estimated from the fitted curve using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis. The following equations were used:

Michaelis-Menten equation: $V = V_{\text{max}} \times [S] / (K_m + [S])$

Substrate inhibition equation: $V = V_{\text{max}} \times [S] / (K_m + [S] + [S]^2 / K_i)$

where $V$ is the velocity of the reaction, $[S]$ is the substrate concentration, $K_m$ is the Michaelis-Menten constant, $V_{\text{max}}$ is the maximum velocity, and $K_i$ is the substrate inhibition constant. Data are expressed as the means ± SD of three independent determinations.

Co-immunoprecipitation Assay. The FLAG-PGRMC1 and each Myc-P450 expression plasmid were transiently co-transfected into HEK293 cells. Briefly, the day before transfection, the cells were seeded into a 6-well plate coated with Cell Matrix Type I-C. After 24 h, each 2 µg of FLAG-PGRMC1 and Myc-P450 plasmids were transfected using Lipofectamine 2000. After 48 h, the cells were collected and total cell homogenates were prepared by homogenization with TGE buffer. Five-hundred micrograms of protein were suspended in either buffer A or buffer B in a final volume 0.5 mL and rotated at 4°C for 2 h. Buffer A consisted of 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5 mM (p-amidinophenyl)-methanesulfonyl fluoride hydrochloride, 2 µg/mL aprotinin, and 2 µg/mL leupeptin. The detergents in buffer A were replaced with 0.1% digitonin in buffer B. The lysates were centrifuged at 100,000 g for 30 min. To the supernatants, the anti-FLAG antibody was added and incubated for 8 h followed by precipitation with protein G sepharose beads. The beads were washed with buffer A or B and suspended with Laemmli sample buffer. The eluent was subjected to Western blot analyses with anti-FLAG antibody or anti-c-Myc antibody.

Statistical Analyses. Statistical significance was determined by using unpaired, two-tailed
student’s $t$ test for paired data between two groups, or Shirley-Williams’ test when more than 3
groups were compared. Correlation analyses were performed by Pearson’s product-moment
method. When the $P$ value was less than 0.05, the differences was considered statistically
significant.
Results

Establishment of Co-expression Systems for PGRMC1 and P450s in HepG2 Cells. To establish co-expression systems for PGRMC1 and P450s, AdPGRMC1 was co-transfected with AdCYP3A4, AdCYP2C9, or AdCYP2E1 into the HepG2 cells. Preliminarily, we optimized the MOIs to obtain the expression levels of PGRMC1 and P450s that are close to those in pooled human liver microsomes. When AdPGRMC1 was infected at MOI 3-4, the expressed PGRMC1 levels were close to those in the pooled HLM. Accordingly, we set the MOIs for AdPGRMC1 at 2.5, 5, and 10, which were represented as ×1, ×2, and ×4, respectively (Fig. 1). The MOIs for AdCYP2C9 and AdCYP2E1 were set at 20 and 25, respectively. In the case of AdCYP3A4, MOIs over 10 showed cellular toxicity. Therefore, we set the MOIs for AdCYP3A4 at 5, although the level of CYP3A4 protein produced was lower than that in the pooled HLM (Fig. 1) but was in the range of those in individual HLM, as described below. Thus, we obtained four lines for each P450 with various levels of PGRMC1. Using these systems, we investigated the effects of PGRMC1 on the P450 activities.

Effects of Co-expression of PGRMC1 on CYP3A4 Activities. To investigate the effects of PGRMC1 on the CYP3A4 activity, kinetic analyses of testosterone 6β-hydroxylation and midazolam 1’-hydroxylation were performed using the total cell homogenates from the HepG2 co-expression system. The kinetics of testosterone 6β-hydroxylation followed the Michaelis-Menten equation (Fig. 2A). The $K_m$, $V_{max}$, and $V_{max}/K_m$ values of the homogenates from the cells with no exogenous PGRMC1 were $56.9 \pm 9.1 \mu M$, $54.3 \pm 4.0 \text{ pmol/min/pmol CYP}$, and $0.96 \pm 0.08 \mu L/min/pmol CYP$, respectively (Table 2). The co-expression of PGRMC1 significantly decreased the $V_{max}$ values and increased the $K_m$ values, resulting in a decrease of the $V_{max}/K_m$ values in a PGRMC1 concentration-dependent manner. The kinetics of midazolam 1’-hydroxylation followed the substrate-inhibition equation (Fig. 2B). The $K_m$, $V_{max}$, $K_i$, and $V_{max}/K_m$ values by the homogenates from the cells with no exogenous PGRMC1 were $5.7 \pm 0.9 \mu M$, $9.8 \pm 1.5 \text{ pmol/min/pmol CYP}$, $15.9 \pm 4.1 \mu M$, and $1.72 \pm 0.03 \mu L/min/pmol CYP$,
respectively (Table 2). The co-expression of PGRMC1 significantly decreased the $V_{\text{max}}$ values and increased the $K_m$ values, resulting in a decrease of the $V_{\text{max}}/K_m$ values in a PGRMC1 concentration-dependent manner. The $K_i$ value was not affected by the co-expression of PGRMC1. Thus, it was demonstrated that PGRMC1 has the ability to attenuate the CYP3A4 activity independently of the substrate.

**Effects of Co-expression of PGRMC1 on CYP2C9 Activities.** To investigate the effects of PGRMC1 on the CYP2C9 activity, kinetic analyses of $S$-warfarin 7-hydroxylation and diclofenac 4′-hydroxylation were performed. The kinetics of $S$-warfarin 7-hydroxylation followed the Michaelis-Menten equation (Fig. 3A). The $K_m$, $V_{\text{max}}$, and $V_{\text{max}}/K_m$ values by the homogenates from the cells with no exogenous PGRMC1 were $1.6 \pm 0.1 \mu M$, $5.4 \pm 0.2 \text{ pmol/min/pmol CYP}$, and $3.3 \pm 0.2 \mu L/min/pmol CYP$, respectively (Table 3). The co-expression of PGRMC1 significantly decreased the $V_{\text{max}}$ values in a PGRMC1 concentration-dependent manner but did not affect the $K_m$ values, resulting in a decrease of the $V_{\text{max}}/K_m$ values. The kinetics of diclofenac 4′-hydroxylation followed the Michaelis-Menten equation (Fig. 3B). The $K_m$ and $V_{\text{max}}$, and $V_{\text{max}}/K_m$ values by the homogenates from the cells with no exogenous PGRMC1 were $7.3 \pm 0.3 \mu M$, $91.6 \pm 6.8 \text{ pmol/min/pmol CYP}$, and $12.6 \pm 1.5 \mu L/min/pmol CYP$, respectively (Table 3). The co-expression of PGRMC1 significantly decreased the $V_{\text{max}}$ values in a PGRMC1 concentration-dependent manner but did not affect the $K_m$ values, resulting in a decrease of the $V_{\text{max}}/K_m$ values. Thus, it was demonstrated that PGRMC1 has the ability to attenuate the CYP2C9 activity independently of the substrate.

**Effects of Co-expression of PGRMC1 on CYP2E1 Activities.** To investigate the effects of PGRMC1 on the CYP2E1 activity, kinetic analyses of chlorzoxazone 6-hydroxylation and 7-ethoxycoumarin $O$-deethylation were performed. The kinetics of chlorzoxazone 6-hydroxylation followed the Michaelis-Menten equation (Fig. 4A). The $K_m$, $V_{\text{max}}$, and $V_{\text{max}}/K_m$ values by the homogenates from the cells with no exogenous PGRMC1 were $67.1 \pm 5.6 \mu M$, $618.3 \pm 18.2 \text{ pmol/min/pmol CYP}$, and $9.3 \pm 0.5 \mu L/min/pmol CYP$, respectively (Table 4). The
co-expression of PGRMC1 did not affect the kinetic parameters. The kinetics of 7-ethoxycoumarin $O$-deethylation followed the Michaelis-Menten equation (Fig. 4B). The $K_m$, $V_{max}$, and $V_{max}/K_m$ values by the homogenates from the cells with no exogenous PGRMC1 were 40.2 ± 2.5 µM, 16.0 ± 1.2 pmol/min/pmol CYP, and 0.40 ± 0.01 µL/min/pmol CYP, respectively (Table 4). The co-expression of PGRMC1 did not affect the $K_m$ and $V_{max}$ values, but slightly decreased the $V_{max}/K_m$ values. PGRMC1 likely had a small effect on the CYP2E1 activities in comparison with the CYP3A4 and CYP2C9.

**Effects of Overexpression of PGRMC1 on Enzyme Activities in Human Hepatocytes.** To investigate whether PGRMC1 modulates the activities of endogenous human P450s, we sought to overexpress PGRMC1 in human hepatocytes. When the homogenates from the human hepatocytes were subjected to Western blot analysis, the band density of PGRMC1 protein was similar to that in the pooled HLM (Fig. 5A). When AdPGRMC1 was infected, the PGRMC1 protein level was significantly (5.6 fold, $P < 0.001$) increased (Fig. 5A). We confirmed that there was no morphological change by the infection with AdPGRMC1. Using the homogenates from these cells, the midazolam 1'-hydroxylase, $S$-warfarin 7-hydroxylase, and chlorzoxazone 6-hydroxylase activities, at the substrate concentrations of 10, 10, and 500 µM, respectively, were evaluated. Interestingly, we found that the midazolam 1'-hydroxylase and $S$-warfarin 7-hydroxylase activities in the homogenates from the AdPGRMC1-infected cells were significantly lower than those in control (AdGFP-infected cells). In contrast, the chlorzoxazone 6-hydroxylase activity was not affected by the overexpression of PGRMC1 (Fig. 5B). These results suggest that PGRMC1 modulates the endogenous human P450 activity in an isoform-specific manner, supporting the results from the expression systems.

**Co-immunoprecipitation of PGRMC1 and P450s.** To investigate whether PGRMC1 directly interacts with P450s, we employed a co-immunoprecipitation assay. Since commercially available antibodies against PGRMC1 or P450s are not suitable for immunoprecipitation assays, we constructed FLAG-PGRMC1 and Myc-P450 co-expression
systems to perform the immunoprecipitation assay using anti-tag antibodies. We confirmed, by
Western blot analyses using anti-FLAG and anti-c-Myc antibodies, that both FLAG-PGRMC1
and Myc-P450s were successfully expressed (Fig. 6). When the lysates using buffer A were
assayed, the PGRMC1 in the three expression systems was immunoprecipitated to the same
extent by using the anti-FLAG antibody, and only Myc-CYP2E1 was co-immunoprecipitated
(Fig. 6A). When the lysates using buffer B were assayed, all three P450s were
coproportionated (Fig. 6B). These results suggest that PGRMC1 binds directly to these
P450s, although the degree would be different among the isoforms.

**Relationship between P450 Activities and PGRMC1, CPR, and Cytochrome b$_5$ Levels in A Panel of 29 Human Liver Microsomes.** The midazolam 1'-hydroxylase, S-warfarin
7-hydroxylase, and chlorzoxazone 6-hydroxylase activities in a panel of 29 human liver
microsomes were measured at the substrate concentrations of 10, 5, and 500 µM, and the
PGRMC1, CYP3A4, CYP2C9, CYP2E1, CPR, and cytochrome b$_5$ protein levels were
determined by Western blot analysis. The variability of the PGRMC1 protein levels was
~5-fold (Table 5). Although the variability of the CYP3A4 protein levels was large (3 to 72
pmol/mg, 24-fold), those of the CYP2C9 (5 to 17 pmol/mg, ~3-fold) and the CYP2E1 (3 to 16
pmol/mg, ~5-fold) protein levels were relatively small. As shown in supplemental Fig. 1A-C,
the midazolam 1'-hydroxylase, S-warfarin 7-hydroxylase, and chlorzoxazone 6-hydroxylase
activities represented as the metabolite/min/mg protein were significantly correlated with
CYP3A4, CYP2C9, and CYP2E1, respectively. To investigate whether the PGRMC1 would be
a factor modulating the P450 activities, correlation analyses between the ratio of PGRMC1 to
P450 and the midazolam 1'-hydroxylase, S-warfarin 7-hydroxylase, and chlorzoxazone
6-hydroxylase activities represented as the metabolite/min/pmol CYP were performed. We
expected inverse correlations between the PGRMC1/CYP3A4 ratio and midazolam
1'-hydroxylase or PGRMC1/CYP2C9 ratio and S-warfarin 7-hydroxylase activities based on
the results of the co-expression systems in HepG2 cells. However, no inverse correlation was
observed (Supplemental Fig. 1D-F). Additionally, except for the CPR/CYP2E1 ratio, the
CPR/P450 or cytochrome b\textsubscript{5}/P450 ratios did not show a positive correlation with the activities (Supplemental Fig. 1G-L). Next, we determined the relationship between the PGRMC1 protein level and each P450 activity corrected with the CPR protein (Supplemental Fig. 2A-C) or cytochrome b\textsubscript{5} protein (Supplemental Fig. 2D-F) levels. However, no inverse correlation was observed in the CYP3A4 or CYP2C9 activities. Since the PGRMC1 protein levels were significantly correlated with the CPR ($R = 0.50, P < 0.01$) and cytochrome b\textsubscript{5} ($R = 0.76, P < 0.0001$) protein levels (Supplemental Fig. 2G-H), it would be difficult to estimate the contribution of PGRMC1 to the P450 activities in HLM by the correlation analyses.
Discussion

In this study, we investigate the effects of PGRMC1 on the human drug-metabolizing P450 activities, focusing on three major isoforms, CYP3A4, CYP2C9, and CYP2E1. Using co-expression systems for PGRMC1/CYPs in HepG2 cells, we found that PGRMC1 increased the $K_m$ and decreased the $V_{max}$ of the CYP3A4 activities, and decreased the $V_{max}$ of the CYP2C9 activities irrespective of the substrates (Figs. 2 and 3). In contrast to CYP3A4 and CYP2C9, PGRMC1 did not dramatically affect the CYP2E1 activities, indicating the effects of PGRMC1 would be P450 isoform-dependent. During the process of preparing this report, an independent study reported that PGRMC1 commonly decreased the CYP3A4, CYP2C8 and rabbit CYP2C2 activities using co-expression systems in HEK293 cells (Szczesna-Skorupa and Kemper, 2011). They evaluated the enzyme activities by P450-Glo assay at a substrate concentration. The findings that PGRMC1 decreased the activities of drug-metabolizing P450s were similar between our and recent reports, but our new findings are that the effects of PGRMC1 on the kinetics were different between the P450 isoforms and that there exists P450(s) not affected by PGRMC1. When we mixed the homogenates from the single expression system for PGRMC1 and the homogenates from the single expression system for P450s, no changes were observed in the kinetics of each P450 activity (data not shown). Therefore, it was suggested that co-localization on the membrane would be critical for PGRMC1 to exert its effect in modulating the P450s activities. It has been reported that PGRMC1 is predominantly located in the endoplasmic reticulum. However, in a human ovarian cancer cell line, Ovcar-3, PGRMC1 is found in the cytoplasm (Lösel et al., 2008). To analyze the localization of PGRMC1 in human liver, we performed Western blot analysis using cytosol, but PGRMC1 could not be detected. Therefore, the subcellular localization of PGRMC1 appears to be cell type-specific.

To investigate whether PGRMC1 modulates the activities of endogenous P450, we performed experiments using human hepatocytes. First, we sought to investigate the effects of repression of PGRMC1 by siRNA on the P450 activities (data not shown). When siRNA for PGRMC1 (Stealth select RNAi, Invitrogen) was transfected into human hepatocytes, the PGRMC1 mRNA levels were decreased by 70%. However, the PGRMC1 protein level was not
decreased (data not shown). Although we used additional siRNA for PGRMC1 from another supplier (Ambion, Austin, TX), favorable results were not obtained. Hence, we sought to investigate the effects of the overexpression of PGRMC1 in human hepatocytes. The overexpression of PGRMC1 resulted in decreases of the CYP3A4 and CYP2C9 activities, but not CYP2E1 activity, which were the same as with the HepG2 co-expression systems (Fig. 5). These results suggest that PGRMC1 modulates the activities of endogenous P450 in an isoform-specific manner.

Using the co-immunoprecipitation assay (Fig. 6), we found that PGRMC1 interacts with P450s (not only CYP3A4 and CYP2C9 but also CYP2E1). When buffer A containing Nonidet P-40 and sodium deoxycholate, which are relatively strong detergents, was used, only CYP2E1 was co-immunoprecipitated (Fig. 6A). However, when buffer B containing digitonin, which is a relatively weaker detergent, was used, all of the three P450 isoforms were co-immunoprecipitated (Fig. 6B). These results suggested that the binding of PGRMC1 to CYP2E1 might be stronger than the binding to CYP3A4 or CYP2C9. Alternatively, the number of CYP2E1 molecules that bound to a PGRMC1 molecule might be larger than that of CYP3A4 or CYP2C9. In other words, a smaller number of PGRMC1 molecules may bind to CYP2E1. Such differences might explain why PGRMC1 did not affect the CYP2E1 activity but decreased the CYP3A4 and CYP2C9 activities, or the effects of PGRMC1 might not be a simple protein-protein interaction.

It was demonstrated that introduction of a mutation in the cytochrome \(b_5\)-like domain of PGRMC1, to which heme binds, abolishes the binding to CYP7A1 (Mansouri et al., 2008). Min et al. (2005) also reported that a heme-deficient PGRMC1 mutant could not increase the CYP21A2 activity. These observations suggest the importance of heme binding for PGRMC1 in its function. Cytochrome \(b_5\) has a hexacoordinate heme that is capable of transferring an electron. Meanwhile, PGRMC1 has a pentacoordinate heme (Cahill, 2007; Rohe et al., 2009), suggesting that PGRMC1 does not donate electrons to P450. Therefore, PGRMC1 affects the P450 function without direct electron transfer. Szczesna-Skorupa and Kemper (2011) have reported that the PGRMC1-dependent inhibition of P450 activities is partially restored by the
overexpression of CPR. In addition, since the co-immunoprecipitation of CPR and PGRMC1 was observed, they concluded that PGRMC1 binds to CPR and decreases the P450 activities. In contrast to their study, the co-immunoprecipitation of CPR and PGRMC1 was not observed in our system using HEK293 cells without the overexpression of CPR, probably due to the low expression level (data not shown). Alternatively, we measured the cytochrome c reduction and found that PGRMC1 did not alter the CPR activity (Supplemental Table 1). Thus, the decrease of the P450 activities by PGRMC1 might not be due to the decrease of CPR activity, although the possibility that PGRMC1 might influence the electron transferring from CPR to P450, because the $V_{\text{max}}$ values of CYP3A4 and CYP2C9 were decreased, could not be excluded. In the case of CYP3A4, an increase of the $K_m$ values was also observed. Therefore, as another mechanism, it is suggested that PGRMC1 might cause an allosteric change in the CYP3A4 structure that affects the affinity to substrates. Nevertheless, the effects of PGRMC1 on the P450 activity were diverse depending on the P450 isoforms, and further detailed studies are needed to clarify the underlying mechanisms.

In this study, the variability of the PGRMC1 protein levels in HLM was first evaluated. In 29 human liver samples, there was 5-fold variability (Table 5). We sought to estimate the contribution of PGRMC1 to the modulation of the P450 activities by correlation analyses between the P450 activities and P450 or PGRMC1 protein levels. In the analysis, we took into account the other components, the transferring of electrons to P450 such as CPR or cytochrome $b_5$. Although these components positively regulate the enzyme activities of CYP3A4, CYP2C9, and CYP2E1, no clear correlation with the P450 activities was observed except in the case of CYP2E1. It has been reported that the molar ratio of P450:CPR:cytochrome $b_5$ seems to be important to understanding the role of CPR in the modulation of the P450 activities. We can determine the absolute expression levels of P450, CPR, and cytochrome $b_5$, but not PGRMC1. Understanding the absolute expression level of PGRMC1 in HLM would be helpful to determine the relative importance of PGRMC1 in the modulation of the P450 activity.

Earlier studies reported that PGRMC1 increased the activities of CYP21A2 and CYP51A1 that are responsible for steroid or sterol metabolism (Min et al., 2005; Hughes et al., 2007). In
contrast, this study found that PGRMC1 decreased the activity of human drug-metabolizing P450s, which is supported by the study by Szczesna-Skorupa and Kemper (2011), although it is likely isoform-dependent. It has been reported that PGRMC1 is highly expressed in breast and ovary tumors and in cancer cell lines from the colon, thyroid, lung, and cervix (Cruden et al., 2005; Peluso et al., 2008a). Recently, it has been reported that PGRMC1 is highly expressed in human myometrium during pregnancy and may mediate the relaxation effect on myometrium (Wu et al., 2011). The changes of PGRMC1 expression under certain physiological conditions might impact on the metabolism of steroids. Meanwhile, little is known about the factors that affect the PGRMC1 expression level in human liver. Further studies are warranted to clarify the physiological significance of PGRMC1 in the modulation of drug-metabolizing P450 in liver.

In conclusion, we found that PGRMC1 decreases the activities of drug-metabolizing P450s in an isoform-dependent manner. The action was opposite to that for steroid-metabolizing P450s. Thus, PGRMC1 seems to affect P450s depending on their functions. The present study revealed a novel function of PGRMC1 in modulating the drug-metabolizing activity.

Acknowledgements

We acknowledge Mr. Brent Bell for reviewing the manuscript.
Authorship Contributions

Participated in research design: Oda, Nakajima, Fukami, and Yokoi

Conducted experiments: Oda

Contributed new reagents or analytic tools: Oda, Toyoda, and Fukami

Performed data analysis: Oda and Nakajima

Wrote or contributed to the writing of the manuscript: Oda, Nakajima, and Yokoi
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Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, and Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice
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Yamazaki H, Gillam EM, Dong MS, Johnson WW, Guengerich FP, and Shimada T (1997) Reconstitution of recombinant cytochrome P450 2C10 (2C9) and comparison with
cytochrome P450 3A4 and other forms: effects of cytochrome P450-P450 and

cromatographic assay for coumarin 7-hydroxylation and 7-ethoxycoumarin
O-deethylation by human liver cytochrome P450 enzymes. J Chromatogr B Biomed Appl
Footnotes

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E-mail: tyokoi@kenroku.kanazawa-u.ac.jp
Figure Legends

Fig. 1. Establishment of co-expression systems for PGRMC1 and CYP3A4, CYP2C9, or CYP2E1. HepG2 cells were infected with AdPGRMC1, each AdP450, and AdGFP for 72 h at the indicated MOI. Total MOI was adjusted by the infection of AdGFP. Twenty-micrograms of total cell homogenates from the HepG2 or human liver microsomes were subjected to Western blot analyses. The absolute P450 protein levels were determined using a standard curve with each human P450 Supersomes. The PGRMC1 protein levels were expressed relative to the value in the pooled HLM set at 1.0. The values on the membrane are the mean of two independent determinations. MOI, multiplicity of infection.

Fig. 2. Kinetic analyses of testosterone 6β-hydroxylation (A) and midazolam 1’-hydroxylation (B) by recombinant CYP3A4 in single or co-expression systems with PGRMC1. The expression systems were constructed using recombinant adenoviruses as described under Material and Methods. The obtained kinetic parameters are shown in Table 2. Data are the means ± SD of three independent determinations. MOI, multiplicity of infection.

Fig. 3. Kinetic analyses of S-warfarin 7-hydroxylation (A) and diclofenac 4’-hydroxylation (B) by recombinant CYP2C9 in single or co-expression systems with PGRMC1. The expression systems were constructed using recombinant adenoviruses as described under Material and Methods. The obtained kinetic parameters are shown in Table 3. Data are the means ± SD of three independent determinations. MOI, multiplicity of infection.

Fig. 4. Kinetic analyses of chlorzoxazone 6-hydroxylation (A) and 7-ethoxycoumarin O-deethylation (B) by recombinant CYP2E1 in single or co-expression systems with PGRMC1. The expression systems were constructed using recombinant adenoviruses as described under Material and Methods. The obtained kinetic parameters are shown in Table 4. Data are the means ± SD of three independent determinations. MOI, multiplicity of infection.
**Fig. 5.** Effects of overexpression of PGRMC1 on P450 activities in human hepatocytes. Human hepatocytes were infected with AdPGRMC1 or AdGFP at MOI 30. After 48 h, cells were collected and total cell homogenates were prepared. (A) Expression level of PGRMC1 in the homogenates (10 µg) was determined by Western blot analysis. Pooled HLM (20 µg) were also subjected. (B) Midazolam 1’-hydroxylase activity (at 10 µM substrate concentration), S-warfarin 7-hydroxylase activity (at 10 µM substrate concentration), and chlorzoxazone 6-hydroxylase activity (at 500 µM substrate concentration) in the homogenates were measured. *P < 0.05 and **P < 0.001. NS, not significant.

**Fig. 6.** Co-immunoprecipitation of FLAG-PGRMC1 and Myc-P450. FLAG-PGRMC1 plasmid and Myc-P450 (CYP3A4, CYP2C9, and CYP2E1) plasmid were transiently co-transfected into HEK293 cells. Five-hundred micrograms of protein of the total cell homogenates were solubilized with buffer A (A) or buffer B (B) and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were subjected to SDS-PAGE followed by Western blot analyses using anti-FLAG antibody or anti-Myc antibody. Input proteins (20 µg) were also subjected to Western blot. Data are representative of at least three independent experiments. IP, immunoprecipitation; WB, Western blot.
Table 1. Sequence of oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>5’ to 3’ sequence</th>
</tr>
</thead>
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<tr>
<td>For construction of cosmid DNA</td>
<td></td>
</tr>
<tr>
<td>S-PGRMC1</td>
<td>GAGTTCCGGATCCTGCC (The BamH I site is underlined.)</td>
</tr>
<tr>
<td>AS-PGRMC1</td>
<td>ATACCTCGAGAGTATACTTCCACTG (The Xho I site is underlined.)</td>
</tr>
<tr>
<td>S-CYP2E1-1</td>
<td>ATGTCTGCCCTCGAGGTACAC</td>
</tr>
<tr>
<td>AS-CYP2E1-1</td>
<td>CTCATGAGCGGGGAATGACA</td>
</tr>
<tr>
<td>For construction of FLAG-tagged PGRMC1 plasmid</td>
<td></td>
</tr>
<tr>
<td>S-PGRMC1</td>
<td>See above</td>
</tr>
<tr>
<td>AS-FLAG PGRMC1</td>
<td>TAGACTCGAGCTATTTGCTATCTGTCATCTTCTGTAAGACATCATTTTCCCCGGGC ACTC (The Xho I site is underlined. Complementary sequences of FLAG tag are bolded. Complementary sequences of stop codon (TAG) are italicized.)</td>
</tr>
<tr>
<td>For construction of Myc-tagged P450 plasmids</td>
<td></td>
</tr>
<tr>
<td>S-CYP3A4</td>
<td>TCACTCGAGTTGCTCTCATCCAGACTTTG (The Xho I site is underlined.)</td>
</tr>
<tr>
<td>AS-CYP3A4</td>
<td>GGTCTCACTTACCGTGCACTGCCCTCTG (The Kpn I site is underlined.)</td>
</tr>
<tr>
<td>S-CYP2C9</td>
<td>TCACTCGAGAGGATTCTCTTGAGTC (The Xho I site is underlined.)</td>
</tr>
<tr>
<td>AS-CYP2C9</td>
<td>GACAGGAAATGAGCAAGCAGCTC (The Kpn I site is underlined.)</td>
</tr>
<tr>
<td>S-CYP2E1-2</td>
<td>TCACTCGAGCTGCTGCCCTCGGAGTC (The Xho I site is underlined.)</td>
</tr>
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<td>AS-CYP2E1-2</td>
<td>TGACGCGGGGAATGACACAGAG (The Kpn I site is underlined.)</td>
</tr>
<tr>
<td>S-3×Myc</td>
<td>CTCGGTAGAGCACAGAAGCTAGCGAGGACTTGGAAGGACTGCTGGACCGAGGACTGGACAGAG CTGATCGAGGGAGGACCTGAGCAACAGCTGACAGCGAGAGACCTG TCGACGCGGGCA CTGAGCGGGCAGCAGCAGCG (The Kpn I and Not I sites are underlined. Three tandem Myc tags are bolded. The stop codon (TGA) is italicized.)</td>
</tr>
<tr>
<td>AS-3×Myc</td>
<td>CTGCTCGAGCGGCGGCGCTCAAGGTCCTCTCGTACAGCTTCTGCTCC AGTTCCTCCCTCGGTACAGCTTCTTCTGCTGCTCTGAGCGTCTGATCAGCTCCCCT GCTTTGCTGCTACCGAG (The Not I and Kpn I sites are underlined. Complementary sequences of 3 tandem Myc tags are bolded. Complementary sequences of stop codon (TGA) are italicized.)</td>
</tr>
</tbody>
</table>

S: sense; AS: antisense. The start codon is boxed. The deleted stop codon was shown with the mark ‘ˇ’. 
Table 2. Kinetic parameters for testosterone 6β-hydroxylase activity and midazolam 1’-hydroxylase activity by recombinant CYP3A4 in single or co-expression systems with PGRMC1.

<table>
<thead>
<tr>
<th></th>
<th>Testosterone 6β-hydroxylation</th>
<th></th>
<th>Midazolam 1’-hydroxylation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol/min/pmol CYP)</td>
<td>$V_{max}/K_m$ (µL/min/pmol CYP)</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>AdCYP3A4 + AdPGRMC1</td>
<td></td>
<td></td>
<td></td>
<td>$5.7 \pm 0.9$</td>
</tr>
<tr>
<td>-</td>
<td>$56.9 \pm 9.1$</td>
<td>$54.3 \pm 4.0$</td>
<td>$0.96 \pm 0.08$</td>
<td>$10.9 \pm 2.2 \ast$</td>
</tr>
<tr>
<td>× 1</td>
<td>$53.1 \pm 22.3$</td>
<td>$51.2 \pm 5.9$</td>
<td>$1.07 \pm 0.42$</td>
<td>$11.6 \pm 3.8 \ast$</td>
</tr>
<tr>
<td>× 2</td>
<td>$71.4 \pm 14.2$</td>
<td>$43.4 \pm 4.4 \ast$</td>
<td>$0.62 \pm 0.07 \ast$</td>
<td>$12.5 \pm 2.1 \ast$</td>
</tr>
<tr>
<td>× 4</td>
<td>$91.8 \pm 12.2 \ast$</td>
<td>$37.4 \pm 2.7 \ast$</td>
<td>$0.41 \pm 0.04 \ast$</td>
<td>$12.5 \pm 2.1 \ast$</td>
</tr>
</tbody>
</table>

The expression systems were constructed using recombinant adenoviruses as described under Materials and Methods. Kinetic parameters were calculated from curves by non-linear regression. Data are mean ± SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with control by Shirley-Williams’ test.
Table 3. Kinetic parameters for S-warfarin 7-hydroxylase activity and diclofenac 4’-hydroxylase activity by recombinant CYP2C9 in single or co-expression systems with PGRMC1.

<table>
<thead>
<tr>
<th></th>
<th>S-Warfarin 7-hydroxylation</th>
<th></th>
<th>Diclofenac 4’-hydroxylation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (µM)</td>
<td>( V_{max} ) (pmol/min/pmol CYP)</td>
<td>( V_{max}/K_m )</td>
<td>( K_m ) (µM)</td>
</tr>
<tr>
<td>AdCYP2C9 + AdPGRMC1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>× 1</td>
<td>1.6 ± 0.1</td>
<td>5.4 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>× 2</td>
<td>1.7 ± 0.2</td>
<td>2.4 ± 0.2 **</td>
<td>1.5 ± 0.1 **</td>
<td>7.1 ± 2.7</td>
</tr>
<tr>
<td>× 4</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.1 **</td>
<td>1.0 ± 0.1 **</td>
<td>8.4 ± 0.6</td>
</tr>
</tbody>
</table>

The expression systems were constructed using recombinant adenoviruses as described under Materials and Methods. Kinetic parameters were calculated from curves by non-linear regression. Data are mean ± SD of three independent experiments. * \( P < 0.05 \) and ** \( P < 0.01 \) compared with control by Shirley-Williams’ test.
Table 4. Kinetic parameters for chlorzoxazone 6-hydroxylase activity and 7-ethoxycoumarin O-deethylase activity by recombinant CYP2E1 in single or co-expression systems with PGRMC1.

<table>
<thead>
<tr>
<th>AdCYP2E1 + AdPGRMC1</th>
<th>Chlorzoxazone 6-hydroxylation</th>
<th>7-Ethoxycoumarin O-deethylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol/min/pmol CYP)</td>
</tr>
<tr>
<td>-</td>
<td>67.1 ± 5.6</td>
<td>618.3 ± 18.2</td>
</tr>
<tr>
<td>× 1</td>
<td>85.5 ± 21.6</td>
<td>667.8 ± 112.7</td>
</tr>
<tr>
<td>× 2</td>
<td>56.1 ± 8.2</td>
<td>617.9 ± 56.1</td>
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<td>× 4</td>
<td>65.6 ± 7.2</td>
<td>723.1 ± 52.6</td>
</tr>
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The expression systems were constructed using recombinant adenoviruses as described under Materials and Methods. Kinetic parameters calculated from curves by non-linear regression. Data are mean ± SD of three independent experiments. * $P < 0.05$ compared with control by Shirley-Williams’ test.
Table 5. Expression levels of PGRMC1, P450s, CPR and cytochrome b₅, and enzyme activities in human liver microsomes.

<table>
<thead>
<tr>
<th>Relative expression level</th>
<th>Expression level (pmol/mg)</th>
<th>Enzyme activity</th>
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</thead>
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<tr>
<td>PGRMC1ᵃ</td>
<td>CPRᵇ</td>
<td>Cyt b₅ᶜ</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.62 ± 0.25</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>(Range)</td>
<td>(0.23-1.23)</td>
<td>(1.0-2.6)</td>
</tr>
</tbody>
</table>

Mean ± SD and Range of each values from 29 individual human liver microsomes were shown. ᵃPGRMC1 protein level was expressed as relative to the pooled HLM set at 1.0; ᵇCPR and ᶜcytochrome b₅ protein levels are indicated as relative to the lowest set at 1.0; ᵈmidazolam 1'-hydroxylase activity (nmol/min/mg); ᵉS-warfarin 7-hydroxylase activity (pmol/min/mg); ᶠchlorzoxazone 6-hydroxylase activity (nmol/min/mg). Each data are the mean of duplicate determinations. Cyt b₅, cytochrome b₅.
Fig. 2

A

- AdCYP3A4
- AdCYP3A4 + AdPGRMC1 x1
- AdCYP3A4 + AdPGRMC1 x2
- AdCYP3A4 + AdPGRMC1 x4

Testosterone 6β-hydroxylation (pmol/min/pmol CYP)

Testosterone (µM)

0 50 100 150 200 250 300

B

Midazolam 1'-hydroxylation (pmol/min/pmol CYP)

Midazolam (µM)

0 5 10 15 20 25 30
Fig. 3

A

- AdCYP2C9
- AdCYP2C9 + AdPGRMC1 x1
- AdCYP2C9 + AdPGRMC1 x2
- AdCYP2C9 + AdPGRMC1 x4

S-Warfarin 7-hydroxylation (pmol/min/pmol CYP)

S-Warfarin (μM)

B

Diclofenac 4'-hydroxylation (pmol/min/pmol CYP)

Diclofenac (μM)
Fig. 5

A

<table>
<thead>
<tr>
<th></th>
<th>AdGFP</th>
<th>AdPGRMC1</th>
<th>Pooled HLM</th>
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<tr>
<td>Anti-PGRMC1</td>
<td>1.0 ± 0.1</td>
<td>5.6 ± 0.4</td>
<td>***</td>
</tr>
</tbody>
</table>

B

- **Midazolam 1'-hydroxylase activity (pmol/min/mg)**
  - AdGFP: 8
  - AdPGRMC1: 4

- **S-Warfarin 7-hydroxylase activity (fmol/min/mg)**
  - AdGFP: 120
  - AdPGRMC1: 80

- **Chlorzoxazone 6-hydroxylase activity (pmol/min/mg)**
  - AdGFP: 25
  - AdPGRMC1: 25

*NS: Not Significant*

*Significant differences indicated by asterisks.*
Fig. 6

A

<table>
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<th>IP: anti-FLAG</th>
<th>IP: Mouse IgG</th>
<th>Plasmid:</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Myc-P450</td>
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<tr>
<td>2C9 2E1 3A4</td>
<td>+</td>
<td>+</td>
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kDa

25 |

50 |

WB: Anti-FLAG

Anti-c-Myc

B

<table>
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<tr>
<th></th>
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<th>IP: Mouse IgG</th>
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</tbody>
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WB: Anti-FLAG

Anti-c-Myc