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Characterization of chicken cytochrome P450 1A4 and 1A5: Inter-paralog comparisons of substrate preference and inhibitor selectivity

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ABSTRACT

The chicken (*Gallus gallus*) is one of the most economically important domestic animals and also an avian model species. Chickens have two CYP1A genes (CYP1A4 and CYP1A5) which are orthologous to mammalian CYP1A1 and CYP1A2. Although the importance of chicken CYP1As in metabolism of endogenous compounds and xenobiotics is well recognized, their enzymatic properties, substrate preference and inhibitor selectivity remain poorly understood. In this study, functional enzymes of chicken CYP1A4 and CYP1A5 were successfully produced in *Escherichia coli* (*E. coli*). The substrate preference and inhibitor specificity of the two chicken CYP1As were compared. Kinetic results showed that the enzymatic parameters (K_m, V_{max}, V_{max}/K_m) for ethoxyresorufin O-deethylase (EROD) and benzyloxyresorufin O-debenzylase (BROD) differed between CYP1A4 and CYP1A5, while no significant difference was observed for methoxyresorufin O-demethylase (MROD). Lower K_m of CYP1A4 for BROD suggests that CYP1A4 has a greater binding affinity to benzyloxyresorufin than either ethoxyresorufin or methoxyresorufin. The highest V_{max}/K_m ratio was seen in BROD activity for CYP1A4 and in MROD for CYP1A5 respectively. These results indicate that substrate preference of chicken CYP1As is more notably distinguished by BROD activity and CYP1A5 prefers shorter alkoxyresorufins resembling its mammalian ortholog CYP1A2. Differential patterns of MROD inhibition were observed between CYP1As and among the five CYP inhibitors (α-naphthoflavone, furafylline, piperonyl butoxide, erythromycin and ketoconazole). α-Naphthoflavone was determined to be a potent MROD inhibitor of both CYP1A4 and CYP1A5. In contrast, no or only a trace inhibitory effect (<15%) was observed by erythromycin at a concentration of 500 μM. Stronger inhibition of MROD activity was found in CYP1A5 than CYP1A4 by relatively small molecules α-naphthoflavone, piperonyl butoxide and furafylline. AROD kinetics and inhibition profiles between chicken CYP1A4 and CYP1A5 demonstrate that the two paralogous members of the CYP1A subfamily have distinct enzymatic properties, reflecting differences in the active site geometry between CYP1A4 and CYP1A5. These findings suggest that CYP1A4 and CYP1A5 play partially overlapping but distinctly different physiological and toxicological roles in the chicken.

1. Introduction

Cytochrome P450 monooxygenases (CYPs) comprise a large superfamily of heme-containing proteins that are responsible for catalyzing the oxidation of a wide variety of endogenous and exogenous compounds (Meunier et al., 2004). Mammals generally possess two paralogous CYP1A genes (CYP1A1, CYP1A2), and they are the focus of intense scrutiny in view of toxicology, mostly because these enzymes are inducible by xenobiotics and play important roles in the detoxification of foreign compounds, and bioactivation of a variety of drugs and procarcinogenic polycyclic aromatic hydrocarbons (Ma and Lu, 2007).

Similar to mammals, chickens have two CYP1As (CYP1A4 and CYP1A5) (Gilday et al., 1996) which are orthologous to mammalian CYP1A1 and CYP1A2 respectively (Goldstone and Stegeman, 2006). Previous studies have largely focused on xenobiotic induction of CYP1A activities (Nakai et al., 1992; Rifkind et al., 1994; Gilday et al., 1996; Verbrugge et al., 2001; Head and Kennedy, 2007; Lee et al., 2009). Chicken CYP1As are constitutively expressed in chicken liver (Rifkind et al., 1994; Gilday et al., 1996), and inducible by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Rifkind et al., 1994; Mahajan and Rifkind, 1999; Head and Kennedy, 2007; Watanabe et al., 2009)

Abbreviations: AROD, alkoxyresorufin O-dealkylase; BROD, benzyloxyresorufin O-debenzylase; CPR, NADPH-cytochrome P450 reductase; CYP(s), cytochrome P450(s); DMSO, dimethyl sulfoxide; EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; PBO, piperonyl butoxide.

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and other chemicals (Gilday et al., 1996; Verbrugge et al., 2001; Mundy et al., 2010).

Catalytic activity of chicken CYP1As has been investigated with limited substrates by reconstituted systems with purified CYPs or by immunochemical or chemical inhibition approaches (Nakai et al., 1992; Kupfer et al., 1994; Rifkind et al., 1994; Verbrugge et al., 2001; Diaz et al., 2010a). These two CYP1A isoforms exhibit catalytic selectivity either for endogenous compound metabolism or for xenobiotic transformation. Chicken CYP1A4 is mainly responsible for ethoxyresorufin O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH), as evidenced by the observations that the mean turnover numbers for both EROD and AHH metabolism are much greater for reconstituted purified CYP1A4 than CYP1A5 (Rifkind et al., 1994). In contrast, CYP1A5 preferentially catalyzes arachidonic acid epoxygenation, the 4-hydroxylation of tamoxifen and uroporphyrinogen oxidation (Kupfer et al., 1994; Sinclair et al., 1997), but is inactive in EROD or AHH activity (Nakai et al., 1992; Rifkind et al., 1994). Inhibition studies suggest that CYP1A1 ortholog is a key phase I enzyme responsible for the bioactivation of aflatoxin B1 (AFB1) into toxic aflatoxin-8,9-epoxide (AFBO) in the chicken microsome (Diaz et al., 2010a).

Understanding the differences of paralogous isoforms in substrate preference and inhibitor specificity is of significant toxicological and evolutionary importance. However, no comprehensive data is available in this regard for chicken CYP1As so far. Although catalytic analyses have shown the resemblance of catalytic specificities between CYP1A4 and CYP1A1, and between CYP1A5 and CYP1A2 (Rifkind et al., 1994; Gilday et al., 1996; Sinclair et al., 1997), enzymatic function of chicken CYP1As should not be simply deduced from mammalian CYP1 orthologs due to species differences. In fact, species variations in enzymatic function and other aspects of CYP1A enzymes have been increasingly documented (Sinclair et al., 1997; Kubota et al., 2009; Diaz et al., 2010a, 2010b, 2010c). In this study, as a first step towards understanding the biochemistry and toxicology of chicken CYPs, we firstly produced functional CYP1A enzymes using the *Escherichia coli* (*E. coli*) expression system, considering the fact that it is difficult to obtain active, individual CYP enzymes by traditional biochemical techniques. Then we compared the enzymatic properties of the two paralogous CYP1As using prototype substrates and commonly used CYP inhibitors. Our results revealed the differences in enzymatic kinetics and response to inhibitors between the two paralogous chicken CYP1As. In addition, the efficient functional expression of chicken CYP1As and reconstitution with chicken NADPH-cytochrome reductase (CPR) described in this study provides a completely chicken-specific enzyme system applicable for further investigation of physiological, pharmacological and toxicological roles of chicken CYP1As.

2. Materials and methods

2.1. Chemicals

PrimeScript™ Reverse Transcriptase, Prime Star DNA polymerase, LA *Taq* DNA Polymerase, r-Taq DNA Polymerase, T4 DNA Ligase, DNase I and pMD19-T simple vector were purchased from Takara (Dalian, China). Easy Pure Quick Gel Extraction Kit was obtained from Tiangen (Beijing, China). Restriction endonucleases *Nde* I, *Eco*R I, *Sal* I, *Bam*H I were from New England Biolabs (Beijing, China). Protein molecular weight standards (Marker II), Trans Easy Pure Plasmid Miniprep Kit, *pEASY*-T1 Simple Cloning Kit were from Transgen Biotech (Beijing, China). Oligonucleotide primers were synthesized by Invitrogen (Beijing, China). The plasmid pCWOri+, used to express CYP1A proteins, was kindly provided by Dr. Thomas Friedberg (University of Dundee, Scotland).

Resorufin (95%), methoxyresorufin (98%), ethoxyresorufin, benzyloxyresorufin, ketoconazole (98%), piperonyl butoxide (PBO, 90%), furafylline (98%), α-naphthoflavone (98%), glucose-6-phosphate dehydrogenase and 5-aminolevulinic acid hydrochloride (ALA, 98%) were purchased from Sigma (St. Louis, MO, USA). Cytochrome c (horse heart, 95%), isopropyl β-D-1-thiogalactopyranoside (IPTG, 99.6%), NADPH Na4 (98%), glucose-6-phosphate (98%), dithiothreitol (DTT) and phenylmethanesulfonyl fluoride (PMSF, 99%) were obtained from Merck (Darmstadt, Germany). All other chemicals commercially obtained were of the highest purity (analytical or reagent grade) unless otherwise specified.

2.2. PCR amplification of CYP1A4 and CYP1A5

Isolation of CYP1A4 and 1A5 was performed by RT-PCR using *Taq* polymerase (LA *Taq*, Takara) and cDNA as templates. Total RNA was extracted from a 35-day-old Roman hen (*Gallus gallus*) liver (College of Veterinary Medicine, China Agricultural University). RNA extraction and cDNA synthesis were conducted as described by Zhou et al. (2011). Gene-specific primers (CYP1A4F, CYP1A4R, CYP1A5F, CYP1A5R, Table 1) spanning start and stop codons of cDNA (GenBank accession no. NM205147 for CYP1A4 and NM205146 for CYP1A5) were designed to obtain the intact open reading frame of chicken CYP1A4 and CYP1A5 genes. The PCR product was extracted from a gel, cloned into pMD19-T vector and sequenced (Invitrogen, Beijing). At least three clones were sequenced from both directions to confirm the nucleotide sequence of the PCR product.

2.3. Construction of recombinant plasmids for expression

It is reported that N-terminal modifications can improve bacterial expression of CYPs (Yip and Coulombe, 2006; Yun et al., 2006). Thus we used N-terminal truncated sequences for constructing the expression plasmids of both CYP1A4 and CYP1A5 according to the protocol of Yip and Coulombe (2006). The N-terminal truncated CYP1A4 lacked codons 3–33, while truncated CYP1A5 lacked codons 3–31 compared with their wild-type sequences. We also mutated Gly to Ala (for CYP1A5) at the second codon and made synonymous substitutions at the 3–8 codons. In addition, a 6× His tag was also added to the C-terminal to facilitate purification if necessary. The resultant N-terminal truncated and 6× His-tagged sequences were ultimately inserted at *Nde* I and *Sal* I sites of pCWOri+ vector in order to optimize translation of CYP1A mRNA. To construct such recombinant plasmids, identical forward primer (tCYP1AF) for both CYP1A4 and CYP1A5, and two gene-specific reverse primers (tCYP1A4R and tCYP1A5R, Table 1) were designed. The plasmids carrying individual CYP1A4 and CYP1A5 (Section 2.2) were used as the templates respectively. High fidelity Prime Star DNA polymerase (Takara) was used for PCR and the PCR conditions were as follows: 94 °C—2 min; 30 cycles of 98 °C—10 s, 65 °C—30s, and 72 °C—1 min–30 s; and 72 °C—10 min. Further 30 min incubation with addition of 1 μL r-Taq DNA Polymerase (Takara) at 72 °C was performed in order to add overhang-A to PCR product for T-cloning. The PCR products were T-cloned using *pEASY*-T1 Simple vector (Transgen) and then ligated into pCWOri+ vector at *Bam*H I and *Sal* I sites. The expression plasmid for each CYP (pCW-CYP1A4 and pCW-CYP1A5) was transformed into *E. coli* DH5α respectively.

2.4. Bacterial expression of CYP1A4 and CYP1A5

2.4.1. CYP1A5

CYP1A5 was functionally expressed in *E. coli* Rossetta (DE3) (Novagen) essentially as described previously (Pritchard et al., 2006) with minor modifications. A single colony carrying the recombinant pCW-CYP1A5 plasmid was picked from a Luria–Bertani (LB) agar plate (containing 100 μg/mL ampicillin and 34 μg/mL chloromycetin) and grown overnight (200 rpm, 37 °C) in LB broth with antibiotics. Overnight cultures were seeded into modified terrific broth (100 mL) containing 100 μg/mL ampicillin, 34 μg/mL chloromycetin and 1 mM thiamine. Cultures were grown to an OD_{600} of 0.7–1.0 (~6 h), then

Table 1
The primers used in this study.

Primer name	Sequence
CYP1A4F	ATGGCAGCGGGGCCGCAGGCT
CYP1A4R	TCACGCAGAGCCCTTGCTGGG
CYP1A5F	ATGGGGCCGGAGGAAGTGAT
CYP1A5R	TTAGTTTGAGCTCTTCATGGAG
tCYP1AF	GCGGATCCATCGATGCTTAGGAGGT*CATATG*GCTTTATTATTAACTCAAACTCGCCGGCAG CACGCACCCAA
tCYP1A4R	AAGGTCGACTCA**GTGATGGTGATGGTGATG**CGCAGAGCCCTTGCTGGGGAAGCGCTT
tCYP1A5R	CGGTCGACTTA**GTGATGGTGATGGTGATG**GTT TGAGCTCTTCATGGAG

Note: The restriction digestion sequence is underlined. The *Nde* I site is in italic. 6× His-tag sequence is indicated in bold.

ALA (75 mM) and IPTG (1 mM) were added. A further 22–24 h growth at 30 °C and 190 rpm was conducted and the cultures were chilled on ice for 10 min. Cells were harvested by centrifugation at 2800 *g* for 20 min at 4 °C, and stored at −80 °C.

2.4.2. CYP1A4

Due to unstable and low expression of CYP1A4 in *E. coli* by the protocol used for CYP1A5 expression (2.4.1), we adopted the chaperone coexpression strategy (Wu et al., 2009) to express CYP1A4. pCW-CYP1A4 was cotransformed into *E. coli* DH5α with the pGro12 ES/EL plasmid encoding the chaperone protein GroEL/ES (Nishihara et al., 1998). A single colony was inoculated in 5 mL LB media (100 μg/mL ampicillin and 50 μg/mL kanamycin) and grown overnight at 37 °C with rotation at 200 rpm. The overnight culture was then seeded into TB medium and incubated at the same conditions. When the OD_{600} of culture was 0.7–1.0 (~6 h),1 mM IPTG, 1 mg/mL L-arabinose and 75 mM ALA were added and incubated at 30 °C and 190 rpm for 24 h.

2.5. Isolation of membrane and measurement of CYP1A contents

The preparation of spheroplasts and isolation of membrane was performed according to the protocol described by Pritchard et al. (2006). Cells from 100 mL culture were re-suspended with 5 mL ice-cold 2× TSE buffer (100 mM Tris–acetate, pH 7.6 containing 500 mM sucrose and 0.5 mM EDTA), and then diluted with 5 mL ice-cold water. The re-suspended cells were added with lysozyme to a final concentration of 0.25 mg/mL. After shaking the re-suspended cells at 4 °C (140 rpm) for 40 min, the spheroplasts were pelleted (2800 *g*, 4 °C for 20 min) and supernatant discarded. The spheroplast pellet was re-suspended in ice cold spheroplast resuspension buffer, (100 mM potassium phosphate, pH 7.6 containing 6 mM magnesium acetate, 20% (v/v) glycerol, 1 mM PMSF and 0.1 mM DTT). The spheroplast resuspension was subsequently sonicated on ice (pulse 5 s on, 10 s off) at 20% of maximal power for 2 min, 25%—2 min, 30%—2 min, and 35%—1 min (Scientz-IID, China). The sonicated resuspension was centrifuged at 4 °C for 20 min at 12,000 *g*. The membranes were prepared by centrifuging the 12,000 *g* supernatant at 180,000 *g* for 60 min and the pellet was re-suspended with ice-cold 1× TSE buffer.

Protein concentrations were determined using bovine serum albumin as a standard (Bradford, 1976). Concentrations of CYP1A proteins in whole cells or cell fractions were estimated by UV–visible difference spectroscopy for the CO-complex of the dithionite-reduced enzyme versus the reduced enzyme (Omura and Sato, 1964).

2.6. AROD assay

AROD assays including methoxyresorufin *O*-demethylase (MROD), ethoxyresorufin O-deethylase (EROD) and benzyloxyresorufin *O*-debenzylase (BROD), were performed basically according to the method described by Yip and Coulombe (2006), using Hitachi 4500 fluorospectrophotometer (Hatachi, Japan). The excitation wavelength was set at 530 nm, and the emission wavelength at 585 nm. The reaction mixture contained 25 mM $MgCl_2$, 50 nM CYP1A and 50 nM CPR in 50 mM Tris–HCl buffer (pH 7.5) and each substrate (dissolved in DMSO, 0 to 2 μM for methoxyresorufin, 0 to 5 μM for ethoxyresorufin and 0 to1.6 μM for benzyloxyresorufin) in a total volume of 750 μL. For inhibition experiment, each inhibitor dissolved in dimethyl sulfoxide (DMSO) was added 10 min prior to application of the substrate methoxyresorufin (1.5 μM). For control, DMSO alone of the same volume was added. The amount of DMSO in the reaction mixtures was 0.5% in the kinetic studies, and 1.0% in the inhibition experiments. Reaction was initiated by adding NADPH (0.25 mM final concentration) and allowed to proceed for 5 min at 37 °C. The fluorescence of the product (resorufin) was recorded. A standard curve was generated using identically handled resorufin and was used to quantify the amount of resorufin. Velocities were calculated based on fluorescence values within linear range and presented as the formation of resorufin per minute per nanomole of CYP. Three separate determinations, each in duplicate were preformed for kinetic assays. For inhibition assays, two separate determinations, each in duplicate, were conducted.

2.7. Statistical analysis

Kinetic parameters were determined by nonlinear Michaelis–Menten plots using GraphPad Prism 5 (San Diego, CA, USA). Data were presented as mean ± SE. Significance of differences in each kinetic parameter among three AROD activities for each CYP1A enzyme was evaluated by one-way ANOVA following LSD test. Significant differences in each kinetic parameter between CYP1A4 and CYP1A5 were determined by Student's t-test. Because the IC50 parameters were not normally distributed, nonparametric method (Mann–Whitney U test) was used to test the statistical significance in comparisons between CYP1A4 and CYP1A5. A probability of <0.05 was considered significant.

3. Results

3.1. CYP1A sequences

Based on the sequences of CYP1A4 and CYP1A5 genes in GenBank (GenBank no. NM205147 for CYP1A4 and NM205146 for CYP1A5), the cDNA sequences of the open reading frame of chicken CYP1A genes were cloned and sequenced. Compared with corresponding GenBank sequence data, we identified five amino acid substitutions, including two conservative replacements in CYP1A4 (E191G, R304K, P435A, P436S, K511E) (GenBank no. NM205147), and three conservative or semi-conservative substitutions in CYP1A5 (T134A, S173T, T174K) (GenBank no. NM205146) (Figs. S-1 and S-2).

Recombinant chicken CYP1A4 and CYP1A5 differed by 23% amino acids, including several residues predicted to line the active site cavity of human CYP1A2 (Fig. S-3). Compared with human CYP1A2 (pdb: 2HI4) (Sansen et al., 2007), higher degree of residue similarity occurred in chicken CYP1A5 (62%) than in CYP1A4 (55.6%).

3.2. Heterologous expression of chicken CYP1A4 and CYP1A5 proteins in E. coli

The biochemical characterization of individual CYP is heavily hindered by technical difficulties in preparing active, individual CYP enzymes from target organisms, given that CYPs are membrane-bound proteins and multiple CYP isozymes co-occur in eukaryotic organisms. To obtain specific CYP1A enzyme, bacterial expression system was used in this study. To increase the solubility and enhance the expression level of chicken CYP1As, 31 and 29 amino acids were truncated from the N-terminal of CYP1A4 and CYP1A5, respectively. Coexpression of *E. coli* molecular chaperones (GroEL/ES) was also adopted in CYP1A4 expression. Applying these strategies, high yield of CYP1As (1000–1200 nmol for CYP1A5, and ~500 nmol for CYP1A4) per liter culture was produced as determined using whole cells. CYP1A5 was detected in both the membrane and cytosol fractions with a ratio of 2:1 in amount, while CYP1A4 mostly recovered from membrane fraction. The CYP content of CYP1A4 and CYP1A5 in membrane preparations was 0.18–0.34 and 2.4–3.9 nmol/mg protein respectively. The membranes prepared from *E. coli* cells expressing chicken CYP1A4 or CYP1A5 showed a typical CO difference spectrum at ~450 nm (Fig. 1). Greater amount of inactive form (with absorption peak at 420 nm) was observed in CYP1A4 (Fig. 1B) than in CYP1A5 where only a trace amount was observable (Fig. 1A). The membrane fractions containing expressed CYP1As were used for the kinetic analysis and inhibition assays in this study.

3.3. Kinetic analysis of AROD activities of chicken CYP1A4 and CYP1A5

AROD activities are well-known typical catalytic markers for a variety of CYP enzymes, especially CYP1A subfamily (Parente et al., 2011). Therefore, AROD assays were used to compare the enzymatic traits between CYP1A4 and CYP1A5 in this study.

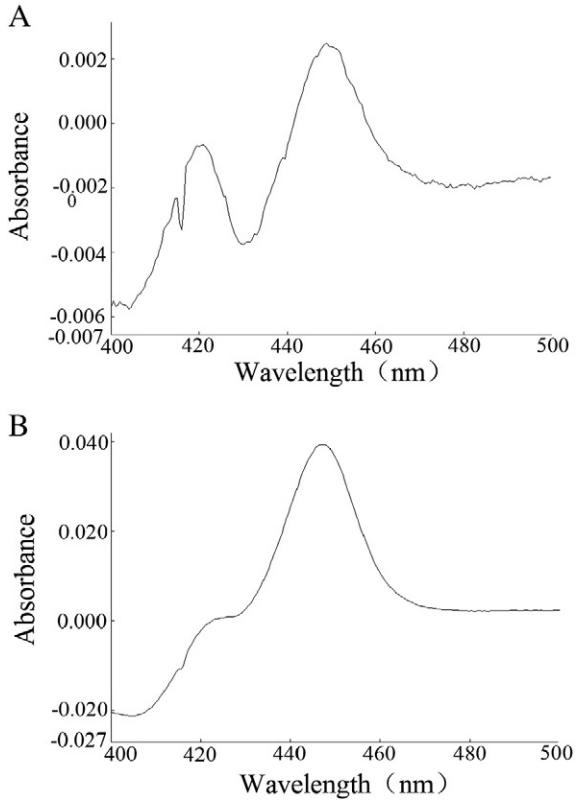

Fig. 1. Reduced CO-difference spectra of *E. coli* membranes containing chicken CYP1A4 (A) and CYP1A5 (B).

Kinetic analysis of AROD activities showed that AROD reactions of CYP1A4 and CYP1A5 followed typical Michaelis–Menten kinetics (Fig. 2, Table 2). For CYP1A4, there were no significant differences in K_m, V_{max} and V_{max}/K_m between MROD and EROD. However, K_m and V_{max} values of BROD for CYP1A4 were lower than those of MROD and EROD, while the V_{max}/K_m ratio was significantly greater (1.6- to 2.1-fold) than those of MROD and EROD. For CYP1A5, K_m value for EROD was greater than MROD (2.4-fold) and BROD (3.7-fold), while no difference was observed between MROD (1.39 ± 0.10 μM) and BROD (0.91 ± 0.30 μM). CYP1A5 V_{max} values were in the order of MROD > EROD > BROD. V_{max}/K_m value of MROD activity was the highest for CYP1A5, but no significant difference was detected between EROD and BROD.

The three parameters were significantly different between the CYP1A paralogs for both EROD and BROD, while comparable values were exhibited in MROD. CYP1A4 showed 3.4- and 6.3-fold greater V_{max} and V_{max}/K_m values for BROD than CYP1A5 respectively, whereas relatively small differences (1.4-fold) in K_m values were observed between CYP1A4 and CYP1A5.

3.4. Effect of CYP inhibitors on MROD activity of chicken CYP1A4 and CYP1A5

Functional divergence between CYP1As is also reflected by inhibitory profiles. In this study, MROD activity was adopted to examine the inhibitory effect of several known CYP inhibitors considering that there are no differences in the kinetic parameters of MROD between chicken CYP1A4 and CYP1A5. Considering the possibility that the chicken CYP1A may behave differently from mammalian CYP1A enzymes, we included two mammalian CYP1A specific inhibitors (α-naphthoflavone and furafylline) and three non-CYP1A specific CYP inhibitors (PBO, erythromycin and ketoconazole) in this study. The effects of five CYP inhibitors on MROD activity of chicken CYP1A4 and CYP1A5 are shown in Fig. 3.

Among the five chemicals, α-naphthoflavone was the most potent inhibitor of both CYP1A4 and CYP1A5. Incorporation of 1 μM α-naphthoflavone in the reaction mixture resulted in >70% inhibition of MROD activity of CYP1A4. The inhibitory effect of α-naphthoflavone to CYP1A5 was stronger than to CYP1A4 ($p < 0.01$), with IC50 values of 0.06 ± 0.01 μM and 0.29 ± 0.04 μM for CYP1A5 and CYP1A4 respectively. Ten μM ketoconazole caused greater than 90% inhibition of MROD for both CYP1A4 and CYP1A5 with an IC50 of around 1 μM (0.9 ± 0.11 μM vs 1.5 ± 0.48 μM, $p < 0.05$). PBO and furafylline displayed no or weak inhibition to MROD activity of CYP1A4, while these two chemicals inhibited MROD activity of CYP1A5 with an IC50 of 1.8 ± 0.4 μM and 375 ± 95 μM respectively. No or only a trace inhibitory effect (<15%) was observed by erythromycin at a concentration of 500 μM.

4. Discussion

Although the importance of CYPs is highly recognized, the characterization of structure and function of individual CYP is heavily hindered by technical difficulties in preparing active, individual enzyme from target organisms. Following the rapid advance in molecular biology, various strategies have been introduced in order to enhance the expression of CYPs (Pritchard et al., 2006; Yip and Coulombe, 2006; Wu et al., 2009). Recombinant expression and enzyme reconstitution have greatly facilitated the biochemical characterization of many CYPs for decades (e.g. Sakaki et al., 1994; Yip and Coulombe, 2006; Kubota et al., 2009; Locuson et al., 2009). Due to ease of use, low cost and high protein production, bacterial expression system is often used as a valuable tool to produce active CYP enzymes for further structural and functional studies. However, functional expression in *E. coli* remains a challenge for CYPs of eukaryotic origins which are all membrane-bound proteins (Wu et al., 2009). There is no completely

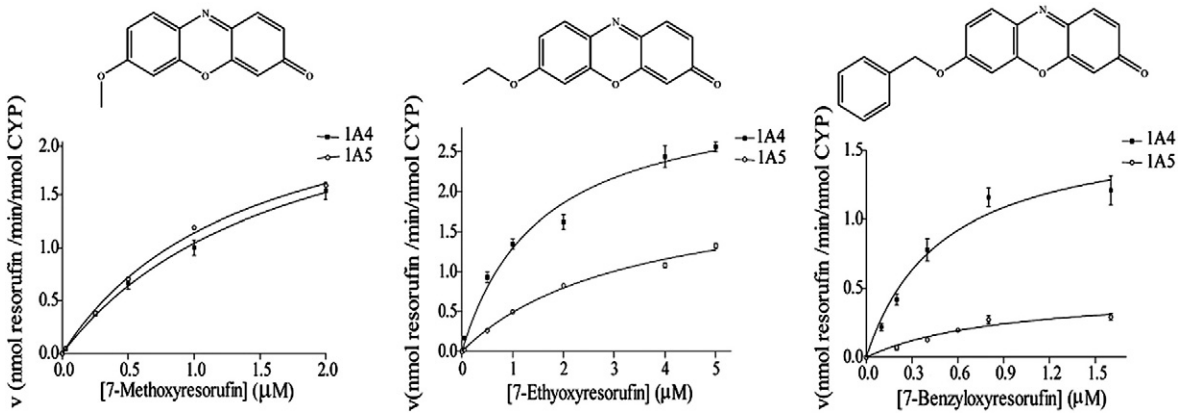

Fig. 2. Kinetic analysis of *E. coli* expressed CYP1A4 and CYP1A5 for MROD, EROD and BROD activities. Results are presented as mean ± SE and are from three independent determinations with each in triplicate.

chicken-specific CYP enzyme system available until now. In this study, N-terminal modification, codon optimization and codon-bias *E. coli* strain Rossetta (DE3) were employed, leading to an efficient expression of active chicken CYP1A5 with a high yield (1000–1200 nmol per liter culture). Functional expression of CYP1A4 was improved when coexpressed with GroEL/ES chaperone, with a production of ~500 nmol per liter culture. Similarly, N-terminal deletion of 29 amino acids resulted in a relatively high level of expression of turkey CYP1A5 in *E. coli*, with 200–300 nmol active truncated CYP1A5 protein per liter culture (Yip and Coulombe, 2006). To our experience, the success in expressing eukaryotic CYP enzymes is largely dependent on appropriate N-terminal modifications and the adoption of "ATG" of the *Nde* I site in the polylinker of the expression vector pCWOri + as the initiation codon (Pritchard et al., 2006). Using such strategies, we are able to produce active chicken CYP3A37 and several insect CYPs in sufficient quantities (our unpublished data).

Limited studies focusing on enzymatic activity of CYP1As suggested that the AROD profiles differed even within avian species (Verbrugge et al., 2001; Watanabe et al., 2005; Kubota et al., 2006, 2009). Our kinetic results showed that both chicken CYP1A4 and CYP1A5 exhibit catalytic activities towards the three substituted resorufins with distinct substrate preference. The enzymatic parameters for EROD and BROD differed between CYP1A4 and CYP1A5. However, no significant difference was observed for MROD. This observation is different from that of Kubota et al. (2009) who reported that MROD activity differed in yeast expressed CYP1A4 and CYP1A5 from common cormorant (*Phalacrocorax carbo*). Our data showed that chicken CYP1A4 had lower K_m values and greater V_{max}/K_m ratios for BROD and EROD than CYP1A5. The highest V_{max}/K_m ratio was seen in BROD activity for CYP1A4. Conversely, greatest V_{max}/K_m was observed in MROD for CYP1A5. These results added support to the notion that CYP1A5 preferred shorter alkoxyresorufins as its mammalian ortholog CYP1A2 (Burke et al., 1985; Lubet et al., 1990; Nakajima et al., 1991; Nerurkar et al., 1993). With regard to EROD, chicken CYP1A4 has a greater V_{max}/K_m (3.4-fold) value, which is consistent with the prior study showing CYP1A4 has a greater capacity than CYP1A5 in common cormorant (Kubota et al., 2009). Similar profile of catalytic specificity for EROD was previously documented using purified and reconstituted chicken CYP1A4 and CYP1A5, being reflected by a greater turnover number of EROD for CYP1A4 than CYP1A5 (Rifkind et al., 1994). In addition, substrate selectivity between chicken CYP1A4 and CYP1A5 is most notable for BROD among the three AROD activities; CYP1A4 exhibits highly specific preference for BROD, with greater V_{max} value (3.4-fold) and V_{max}/K_m ratio (6.3-fold) than that of CYP1A5 respectively. Our results are in keeping with those of studies on birds (Head and Kennedy, 2007; Kubota et al., 2009) and fish (Parente et al., 2011) suggesting that BROD presents a more specific biomarker for CYP1A4 than the most commonly used EROD activity.

Table 2
Kinetic parameters of AROD activities in *E. coli* membranes containing chicken recombinant CYP1A enzymes.

Activities	Parameters	CYP1A4	CYP1A5
MROD	K_m	1.71 ± 0.32a	1.39 ± 0.10a
	V_{max}	2.83 ± 0.30a	2.72 ± 0.10a
	V_{max}/K_m	1.66 ± 0.30a	1.98 ± 0.05a
EROD	K_m	1.54 ± 0.24a, *	3.35 ± 0.41b
	V_{max}	3.29 ± 0.19a, *	2.12 ± 0.13b
	V_{max}/K_m	2.20 ± 0.40a, *	0.64 ± 0.06b
BROD	K_m	0.50 ± 0.11b, *	0.91 ± 0.30a
	V_{max}	1.69 ± 0.16b, *	0.49 ± 0.08c
	V_{max}/K_m	3.42 ± 0.51b, *	0.54 ± 0.06b

Values present mean ± SE from three independent experiments with each determination made in triplicate. The unit of K_m is μM, and V_{max} is nmol resorufin/nmol CYP/min. Significant differences in each kinetic parameter among three AROD activities for each CYP were determined by one-way ANOVA followed by LSD test and marked by different letters ($p < 0.05$). Significant differences in each kinetic parameter between CYP1A4 and CYP1A5 were determined by Student's t-test ($p < 0.05$), and marked by an asterisk.

Differential patterns of inhibition were observed between chicken CYP1As and among chemicals (Table 2, Fig. 3). Data in Fig. 3C showed that α-naphthoflavone, a mammalian CYP1A1 specific inhibitor, strongly inhibited MROD activity of both CYP1A4 and CYP1A5. Stronger inhibition by α-naphthoflavone was observed for CYP1A5 than CYP1A4. Furafylline has been reported to be an inhibitor of CYP1A2 but not of CYP1A1 in humans, rats (Sesardic et al., 1990) and mice (Tsyrlov et al., 1993). Varying inhibitory effect of furafylline on AROD activities was documented in three avian species including chickens (Verbrugge et al., 2001). We also observed this selectivity in chicken CYP1A4 and CYP1A5 (Fig. 3D). Notably, furafylline showed only a weak inhibitory effect on CYP1A5-dependent MROD activity. The furafylline sensitivity of chicken CYP1A5 is far less than that of human CYP1A2 (Sesardic et al., 1990), but similar to that of rodents, supporting that the potency of furafylline as a CYP1A2 inhibitor differs dramatically among species (Sesardic et al., 1990; Verbrugge et al., 2001).

Both ketoconazole and erythromycin are human CYP3A inhibitors. Whether these chemicals inhibit chicken CYP1A is poorly understood. In this study, we found that MROD activity of both chicken CYP1A4 and CYP1A5 was not obviously reduced by the addition of erythromycin at concentrations ranging from 10 μM to 500 μM (Fig. 3B). This result is in keeping with that of turkey CYP1A5 (Yip and Coulombe, 2006). However, inhibition of the MROD activity by erythromycin was seen for heterologously expressed zebrafish CYP1A (Smith et al., 2012). In contrast to erythromycin, ketoconazole strongly inhibited MROD activity of chicken CYP1As, abolishing MROD activity at 10 μM

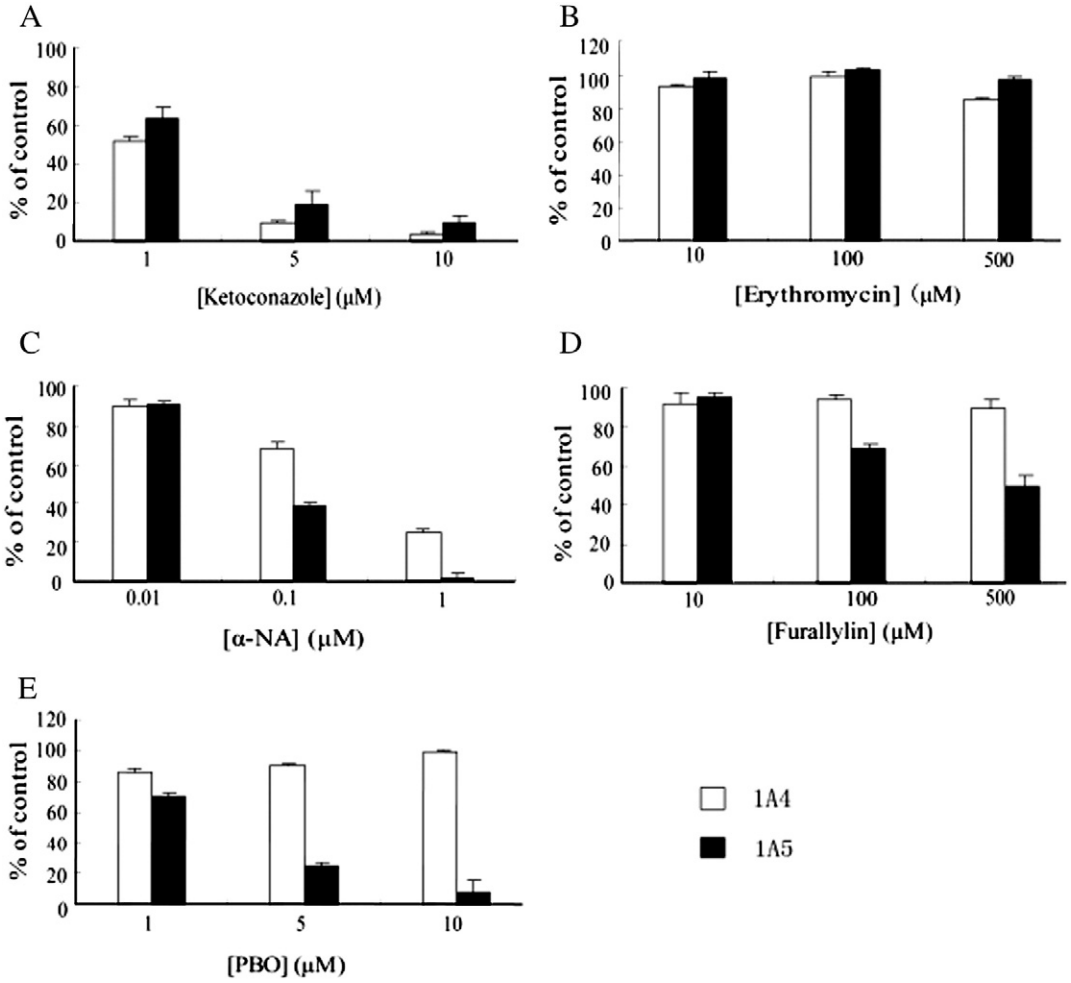

Fig. 3. Percent inhibition (mean ± SE) of MROD activities of chicken CYP1A4 and CYP1A5 by five CYP inhibitors. α-NA = α-naphthoflavone; PBO = piperonyl butoxide.

(Fig. 3A). In addition, ketoconazole inhibition appeared nonspecific for either CYP1A4- or CYP1A5-dependent MROD. Consistent with this observation, Sinclair et al. (1997) reported that 50 μM ketoconazole decreased chicken CYP1A4- and CYP1A5-catalyzed uroporphyrinogen oxidation activity to similar extent (89% vs 94%).

Piperonyl butoxide (PBO), a synthetic analog of the natural methylenedioxyphenyl (MDP) compounds and commonly used as pesticide synergist, is an effective inhibitor of many CYPs (Murray, 2000). It was assumed previously that MDP compounds were nonspecific inhibitors. However, increasing evidences have demonstrated that MDP compounds may exhibit selectivity in their interactions with different CYPs (cf. Murray, 2000). Under the conditions of this study, selective inhibitory effect of PBO on CYP1A-catalyzed MROD activity was observed at concentrations ranging from 1 to 10 μM; 10 μM PBO maintained the MROD activity of CYP1A4, but almost abolished that of CYP1A5 (Fig. 3E).

Overall, chicken CYP1A5 exhibits stronger activity to relatively small substrates (e.g. methoxyresorufin, Fig. 2) than CYP1A4, and small chemicals (α-naphthoflavone, PBO, furafylline, showing in Fig. S-5) show stronger inhibition of MROD activity of CYP1A5 than that of CYP1A4 (Fig. 3). The observed differences in the substrate specificity and inhibitor selectivity are determined by the variations in their amino acid sequences, which may be partially explained by the structural alignment of homology models exhibiting a more compact active site topology of CYP1A5 than that of CYP1A4 (Fig. S-4). Given that the structure–function relationship of CYPs is very complicated (Liu et al., 2004; Lewis et al., 2007; Tu et al., 2008; Parente et al., 2011), we could not conclude what residues define functional differences between chicken CYP1A4 and CYP1A5 without a mutagenesis study. Key amino acids determining the inter-paralog functional differences between chicken CYP1As are worthy of further investigation.

In conclusion, we have demonstrated the differences in kinetics and response to inhibitor between chicken CYP1As using *E. coli* produced enzymes. Although the activity measured using recombinant CYP enzymes may not represent absolute enzyme activity in vivo, the obvious differences in AROD kinetics and inhibition profiles between chicken CYP1A4 and CYP1A5 suggest that the two members of the CYP1A subfamily have distinct enzymatic properties, reflecting differences in the active site geometry between CYP1A4 and CYP1A5. Further studies are required to elucidate the structural bases underlying catalytic differences between CYP1A paralogs/orthologs, which will allow a mechanistic explanation of enzymatic differences and provide a more comprehensive insight into the functional evolution of CYP1A genes. The recombinant expression system for producing CYP1A presented here shows promising applications for further investigation of the physiological or toxicological roles of chicken CYPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2013.02.005.

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