Carbonyl Reduction of Mequindox by Chicken and Porcine Cytosol and Cloned CBR1

Xianqing Tang, Peiqiang Mu, Jun Wu, Jun Jiang, Caihui Zhang, Ming Zheng, Yiqun Deng

College of Life Sciences, South China Agricultural University, Guangzhou 510642, China
Running title: Carbonyl Reduction of Mequindox

Corresponding author: Yiqun Deng; Tel: 86-20-38604967; Fax: 86-20-38604967;
E-mail address: yqdeng@scau.edu.cn

16 pages of text
4 tables
8 figures
1 supplemental table
32 references
249 word in the Abstract
597 words in the Introduction
1075 words in the Discussion

Abbreviations:
MEQ, mequindox; CBR, carbonyl reductase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyl tetrazolium bromide; MEN, menadione; QUE, quecertin; NADPH, nicotinamide adenine dinucleotide phosphate; IPTG, isopropyl-β-D-(-)thiogalacto-pyranoside; HPLC, High-performance liquid chromatography.
Abstract

Mequindox (MEQ) is a novel synthetic quinoxaline 1,4-dioxides derivative, which is widely used as veterinary drug and animal feed additive. However, the metabolic mechanism of MEQ is rarely reported. The N-oxide reduction mechanism of MEQ was reported in our previous work. In this paper, the toxicity and the reduction of the carbonyl of MEQ were studied. The MTT assays demonstrated that the carbonyl reduced MEQ, 2-isoethanol mequindox was much less toxic than MEQ. HPLC analysis showed that the cytosol extracts of chicken and pig livers were able to reduce MEQ to 2-isoethanol mequindox and the reaction was NADPH-dependent. Further study via enzyme inhibitory experiment revealed that CBR1 participated in this metabolism. The enzyme activity analysis showed that both chicken CBR1 (cCBR1) and porcine CBR1 (pCBR1) were capable of catalyzing the carbonyl reduction of MEQ and its N-oxide reductive metabolite, 1-deoxymequindox. By comparison of the kinetic constants, we observed that the activity of cCBR1 was higher than pCBR1 to MEQ and the standard substrate of CBR1, menadione (MEN). On the other hand, both two CBR1s exhibited higher activity to 1-deoxymequindox than MEQ. Mutation analysis suggested that the difference of amino acid at position 141/142 may be one possible reason which caused the activity difference between cCBR1 and pCBR1. So far, the CBR1 was first reported to participate in the carbonyl reduction of MEQ. Our results will be helpful to recognize the metabolic pathways of quinoxaline drugs deeply and provide theoretical basis for controlling the negative effects of these drugs.
Introduction

Mequindox (MEQ), namely 3-methyl-2-acetyl-1,4-dioxyquinoxaline (C₁₁H₁₀N₂O₃), is a novel synthetic quinoxaline derivative, developed by Lanzhou Institute of Animal Husbandry and Veterinary Drugs, Chinese Academy of Agricultural Sciences (Lanzhou, China). As a new member of the quinoxaline-1,4-dioxides, the MEQ has a strong inhibitory effect against several gram-positive and negative bacteria, such as *E. coli*, *Salmonella* sp. and *Pasteurella* sp. through inhibiting bacterial DNA synthesis (Ihsan et al., 2010). Owing to the antimicrobial and growth-promoting effects (Vicente et al., 2009), MEQ has been widely used in animal breeding and as a veterinary medication to treat livestock diseases including swine dysentery and piglet white diarrhea since its discovery in the 1980s.

MEQ shares structural and pharmacologic properties similar to other well-known quinoxaline family members including carbadox and olaquindox (Figure 2). The carbadox and olaquindox are mutagenic, developmental and reproductive toxic and carcinogenic, therefore, which have been prohibited to be used as feed additives by the European Union and Health Canada since 1999 and 2001, respectively (Beutin et al., 1981; Yoshimura, 2002; Chen et al., 2009). In comparison to these two drugs, MEQ exhibits less toxicity and greater growth-promoting effect. Consequently, MEQ is used as a substitute for carbadox and olaquindox in poultry and livestock husbandry in China for several years. Researches on the metabolism of other members of quinoxalines have revealed that MEQ maybe mainly metabolized through three pathways including carbonyl reduction, N-oxide reduction (Liu. et al., 2008; Zheng et al., 2011) and hydroxylation of side chains (Liu. et al., 2011). However, there is little information concerning the carbonyl reduction of MEQ at present. The enzymes involved in the carbonyl reduction of MEQ are still unknown, especially in chickens and pigs.

The carbonyl group (aldehyde or ketone group) exists frequently in endogenous compounds such as...
hormones (e.g., steroids), and lipid aldehydes derived from oxidative stress (e.g., 4-hydroxy-2-nonenal) (Oppermann, 2007). Furthermore, it is widely found in xenobiotics such as food ingredients, drugs, and environmental pollutants (e.g. warfarin, aflatoxin B1, the nitrosamine NNK) (Oppermann and Maser, 2000; Oppermann, 2007). Reactive carbonyl compounds can promote oxidative stress and are able to covalently modify DNA or amino acids. The products of which are thought to be an important initiating factor in degenerative diseases or cancer (Matsunaga et al., 2006). The carbonyl moiety often is a determining factor for the biological activity of a molecule. Consequently, metabolic conversion of a carbonyl group can be an essential metabolic route in the phase biotransformation of a great variety of xenobiotic carbonyl compounds (Maser, 1995). Organisms have evolved several enzyme systems for detoxifying reactive carbonyl compounds. Such well established pathways include the oxidation of aldehydes to the corresponding carboxylic acids by aldehyde dehydrogenases and aldehyde oxidases, and the reduction of aldehydes and ketones into the corresponding alcohols by NADPH-dependent reductases with broad substrate specificity (Matsunaga et al., 2006). These reductases with broad substrate specificity for xenobiotic carbonyl compounds belong to two fundamentally distinct enzyme families, namely the short-chain dehydrogenase/reductase (SDR) and the aldo-keto reductase (AKR) (Matsunaga et al., 2006). One subfamily of SDR, the CBRs (EC1.1.1.184), has been proved to have broad substrate specificity for many endogenous and xenobiotic carbonyl compounds (endogenous prostaglandins, steroids, and other aliphatic aldehydes and ketones) (Oppermann and Maser, 2000; Doorn et al., 2004; Gonzalez-Covarrubias et al., 2007; Oppermann, 2007; Bains et al., 2009), and most of which are NADPH-dependent and localized in cytosol (Forrest and Gonzalez, 2000).

In this study, we tested whether the liver cytosol extracts of two important livestocks, pigs and chickens, were able to reduce the carbonyl of MEQ, and identified the possible main enzymes which were
responsible for the reduction.

Materials and Methods

Chemicals

Mequindox (C_{11}H_{10}N_{2}O_{3}, mol.wt 218.21, purity 99.8%), N1-deoxymequindox, 2-isoethanol mequindox and 2-isoethanol N1-deoxymequindox were synthesized at the institute of veterinary pharmaceuticals (Wuhan, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyl tetrazolium bromide (MTT) and menadione was purchased from Sigma-Aldrich Co. (S. Louis, MO, USA). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Roche Chemical Co. (Guangzhou, China). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Guangzhou, China). Water was purified using a Milli-Q system (Millipore, MA, USA). Isopropyl-β-D-(-)thiogalacto-pyranoside (IPTG) was obtained from Gen-View Scientific Inc.. All primers were synthesized in Invitrogen Co. (Guangzhou, China). All other chemicals and reagents were of the highest analytical grade available.

Animals

Danish Landrace×Yorkshire×Duroc cross-breeds pigs (4-5 months old) were purchased from the College of Veterinary Medicine at South China Agricultural University (SCAU). Chickens (2 months old) were purchased from the Institute of Animal Science, Guangdong Academy of Agricultural Sciences. Both pigs and chickens were fed with commercial standard diet and allowed access to water ad libitum to ensure the absence of therapeutic or illicit treatments before slaughtering.
Methods

HepG2 cell culture and the MTT assay

HepG2 cells were obtained from ATCC (USA) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin/streptomycin (100U/ml) (all purchased from Invitrogen, USA). Cell plates were maintained in a humidified incubator at 37°C with 5% CO₂.

The toxicity of MEQ and 2-isooethanol mequindox to HepG2 Cells were measured by cell viability using the MTT assay. Cells were seeded into 96-well plates (6×10³ cells per well) and cultured for approximate 36h. And then the cells were treated with different concentrations of mequindox and 2-isooethanol mequindox (0–200 μg/ml) for 48 h. Subsequently, 0.5 mg/ml MTT was added to each well, and the plates were incubated at 37°C for an additional 4 h. The medium containing MTT was then removed, and 150 μl DMSO (Sigma, USA) was added to each well to dissolve the formazan crystals. Absorbance at 490 nm was measured using a microplate reader (Bio-Rad, USA). All experiments were performed at least 3 times.

Preparation of pig and chicken liver cytosol extracts

Cytosol extracts were prepared following the procedures described previously (Liu. et al., 2008). The obtained cytosol suspension was stored at -80°C until used. Cytosol protein concentrations were estimated by the Bradford method using bovine serum albumin as the protein concentration reference standard.

Cloning of cCBR1 and pCBR1

Total RNA of pig and chicken livers were isolated using TRIzol reagent (Invitrogen, USA), and were transcribed to cDNA through Reverse-transcription Polymerase Chain Reaction. The cDNAs were stored at -80°C until used. Primers for construction and cloning of pig and chicken CBR1s are displayed in
supplementary table 1. The open reading fragments cCBR1 (Gene ID: 418512) or pCBR1 (Gene ID: 397143) was ligated into invitrogen’s pET-28a vector using the *Nco*I/*Xho*I restriction sites for cCBR1 and the *Nco*I/*Hind*III restriction sites for pCBR1. The recombinant constructs were transformed into calcium chloride competent *Escherichia coli* DH5α cells using the heat shock method. Transformed cells were screened with colony PCR and purified plasmid DNA was sequenced ( invitrogen) for verification.

**Expression and purification of CBR1s**

The cCBR1 and pCBR1 constructs were transfected into *E.coli* BL21 (DE3)-competent cells. Cells were plated on Luria-Bertani broth agar supplemented with kanamycin (30 mg/L) for selection. Colonies were randomly picked and cultured at 37°C in 2 ml of Luria-Bertani broth with kanamycin (30 mg/L) firstly, and then the culture was expanded to 100 mL. Lastly, Cultures were expanded to 1 liter at a volume rate of 1:100 and grown at 37°C until OD$_{600}$ of 0.5-0.7 was reached. IPTG was added at a final concentration of 0.8 mM, and cells were continued to grow for another 4 hours. The *E. coli* cells were harvested by centrifugation (6000 rpm for 10 min at 4°C, Beckman). The precipitate was suspended and lysed in buffer containing 50 mM potassium phosphate, 10 mM imidazole, 300 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. Lysates were supplemented with lysozyme (1 mg/ml) and incubated for 1 hour at 4°C. Subsequently, lysates were sonicated (200 W, 10 sec, 30) and centrifuged for 30 min at 4°C (25,000 g). The 6×His-tagged proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. The cCBR1 and pCBR1 proteins were eluted in buffer containing 50 mM potassium phosphate, pH 7.4, 300 mM NaCl, 100 mM imidazole. Proteins were extensively dialyzed for 12 h in 50 mM potassium phosphate buffer containing 10% glycerine at 4°C, with two buffer changes. After dialysis, proteins were aliquoted and stored at -80°C until used. We used western blotting to detect the
recombinant proteins. Briefly, membrane was first incubated with a polyclonal His-tag monoclonal antibody (1:1000 dilution; Novagen), followed by incubation with a secondary rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (1:3000 dilution; Invitrogen). Biotinylated protein ladder (Cell Signaling Technologies) was used for detecting the molecular mass of the proteins.

**Assay measuring carbonyl reduction of liver cytosol extracts**

The incubation mixture contained 50 mM potassium phosphate buffer (pH 7.4), 200 μM NADPH, cytosol proteins, and one of the following substrates: 50 μM MEQ, 50 μM 1-deoxymequindox. The total volume of the reaction system was 1 ml. The boiled cytosol extracts were used as a negative control of the proteins, and the NADPH was added or not to determine the reaction is NADPH-dependent or not. The enzymes activity inhibitor of CBR1, 10 μM quercetin (QUE) (Forrest and Gonzalez, 2000), was added or not to test whether the carbonyl reductive activity of the liver cytosol extracts was attributed to CBR1. All mixtures were incubated at 37°C for 30 min in water bath. The reaction was stopped by adding 2 ml ethyl acetate, which was also used to extract target chemicals from the incubation mixture. The collected liquid in the organic phase was evaporated dryness under high-purity nitrogen. The residues were dissolved in 100 μl methanol and then subjected to High-performance liquid chromatography (HPLC) for analysis.

**Enzyme kinetic analysis of cCBR1 and pCBR1**

The enzyme activities of the purified CBR1s were measured by following the initial rate of NADPH oxidation at 340 nm at 37 °C using WFZ UV-2802PCS Spectrophotometer (UNICO, ShangHai, China). The assay mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 200 μM NADPH, MEN at various concentrations (10 to 320 μM), and enzyme in total volume of 1.0 ml. The enzyme concentration
was optimized for each enzyme and substrate concentration to ensure that measured rates were in the linear range of the enzyme kinetic curve. The reaction was initiated by the addition of NADPH to the assay mixture. Blanks without enzyme were routinely included. The substrates were dissolved in absolute ethanol. The final concentration of the solvent in the assay mixture did not exceed 4% (v/v). The readings were collected at 1 second intervals for 90 seconds. Enzymatic activity (nanomoles of NADPH consumed per minute per milligram of purified protein) was calculated from these rates using a standard curve constructed from the measurements of solutions of known NADPH concentrations.

The reduction level of the MEQ and 1-deoxymequindox by CBR1 were measured by incubating with purified enzyme in a total volume of 1 ml containing 50 mM potassium phosphate (pH 7.4) and 200 μM NADPH at 37°C for 30 minutes. The reaction was stopped by adding 2 ml ethyl acetate. And the following steps were described as the section of “assay measuring carbonyl reduction of liver cytosol extracts”.

The kinetic constants of maximal rate of reaction ($V_{max}$) and substrate affinity ($K_m$) were determined by fitting rate measurement data using nonlinear least-squares fitting of a Michaelis-Menten hyperbola (version 5.0, GraphPad Prism; GraphPad Software Inc., San Diego, CA). The turnover values ($k_{cat}$) were calculated from $V_{max}$ values using the apparent molecular weight for the CBR1s (31 kDa for cCBR1 and 33 kDa for pCBR1). Catalytic efficiency ($k_{cat}/K_m$) was also calculated.

**High-performance liquid chromatography**

High-performance liquid chromatography (HPLC) was performed using a Waters 2695 System (Waters Alliance, Milford, MA, USA) equipped with a Waters 2487 Dual λ Absorbance Detector. To separate MEQ and its reduced metabolites, the samples (10 μl) were injected into a Hypersil BDS C18 column (4.6×250 mm I.D.; particle size 5 μm; Elite, Dalian, China). The mobile phase consisted of solvent A (0.1% formic
acid in ultrapure water) and solvent B (acetonitrile). The gradient elution program was as follows: 0-5 min, 15% B; 5-15 min, 15% B to 70% B; 15-18 min, 70% B to 100% B; 18-23 min, 100% B; 23-23.1 min, 100% B to 15% B; 23.1-30 min, 15% B. The chromatograph was operated at a flow rate of 1 ml/min at room temperature, with UV detection at 305 nm. Quantitative analysis of reductive products was carried out using the peak area.

**Mutagenesis, expression, purification and activity analysis of variant enzymes.**

The mutations including cCBR1 (V141M), cCBR1 (V141E), pCBR1 (E142M) and pCBR1 (E142V) were introduced using the site-directed mutagenesis method. Primers were displayed in supplementary table 1. The methods of expression and purification of variant enzymes were the same as wild-type CBR1s. Also, the activities of the purified variant enzymes were measured by following the initial rate of NADPH oxidation at 340 nm at 37°C. The assay mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 200 μM NADPH, 100 μM MEN and enzyme in total volume of 1.0 ml.

**Results**

**The toxicity of the carbonyl group of mequindox**

The carbonyl of xenobiotic carbonyl compounds was considered to be the toxic group (Oppermann and Maser, 2000; Matsunaga et al., 2006). To determine the toxic effect of the carbonyl of MEQ, the toxicity of MEQ and the carbonyl reduced MEQ, 2-isoethanol mequindox, to the HepG2 cells was tested by MTT assay. In consistent with previous reports on MEQ toxicity (Liu et al., 2012), the cell survival rates of HepG2 cells were inversely correlated with increasing concentrations of mequindox after 48h treatment. The IC$_{50}$ of MEQ was 58.71 μg/ml (Figure 1). As expected, 2-isoethanol mequindox was much less toxic...
to HepG2 cells (Figure 1). The cell survival rates of 2-isoethanol MEQ were higher than MEQ at all used concentrations, the IC_{50} of which was 260.44 μg/ml. Thus, the results above indicated that the carbonyl of MEQ was one of the toxic group of MEQ and the reduction of which is important for its detoxification.

**Carbonyl reductive activity of chicken and pig liver cytosol extracts to the MEQ.**

To test whether MEQ may be metabolized through carbonyl reduction in pig and chicken livers, the MEQ was incubated with chicken and pig liver cytosol, and the metabolites were detected by HPLC. As shown in Table 1, the metabolites of MEQ, 2-isoethanol mequindox and 2-isoethanol 1-deoxymequindox (Figure 2) were detected after incubation with chicken and pig cytosol extracts. When cytosol was incubated with MEQ only, the liver cytosol extracts showed limited activities towards MEQ (2.93±0.44 pmol/min·mg for chicken and 4.06±0.68 pmol/min·mg for pig). While the cofactor NADPH was added to the incubation system, they showed much higher activities towards MEQ (36.58±5.16 pmol/min·mg for chicken and 22.54±8.55 pmol/min·mg for pig), which indicated that enzymes involved in this process are NADPH-dependent.

The carbonyl reductase 1 (CBR1) of many species, including chicken and pig, were previously proved to have carbonyl reductive activity toward many endogenous and xenobiotic carbonyl compounds. To determine whether CBR1 may be involved in this the carbonyl reduction of MEQ, we used QUE, the potent inhibitor of CBR1, to inhibit the activities of CBR1 (Forrest and Gonzalez, 2000). As expected, the reductive products of MEQ decreased significantly when QUE was added (3.92±2.19 pmol/min·mg for chicken and 4.50±1.73 pmol/min·mg for pig). These results suggested that CBR1s of chicken and pig might participate in the carbonyl reduction of MEQ.

**Cloning, expression and purification of recombinant CBR1s in E.coli**
To confirm the role of CBR1s in the carbonyl reduction of MEQ, we cloned the CBR1s from chicken and pig livers by RT-PCR and expressed them in *E. coli* BL21 (DE3). Expressions of cCBR1 and pCBR1 were efficiently induced by 0.8 mM IPTG at 37°C for 4 h (Figure 3). The recombinant proteins were purified by using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and a high purity recombinant cCBR1 and pCBR1 were obtained (Figure 3). Furthermore, the purified CBR1s were confirmed by Western blot analysis, showing the molecular mass of the His-tagged cCBR1 and pCBR1 were approximately 31 kDa and 33 kDa (Figure 4), which were close to the calculated molecular mass (31.59 kDa for cCBR1 and 33.15 kDa for pCBR1).

**Kinetic characterization of the enzymatic activities of cCBR1 and pCBR1 with MEN**

To determine whether the purified CBR1s were active and, if so, to determine the kinetic properties of the enzymes, we used MEN as a standard substrate to conduct Michaelis-Menten kinetic studies, because of its specific for CBR1 enzyme (Gonzalez-Covarrubias et al., 2007). In the presence of MEN, the $V_{\text{max}}$ of cCBR1 and pCBR1 were 8074.00±346.00 nmol and 279.30±5.14 nmol NADPH consumed/min·mg respectively, and the $K_{m}$ of which were 76.46±8.63 μM and 19.92±1.45 μM respectively (Figure 5, Table 2). These results demonstrated that the purified cCBR1 and pCBR1 were enzymatically active and the cCBR1 had a higher catalytic efficiency than the pCBR1 to the standard substrate, MEN.

**Kinetic characterization of the enzymatic activities of cCBR1 and pCBR1 with MEQ and 1-deoxymequindox**

To confirm whether CBR1s can catalyze the carbonyl reductive reaction of MEQ and its N-oxide reductive metabolite, 1-deoxymequindox, the formation of the major alcohol metabolites after the
incubation of MEQ and 1-deoxymequindox with purified CBR1s was measured *in vitro*. The MEQ, 1-deoxymequindox and their reductive products 2-isoethanol mequindox and 2-isoethanol 1-deoxymequindox were used as standards, and were observed to be eluted approximately at 9.6, 14.3, 6.3 and 9.8 min, respectively (Figure 6). After the incubation of the cCBR1 and pCBR1 with MEQ, a single new chromatographic peak generated, which was identified as 2-isoethanol mequindox (Figure 6A, 6B). Likewise, incubation with 1-deoxymequindox also generated a single new chromatographic peak that was identified as 2-isoethanol 1-deoxymequindox (Figure 6C, 6D). As a control, there were no detectable peaks at the 2-isoethanol mequindox and 2-isoethanol 1-deoxymequindox retention times in the absence of the protein.

To further characterize the CBR1s, Michaelis-Menten kinetic properties were studied, using both MEQ and 1-deoxymequindox as substrates, separately. With MEQ as substrate, the $V_{\text{max}}$ and $K_m$ of cCBR1 were 1.21±0.03 nmol/min·mg and 156.00±12.47 μM, respectively (Figure 7A, Table 3). Otherwise, the pCBR1 showed significantly low $V_{\text{max}}$ (0.49±0.03 nmol/min·mg) and high $K_m$ (469.80±54.76 μM) compared with cCBR1 (Figure 7A, Table 3). In addition, we observed that cCBR1 and pCBR1 exhibited a high activity to 1-deoxymequindox than MEQ as shown by the significant low $K_m$ and high $V_{\text{max}}$. With 1-deoxymequindox as a substrate, the $V_{\text{max}}$ and $K_m$ of cCBR1 were 5.27±0.32 nmol/min·mg and 105.00±22.43 μM, respectively (Figure 7B, Table 3). Interestingly, the pCBR1 showed close $V_{\text{max}}$ (5.26±0.25 nmol/min·mg) and $K_m$ (116.10±19.27 μM) to cCBR1 (Figure 7B, Table 3). Eadie-Hofstee plots for cCBR1 and pCBR1 verified linearity at the same concentration of MEQ and 1-deoxymequindox used to conduct the assays ($r^2 >0.9$ for all plots). Taken together, through enzymatic kinetic characterization, both cCBR1 and pCBR1 showed the carbonyl reduction activity to MEQ and 1-deoxymequindox, and the activity of cCBR1 is higher than pCBR1 to MEQ, but cCBR1 and pCBR1 showed similar activity towards 1-deoxymequindox and higher
activity when MEQ was used as the substrate.

**Activity analysis of variant enzymes.**

To analyze the activity difference between cCBR1 and pCBR1, the amino acids sequence were aligned with hCBR1 and hCBR3. The alignment showed that most of the amino acids sequence in the substrate binding sites and catalytic sites are similar within cCBR1, pCBR1, hCBR1 and hCBR3 (Figure 8). However, it was worth noting that the amino acid at position 141/142 which was proved to affect the activity of CBR significantly, was different between cCBR1 and pCBR1 (Figure 8). To test if the amino acids at position 141/142 affect the activity of cCBR1 and pCBR1 significantly, the activity of variant enzymes at position 141 of cCBR1 and 142 of pCBR1 to MEN were analyzed. As shown in table 4, the velocity of cCBR1 (V141M) (1097.33±15.14 nmol NADPH consumed /min·mg protein) was similar to the wild-type (1018.67±26.63 nmol/min·mg protein), but the cCBR1 (V141E) (825.33±10.07 nmol/min·mg protein) was much lower. Meanwhile, the velocity of pCBR1 (E142M) (15.00±0.47 nmol/min·mg protein) and pCBR1 (E142V) (11.00±0.94 nmol/min·mg protein) were both higher than the wild-type of pCBR1 (8.50±0.24 nmol/min·mg protein).

**Discussion**

The main metabolites, detected after exposed to MEQ in vivo, indicated that MEQ might be mainly metabolized through three pathways including N-oxide reduction, carbonyl reduction, and the methyl hydroxylation (Liu et al., 2010). Our previous work proved that aldehyde oxidase was involved in N-oxide reduction (Zheng et al., 2011) and CYP3A family of CYP450 monoxidases were participated in the hydroxylation at the methyl of the side chains (Liu et al., 2011). The present study demonstrated that MEQ
could be metabolized by carbonyl reduction, and the chicken and pig liver cytosolic CBRs, cCBR1 and pCBR1 were participated in the reaction. Comparative investigation of the metabolism of MEQ and 1-deoxymequindox by cCBR1 and pCBR1 showed that the carbonyl reduction ability of cCBR1 was higher than pCBR1, and the CBR1s preferred to catalyze the carbonyl reduction of the N-oxide reduced MEQ, 1-deoxymequindox.

High-dose of MEQ was observed to lead to cytotoxicity and organ toxicity in liver and spleen (Wang et al., 2011), kidney (Huang et al., 2010), testis (Ihsan et al., 2011) and adrenal gland (Huang et al., 2009) in animals. The N-oxide reductive metabolism of MEQ can lead to the increasing of the intracellular reactive oxygen species (ROS), which cause damage to lipids, sugars, amino acid chains and DNA (Liu et al., 2003; Ellis, 2007). Meanwhile, chemically reactive carbonyl group can interact with the nucleophilic centers of proteins and nucleic acids (Oppermann and Maser, 2000; Matsunaga et al., 2006), that can cause cytotoxicity, genotoxicity, apoptosis and the perturbations in signalling pathways (Forrest and Gonzalez, 2000). Similarly, the carbonyl reduced MEQ, 2-isoethanol mequindox was also showed to be less toxic than MEQ itself in this study (Figure 1). Therefore, the carbonyl reduction is rather important for the detoxification of MEQ. Furthermore, the chicken and pig liver cytosol extracts were found to be capable of catalyzing the carbonyl reduction of MEQ (Table 1). MEQ shares similar structural property with other members in the quinoxaline family (Figure 2), but it is still unknown which enzymes are involved in the carbonyl reduction of them. However, whether other tissues of chicken and pig have the ability of carbonyl reduction to MEQ remains to be further investigated.

To combat the negative effects brought about by carbonyl compounds, organisms have involved several biotransformation enzyme systems, one of them being the CBRs (Oppermann and Maser, 2000; Matsunaga et al., 2006; Oppermann, 2007). CBR1 has been reported to metabolize many endogenous and xenobiotic
carbonyl compounds to their corresponding alcohol. In the study, CBR1 was also found to be capable of metabolizing MEQ and its N-oxide reductive product, 1-deoxymequindox (Figure 7). In many cases, the formation of a hydroxyl group makes the substrate more hydrophilic and provides products that can be conjugated, e.g. via glucuronidation of sulfation, thus facilitation excretion (Maser, 1995; Oppermann and Maser, 2000). Therefore, CBR1 plays a critical role in detoxification of reactive carbonyl compounds. The pCBR1, also known as $3\alpha/\beta,20\beta$-hydroxysteroid dehydrogenase ($3\alpha/\beta,20\beta$-HSD), is ubiquitously distributed and the tertiary structure has been reported (Ghosh et al., 2001; Sugiyama et al., 2004). Meanwhile, cCBR1, namely $20\beta$-hydroxysteroid dehydrogenase ($20\beta$-HSD), shares about 73% sequence homology with pCBR1 and also widely distributed with no tertiary structure data reported (Bryndova et al., 2006). Both of the two CBR1s were reported to metabolize many endogenous and xenobiotic carbonyl compounds (Nakajin et al., 1995; Nakajin et al., 1997; Bryndova et al., 2006). To the best of our knowledge, CBR1 was the first enzyme which was reported to participate in the carbonyl reduction of MEQ. However, we can’t exclude other enzymes which may be also responsible for the reduction.

Comparative study of cCBR1 and pCBR1 showed that the $K_{cat}/K_m$ of cCBR1 to the standard substrate, MEN, was 7.27 fold higher than pCBR1 (Table 2). Similarly, the cCBR1 showed 6.97 fold higher in $K_{cat}/K_m$ than pCBR1 to MEQ (Table 3). The difference of catalytic activity towards MEN was similar to the result reported by other groups (El-Hawari et al., 2009; Pilka et al., 2009). They analyzed the substrate-binding site of human CBR1 and CBR3 by site-directed mutagenesis using isatin and 9,10-phenanthrenequinone as model substrates, and demonstrated the importance of methionine 142 and tryptophan 230 in binding with the substrates. Their results suggested that the substrate was stabilized within the catalytic cleft by hydrophobic interactions with methionine 142 and by non-specific aromatic interaction with tryptophan 230. As shown in figure 8, the alignment showed that both cCBR1 and pCBR1
had a tryptophan at the position 229 and 230, respectively. The cCBR1 has a valine 141 with a nonpolar side chain that is similar to methionine. However, pCBR1 has a glutamic acid 142 with a charged polar side chain that is totally different from methionine. Mutation analysis showed that the methionine at position 141/142 is also important for the activity of cCBR1/pCBR1 (Table 4). Furthermore, MEQ and MEN share almost the same structural characteristics (Figure 2). Consequently, the situations of binding with the catalytic cleft of CBR1 are similar to each other. These results suggested that the amino acid difference at position 141/142 may be one of the reasons which caused the activity difference between cCBR1 and pCBR1. However, the other amino acids which affect the activity significantly remains to be identified.

In this study, both cCBR1 and pCBR1 showed higher catalytic activity toward 1-deoxymequindox than MEQ, especially for pCBR1. It showed a 4.4 fold higher in $V_{\text{max}}$ and 1.5 fold lower in $K_m$ in chicken while a 10.7 fold higher in $V_{\text{max}}$ and 4.3 fold lower in $K_m$ were observed in pig (Table 3). Both MEQ and 1-deoxymequindox share similar structural characteristic property with MEN (Figure 2). The distance between quinone and the catalytically important residue tyrosine 193 and cofactor NADPH determines the catalytic activity to substrate (Sciotti et al., 2006). For this reason, without the interference of N1-oxide, it may make carbonyl more close to tyrosine 193 and NADPH when 1-deoxymequindox is bound with the catalytic cleft of CBR1 than MEQ. In hence, 1-deoxymequindox may be easily bound and catalyzed by CBR1, which indicated that carbonyl reduction might easily occur after N1 deoxidation of MEQ.

In conclusion, this study revealed that the carbonyl group of MEQ is toxic and identified the enzymes which participated in the carbonyl reduction of MEQ in chicken and pig liver cytosols, CBR1s. Moreover, CBR1 was able to catalyze the carbonyl reduction of its N-oxide reductive metabolite, 1-deoxymequindox, with a significant higher catalytic activity compared to MEQ, which suggested that the carbonyl of
1-deoxymequindox may be more easily reduced than MEQ.
Authorship Contributions

Participated in research design: Xianqing Tang, Peiqiang Mu, Jun Wu, Jun Jiang, Yiqun Deng

Conducted experiments: Xianqing Tang, Jun Jiang

Performed data analysis: Xianqing Tang, Peiqiang Mu, Jun Wu, Caihui Zhang, Yiqun Deng

Wrote or contributed to the writing of the manuscript: Xianqing Tang, Peiqiang Mu, Ming Zheng, Yiqun Deng
References

Bains OS, Karkling MJ, Grigliatti TA, Reid RE, and Riggs KW (2009) Two nonsynonymous single
nucleotide polymorphisms of human carbonyl reductase 1 demonstrate reduced in vitro
metabolism of daunorubicin and doxorubicin. Drug Metab Dispos 37:1107-1114.


Chen Q, Tang S, Jin X, Zou J, Chen K, Zhang T, and Xiao X (2009) Investigation of the genotoxicity of


the substrate-binding site of human carbonyl reductases CBR1 and CBR3 by site-directed


reductase. structural basis for a functional monomer in short chain dehydrogenases/reductases. J

polymorphism on human carbonyl reductase 1 (CBR1 V88I) impacts on catalytic activity and NADPH binding affinity. *Drug Metab Dispos* **35:**973-980.


Footnote

*This work was supported by the National Basic Research Program of China (973 Program) grant [2009CB118802], the National Natural Science Foundation of China grant [31172087], the Program for New Century Excellent Talents in University grant [NCET-08-0643], and the Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (2009).
Figure Legends

Figure 1. The effect of mequindox and 2-isoethanol mequindox on cell viability in HepG2 cells. Cells were treated with various (0-200 μg/ml) of mequindox and 2-isoethanol mequindox for 48h. Cell viability was assessed using the MTT assay, and the cells viability of MEQ or 2-isoethanol mequindox treated cells were shown as a percentage of the control cells. Each point represents the mean±S.D. of three duplicated experiments.

Figure 2. The molecular structures of mequindox and its metabolites, two other quinoxaline drugs and menadione.

Figure 3. Over expression and purification of recombinant chicken (A) and pig (B) CBR1 in E.coli BL21 (DE3). The purity of the enzyme is shown in 10% SDS-PAGE: lane 1: the molecular weight ladder; lane 2: cell lysate from uninduced BL21 (DE3) cells; lane 3: cell lysate from 0.8 mM IPTG (4 h) induced BL21 (DE3) cells; lane 4: supernatant after ultracentrifugation; lane 5: cell debris after ultracentrifugation; lane 6: purified recombinant CBR1, eluted by the buffer containing 50 mM potassium phosphate, 300 mM NaCl and 100 mM imidazole.

Figure 4. Immunodetection of purified chicken CBR1 and pig CBR1 with an anti-histidine tag antibody. lane 1: the molecular weight ladder; lane 2, the purified cCBR1; lane 3: the purified pCBR1.

Figure 5. Enzymatic activities of the purified cCBR1 (A) and pCBR1 (B) with menadione as measured by following the initial rate of NADPH oxidation. Assays were performed in triplicate with each batch.
Enzymatic activities were reported as mean±S.D. (n=3). Differences between curves were significant at p < 0.05.

Figure 6. HPLC chromatograms of the carbonyl reductive metabolites of mequindox (A and B) and 1-deoxymequindox (C and D) formed by the CBR1s in the presence of NADPH. Mequindox and 2-isoethanol 1-deoxymequindox were eluted at 9.6 min and 6.3 min; 1-deoxymequindox and 2-isoethanol mequindox were eluted at 14.3 min and 9.8 min.

Figure 7. *In vitro* enzymatic activities of the purified cCBR1 and pCBR1 with (A) 20 to 800 μM mequindox and (B) 20 to 800 μM 1-deoxymequindox. Activities were measured by following the rate of 2-isoethanol mequindox and 2-isoethanol 1-deoxymequindox production. Each point represents the mean±S.D. of three duplicated experiments. Differences between curves were significant at p < 0.05.

Figure 8. The amino acid sequences alignment of CBRs near residues 142 and 230. hCBR1: human CBR1 (GI:49456377); hCBR3: human CBR3 (GI: 49456379); cCBR1: chicken CBR1 (GI:71895267); pCBR1: pig CBR1 (GI:47522960).
Table 1 Carbonyl reduction of mequindox in chicken and porcine cytosol.

<table>
<thead>
<tr>
<th>Addition</th>
<th>2-isoethanol mequindox</th>
<th>2-isoethanol 1-deoxymequindox</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>chicken</td>
<td>pig</td>
</tr>
<tr>
<td>None&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.93±0.44</td>
<td>4.06±0.68</td>
</tr>
<tr>
<td>NADPH</td>
<td>36.58±5.16</td>
<td>22.54±8.55</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.92±2.19</td>
<td>4.50±1.73</td>
</tr>
<tr>
<td>Boiled&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Each value (pmol min<sup>-1</sup>mg protein<sup>-1</sup>) represents the mean±S.D.of three duplicate experiments.

N.D., not detected.

<sup>a</sup> Mequindox was only incubated with cytosol extracts only.

<sup>b</sup> Cytosol extracts were heated in boiling water bath for at least 5 min.
Table 2 The kinetic constants of recombinant cCBR1 and pCBR1 for menadione

<table>
<thead>
<tr>
<th>Kinetic Parameter</th>
<th>cCBR1</th>
<th>pCBR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (nmol/min·mg protein)</td>
<td>8074.00±346.00</td>
<td>279.30±5.14</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>76.46±8.63</td>
<td>19.92±1.45</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (min⁻¹)</td>
<td>250.20</td>
<td>9.00</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_m$ (min⁻¹·μM⁻¹)</td>
<td>3.27</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Values represent the mean±S.D. of three duplicate experiments.

*a $k_{\text{cat}}$ calculated from $M_r$ 31000 (cCBR1) and $M_r$ 33000 (pCBR1).
Table 3 The kinetic constants of recombinant cCBR1 and pCBR1 for the substrates of mequindox and 1-deoxymequindox.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic Parameter</th>
<th>cCBR1</th>
<th>pCBR1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$(nmol/min·mg protein)</td>
<td>1.21±0.03</td>
<td>0.49±0.03</td>
</tr>
<tr>
<td></td>
<td>$K_m$(µM)</td>
<td>156.00±12.47</td>
<td>469.80±54.76</td>
</tr>
<tr>
<td>Mequindox</td>
<td>$k_{\text{cat}}$(min$^{-1}$)$^a$</td>
<td>0.0375</td>
<td>0.0162</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{cat}}/K_m$(min$^{-1}$µM$^{-1}$)</td>
<td>2.40×10$^{-4}$</td>
<td>3.45×10$^{-5}$</td>
</tr>
<tr>
<td>1-deoxymequindox</td>
<td>$V_{\text{max}}$(nmol/min·mg protein)</td>
<td>5.27±0.32</td>
<td>5.26±0.25</td>
</tr>
<tr>
<td></td>
<td>$K_m$(µM)</td>
<td>105.00±22.43</td>
<td>116.10±19.27</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{cat}}$(min$^{-1}$)$^a$</td>
<td>0.1634</td>
<td>0.1736</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{cat}}/K_m$(min$^{-1}$µM$^{-1}$)</td>
<td>1.56×10$^{-3}$</td>
<td>1.50×10$^{-3}$</td>
</tr>
</tbody>
</table>

Values represent the mean±S.D. of three duplicate experiments.

$^a$ $k_{\text{cat}}$ calculated from $M_r$ 31000 (cCBR1) and $M_r$ 33000 (pCBR1).
Table 4 The velocity of NADPH oxidation of recombinant CBR1s and their mutants for the substrates of 100 μM MEN.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>NADPH oxidation (nmol/min·mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cCBR1</td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>1018.67±26.63</td>
</tr>
<tr>
<td>V141M</td>
<td>1097.33±15.14</td>
</tr>
<tr>
<td>V141E</td>
<td>825.33±10.07**</td>
</tr>
<tr>
<td>pCBR1</td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>8.50±0.24</td>
</tr>
<tr>
<td>E142M</td>
<td>15.00±0.47*</td>
</tr>
<tr>
<td>E142V</td>
<td>11.00±0.94</td>
</tr>
</tbody>
</table>

Values represent the mean±S.D. of three duplicate experiments.

*p < 0.05, **p < 0.01
Figure 2

Mequindox

2-isoethanol mequindox

1-deoxymequindox

2-isoethanol 1-deoxymequindox

Menadione

Olaquindox

Carbadox
Figure 8

<table>
<thead>
<tr>
<th>Protein</th>
<th>Start</th>
<th>Sequence 1</th>
<th>Alignment</th>
<th>Sequence 2</th>
<th>Alignment</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCBR1</td>
<td>140</td>
<td>SIMSVRALKS CSEP</td>
<td>·····DKILLNACCPG WVRTDMA GPKA</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hCBR3</td>
<td>140</td>
<td>SLQCLRAFE NCSED L</td>
<td>·····DRILVNACCPG PVKTDM GDKDS</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cCBR1</td>
<td>139</td>
<td>SMVSISALGGCSQEL</td>
<td>·····DHILLNACCPG WVRTDMAGPKA</td>
<td>239</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCBR1</td>
<td>140</td>
<td>STEGVRALNEC S PEL</td>
<td>·····DKILLNACCPG WVRTDMGGPKA</td>
<td>240</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>