N-oxide reduction of quinoxaline-1, 4-dioxides catalyzed by porcine aldehyde oxidase SsAOX1

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ABBREVIATIONS: QdNOs, quinoxaline-1,4-dioxides; AO, aldehyde oxidase; XO, xanthine oxidoreductase; CD, circular dichroism.
ABSTRACT

Quinoxaline-1,4-dioxides (QdNOs) are a class of quinoxaline derivatives that are widely used in humans or animals as drugs or feed additives. However, the metabolic mechanism, especially the involved enzymes, has not been reported in detail. In this study, the N-oxide reduction enzyme, porcine aldehyde oxidase SsAOX1 was identified and characterized. The SsAOX1 gene was cloned from pig liver through RT-PCR using degenerate primers, which encodes a 147-kDa protein with typical aldehyde oxidase motifs, two [2Fe-2S] center, an FAD-binding domain and a molybdenum cofactor domain. After heterologous expression in a prokaryote, purified SsAOX1 formed a functional homodimer under native conditions. Importantly, the SsAOX1 catalyzed the N-oxide reduction at the N1 position of three representative QdNOs (quinocetone, mequindox and cyadox), which are commonly used as animal feed additives. SsAOX1 has the highest activity towards quinocetone, followed by mequindox and cyadox, with \( k_{cat}/K_m \) values of 1.94±0.04, 1.27±0.15 and 0.43±0.09 min\(^{-1}\) \( \mu \)M\(^{-1}\), respectively. However, SsAOX1 has the lowest substrate affinity for quinocetone, followed by the cyadox and mequindox, with \( K_m \) values of 4.36±0.56, 3.16±0.48 and 2.96 ±0.51 \( \mu \)M, respectively. In addition, using site-directed mutagenesis, we found that substitution of glycine 1019 with threonine endows SsAOX1 with the N-oxide reductive activity at N4 position. The goal of this study was to identify and characterize the N-oxide reduction enzyme for a class of veterinary drugs, QdNOs, which will aid in the elucidation of the metabolic pathways.
of QdNOs and will provide a theoretical basis for their administration and new veterinary drug design.
Introduction

The quinoxaline-1, 4-di-N-oxides (QdNOs) are a class of quinoxaline derivatives that possess two N-O bonds at positions N1 and N4, respectively. QdNOs have a wide range of biological properties, such as antibacterial, antiviral, antifungal, antiprotozoal and anticancer activities (Carta et al., 2005), and have been widely used as human drugs or animal feed additives since the 1940s (Vicente et al., 2011).

The N-oxide groups are considered to be the main functional moiety of QdNOs. The presence of N-oxide groups in the molecular structures of the QdNOs leads to their antibacterial, antifungal or antitumor effects in humans and animals (Carta et al., 2005). The presence of N-oxide groups in the quinoxaline ring has been shown to be necessary for the antitubercular activity of 3-chloro-2-quinoxalinecarbonitrile (Ortega et al., 2001). The N-oxide reduced products of quindoxin, have been shown to lose nearly all of the antibacterial activity (Suter et al., 1978). However, the N-oxide groups have also been shown to result in the toxicity of QdNOs. The mutagenic potency of QdNOs depends on the number of N-O bonds in their structure, because the reduced derivative of quindoxin, quinoxaline, was not mutagenic, and the partially reduced derivative, quinoxaline 1-oxide, exhibited lower mutagenic activity than quindoxin (Beutin et al., 1981). The N-oxide reduced product of mequindox, quinoxaline-2-carboxylic acid, did not cause cell death, whereas mequindox produced significant ROS and caused cell death to porcine adrenocortical cells (Huang et al., 2010).
Because the vital roles of N-oxide groups in their activity and toxicity, the elucidation of the metabolic mechanism of the N-oxide groups in QdNOs is helpful for their administration and new veterinary drug design. Numerous reports have showed that the N-oxide groups can be reduced back to their parent amines (Bickel, 1969; Kitamura et al., 1999; Liu et al., 2010a; Liu et al., 2010b; Liu and Sun, 2013; Takekawa et al., 2001). Cyadox was has been shown to be metabolized primarily into N-oxide reduced products in rat, chicken and swine (Beedham, 1987; Garattini et al., 2008; Takekawa et al., 1997; Zheng et al., 2011). The N-oxide reduction was also one of the main metabolic pathways of quinocetone in swine (Wu et al., 2012), and the N1 reduced quinocetone, 1-deoxyquinocetone, was significantly more abundant than other metabolites in swine urine (Shen et al., 2010). A similar phenomenon was also observed with mequindox. The 1-deoxymequindox, which is the N1 reduced mequindox, has been reported to be significantly higher than N4 reduced mequindox and other metabolites in chicken (Shan et al., 2012). Although it was widely reported that N-oxide reduction is the main metabolic pathway of QdNOs, the responsible enzyme, especially the specific isoforms of the enzyme, has been rarely reported. The liver has been found to play the dominant role in this reductive transformation. Several enzymes in the liver have been shown to be capable of catalyzing the N-oxide reduction. Brucine N-oxide can be reduced back to its parent tertiary amine by aldehyde oxidase (AO) (Kitamura et al., 1999; Takekawa et al., 2001; Takekawa et al., 1997). Using enzyme inhibition approach, we demonstrated previously that the
N-oxide reduction of cyadox might be catalyzed by AO and xanthine oxidoreductase (XO) (Zheng et al., 2011). Thus, it is interesting whether AO has a function in the N-oxide reduction of QdNOs.

AOs (EC 1.2.3.1) are a small group of structurally conserved proteins belonging to the large family of molybdo-flavoenzymes, which play important physiological and pharmacological roles in various plants and animals (Beedham, 1987; Garattini et al., 2008; Hille, 1996). AO is composed of two homogenous monomers with each subunit having a molecular mass of approximately 150 kDa (Hille, 2005). Each subunit contains two non-identical 2Fe–2S redox centers in the N-terminus, an FAD-binding domain at the center, and a substrate-binding pocket in close proximity to the molybdenum cofactor in the C-terminus (Garattini and Terao, 2013; Pryde et al., 2010). AO is capable of oxidizing numerous aldehydes, such as benzaldehyde and 2-hydroxyprimidine, and a variety of N-heterocyclic compounds in the presence of an electron donor, such as sulfoxides and azo dyes (Garattini et al., 2008; Hille, 2002).

Many cDNAs coding for AO from various species have been cloned. Pigs, which are an important food source of humans, are exposed to significant amounts of drugs and toxins, including QdNOs as a feed additive. However, the AO for QdNOs has not been identified and reported.

In this study, a gene encoding AO from pig liver has been cloned and tested for its ability to catalyze the N-oxide reduction of three widely used QdNOs (quinocetone,
mequindox and cyadox) (Fig. 1), and the catalytic mechanism was analyzed by site-directed mutagenesis.
Materials and Methods

Chemicals and Reagents

Quinocetone (C\textsubscript{18}H\textsubscript{14}N\textsubscript{2}O\textsubscript{3}, 99.8%), 1-deoxyquinocetone (C\textsubscript{18}H\textsubscript{14}N\textsubscript{2}O\textsubscript{2}, 99.8%), 4-deoxyquinocetone (C\textsubscript{18}H\textsubscript{14}N\textsubscript{2}O\textsubscript{2}, 99.8%), 1,4-dideoxyquinocetone (C\textsubscript{18}H\textsubscript{14}N\textsubscript{2}O, 99.8%), Mequindox (C\textsubscript{11}H\textsubscript{10}N\textsubscript{2}O\textsubscript{3}, 99.8%), 1-deoxymequindox (C\textsubscript{11}H\textsubscript{10}N\textsubscript{2}O\textsubscript{2}, 99.8%), 4-deoxymequindox (C\textsubscript{11}H\textsubscript{10}N\textsubscript{2}O\textsubscript{2}, 99.8%), 1,4-dideoxymequindox (C\textsubscript{11}H\textsubscript{10}N\textsubscript{2}O, 99.8%), Cyadox (C\textsubscript{12}H\textsubscript{9}N\textsubscript{5}O\textsubscript{3}, 99.8%), 1-deoxycyadox (C\textsubscript{12}H\textsubscript{9}N\textsubscript{5}O\textsubscript{2}, 99.8%), 4-deoxycyadox (C\textsubscript{12}H\textsubscript{9}N\textsubscript{5}O\textsubscript{2}, 99.8%), and 1,4-dideoxycyadox (C\textsubscript{12}H\textsubscript{9}N\textsubscript{5}, 99.8%) were synthesized at the Institute of Veterinary Pharmaceuticals (Wuhan, China), and the HPLC graphs of which were showed in supplemental Fig. 1, 2 and 3. Benzaldehyde was purchased from the Sigma-Aldrich Company (St. Louis, MO, USA). HPLC-grade methanol was purchased from Thermo Fisher Scientific (Fairlawn, NJ, USA). Ultra-purified water was obtained from a Milli-Q ultra-purification system (Millipore, Bedford, MA, USA). The restriction enzymes were purchased from Takara (Dalian, China). All of the other chemicals and reagents commercially available were of the highest analytical grade.

Animals

The animal studies were approved by the Institute of Animal Care and Use Committee of South China Agricultural University, and adhered to the Chinese
Guidelines for the Proper Conduct of Animal Experiments for the use of laboratory animals. Five-month-old female Danish Landrace x Yorkshire x Duroc cross-breed pigs were purchased from the College of Veterinary Medicine at the South China Agricultural University. The pigs were fed commercial standard diets and had access to water *ad libitum* before being sacrificed. Immediately after the pigs were sacrificed by bleeding from the carotid artery, the liver was isolated and frozen in liquid nitrogen.

**cDNA Cloning of Aldehyde Oxidase from Pig Liver**

Total RNA from pig liver was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. To obtain total cDNA for gene amplification, random primers were used to perform RT-PCR using first-strand cDNA, which was synthesized using the SMART MMLV Reverse Transcriptase (Clontech). Based on the available sequenced genome of *Sus scrofa*, four AO genes originating from *Mus musculus* were used as probes to screen the corresponding homologues. Considering the specificity and degeneracy, the alignment of the four AOX1s originating from human, cow, mouse and rat samples was performed. Three pairs of degenerate primers were designed by Genefisher2 (http://bibiserv.techfak.uni-bielefeld.de/genefisher2). The primer sequences were listed in Supplemental Table 1. PCR was conducted in a 50 μL solution containing KOD FX buffer, 200 mM dNTP solution, 2 U of KOD FX DNA polymerase (Toyobo,
Japan), 2 mM MgCl₂, 1 mM of each primer, and 1 μL of total cDNA as a template.

PCR amplifications were conducted as follows: initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 2 min, and a final extension at 68 °C for 10 min. The PCR products were cloned into the pMD20-T vector (TaKaRa, Qingdao, China) and verified by sequencing. All three sequences of the AO fragment contained an overlapping region for assembling the full-length sequence of the porcine AO. The deduced full-length sequence was used to derive the unique primers for the porcine AO. The forward and reverse primers were 5’-ATGGACGGGGCGGCGGAGCTG-3’ and 5’-TCATATGGGTACACTCCAAGG-3’, respectively. The reaction was conducted in a 50 mL solution containing KOD-plus buffer, 200 mM dNTP solution, 2 U of KOD-plus polymerase (Toyobo, Japan), 2 mM MgCl₂, 1 mM of each primer, and 1 μL of the template. PCR amplifications were performed as follows: initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 4 min, and a final extension at 68 °C for 5 min. The PCR product was cloned into the pMD20-T vector and sequenced.

Bioinformatics and Statistical Analysis

The BLAST algorithm (http://blast.ncbi.nlm.nih.gov/) was used to retrieve nucleotide sequences highly similar to the porcine AOX 1 gene (GenBank accession no. JX977168). The exon-intron junctions were analyzed with an internet splice
program (http://www.fruitfly.org/seq_tools/splice.html). The amino acid sequence
analysis was performed using the EMBOSS Transeq program for publication
(http://www.ebi.ac.uk/Tools/st/emboss_transeq). Motif prediction was performed by
NCBI Conserved domain search
(http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Sequence alignments were
performed using the ClustalW algorithms (http://www.ebi.ac.uk/Tools/msa/clustalw2).
Statistical analyses were performed using the GraphPad Prism program, version 5.0
for Windows XP.

**Construction of the SsAOX1 Expression Vector**

The obtained cDNA was used as a template for the synthesis of PCR fragments
with an expected size of 4017 bp. The primers used for amplification were
5’-CAAGCTTCTATGGACGGGGCGGAG-3’ (forward), with an introduced
**Hind**III restriction site, and 5’-ATAAGAATGCGGCCGC-3’ (reverse), with an introduced **Not**I restriction site.
The reaction was conducted in a 50 mL solution containing KOD-plus buffer, 200
mM dNTP solution, 2 U of KOD-plus polymerase (Toyobo, Japan), 2 mM MgCl₂, 1
mM of each primer, and 1 μL of the template. PCR amplifications were conducted as
follows: initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for
30 s, 68 °C for 4 min, and a final extension at 68 °C for 5 min. The amplified DNA
(20 mg) was cleaved with **Hind**III and **Not**I (New England BioLabs, Beverly, MA,
U.S.A.). The resulting DNA fragment was cloned into the pQE-30 Xa vector (20 mg, QIAGEN, Venlo, The Netherlands) using the TaKaRa DNA Ligation Kit Ver. 2 (TaKaRa) according to the manufacturer’s protocol. Subsequently, 5 μL of the diluted ligation reaction was transformed into 50 μL of DH5α competent cells. The transformants were plated on LB agar plates containing 100 μg/mL ampicillin and grown overnight at 37 °C. Plasmid DNA from several colonies was isolated using a TIANGEN Spin Miniprep Kit (TIANGEN), and screened by restriction digestion with HindIII and NotI. A positive colony was replated and grown overnight at 37 °C. A single colony was then selected and grown overnight in LB broth containing 100 μg/mL ampicillin, and the plasmid DNA was isolated and verified by sequencing.

**Prokaryotic Expression and Purification of Recombinant SsAOX1**

The pQE-30 plasmid containing the SsAOX1 cDNA was transformed into M15 competent cells and grown on LB agarose plates containing 100 μg/mL ampicillin. A single colony was selected and grown overnight in 100 mL of supplemented LB broth (100 μg/mL ampicillin, 1 μg/mL riboflavin, and 50 μM sodium molybdate). The overnight culture (10 mL) was used to inoculate 500 mL of supplemented Terrific broth (100 μg/mL ampicillin, 250 μL of trace element solution containing 2.7 g of FeCl₃·6H₂O, 0.2 g of ZnCl₂·4H₂O, 0.2 g of CoCl₂·6H₂O, 0.2 g Na₂MoO₄·2H₂O, 0.2 g of CaCl₂·H₂O, 0.1 g of CuCl₂, 0.05 g of H₃BO₃, and 10 mL of concentrated HCl autoclaved in a total volume of 100 mL of double-distilled H₂O, 1 μg/mL riboflavin,
and an additional 50 μM sodium molybdate). Cultures were grown at 37 °C and 250 rpm until an absorbance of 0.4 at 600 nm was reached for 60 to 90 min; the addition of 1 mM IPTG was performed for induction, and the cells were allowed to continue growing at room temperature for 72 h at 150 rpm. The cells were chilled on ice and harvested by centrifugation at 3500 × g at 4 °C for 30 min. The cell pellet was resuspended in 100 mM Tris acetate buffer (pH 7.6) containing 500 mM sucrose and 0.5 mM EDTA, and diluted with an equal volume of ice-cold water. Lysozyme was then added to the resuspended cells to a final concentration of 0.25 mg/mL. The suspension was placed on a shaker at 4 °C for 45 min. The spheroplasts were pelleted at 2,800 × g for 20 min at 4 °C and resuspended in 100 mM potassium phosphate buffer (pH 7.6) containing 6 mM magnesium acetate, 20% glycerol (v/v), and 0.1 mM DTT. The suspensions were sonicated on ice and centrifuged at 75,000 × g for 20 min at 4 °C. The 6×-His-tagged proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. The AO proteins were eluted in buffer containing 50 mM potassium phosphate (pH 7.4), 300 mM NaCl, and 500 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, the proteins were aliquoted and stored at -80 °C until use. The protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard.
Western Blot Analysis

Protein samples from the induced bacteria were separated on 7.5% SDS-PAGE gels and then electrophoretically transferred to a PVDF membrane (PALL, Ann Arbor, MI, USA). The membrane was blocked with freshly prepared TBST buffer (25 mM of Tris-HCl (pH 7.5), 150 mM of NaCl and 0.1% Tween-20) containing 5% nonfat dry milk for 1 h at room temperature and incubated for 1 h with a rabbit polyclonal antibody against AOX1 (1:1000 dilution; sc-98500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST buffer containing 1% milk. After being three times 10-min washes with TBST, the membranes were incubated with an HRP-rabbit anti-mouse IgG (Gamma) (Invitrogen, Carlsbad, CA, USA) at 1:4000 for 1 h at room temperature and then washed for another 30 min with TBST buffer. Band detection was performed using the LumiGLO® Chemiluminescent Substrate Kit (CST, Beverly, MA, USA), according to the manufacturer’s instructions. Chemiluminescence was quantified using the Quantity Tools of Image Lab™ software (Bio-Rad, Hercules, CA, USA).

Circular Dichroism Spectroscopy

Wide type AO was tested at 5 μM in 100 mM potassium phosphate buffer (pH 7.4). The circular dichroism (CD) spectra of these samples were obtained on a Chirascan apparatus (Applied Photophysics Limited, Leatherhead, Surrey, UK) at 25 ºC using a bandwidth of 1 nm, a cell path length of 1 mm, a step of 1.0 nm, a time-per-point of 0.5 s and a time interval of 1.0 min. The value for the buffer alone was measured and
subtracted from the protein spectra. All of the spectra were collected using a protein concentration of 0.05 mg/ml. The data were converted and normalized to the molar ellipticity ([θ]), in degrees.cm².dmolt⁻¹.

Native PAGE

The purified SsAOX1 in native PAGE sample buffer (50 mM Tris-HCl (pH 6.8), 10% glycerol and 0.01% bromophenol blue) and boiled SsAOX1 in denatured buffer (50 mM Tris-HCl (pH 6.8), 1% SDS, 1% β-Mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) were separated by 7.5% native PAGE under cool conditions and then immunoblotted with antibodies against aldehyde oxidase.

Enzyme Activity Assays Using Quinoxaline-1,4-dioxides

The enzymatic activities were measured using three quinoxaline-1,4-dioxides, quinocetone, mequindox, and cyadox and their derivatives, 1-deoxyquinocetone, 1-deoxy mequindox, and 1-deoxy cyadox. SsAOX1 (50 μg/ml) in a buffer containing 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA was incubated with 200 mM benzaldehyde in a final volume of 1 mL at 37°C under anaerobic conditions (99.999% N²) for 5 min. Then, 2-60 μM QdNOs was added into the mixtures and maintained under anaerobic conditions (99.999% N²) at 37°C for 60 min. The reactions were stopped by boiling for 10 min followed by adding 3 ml ethyl acetate, vortexing and centrifuging at 500 × g for 10 min. The supernatants were then dried under N², redissolved in 200 μL methanol and subjected to HPLC for analysis. HPLC was
performed using a Waters 2695 HPLC System equipped with a Waters 2487 Dual λ absorbance detector. To separate QdNOs and its reduced metabolites, 20 μL of the samples were injected into an Agilent TC18 (2) column (250 mm ×4.6 mm, 5μm). The mobile phase consisted of solvent A (0.1% formic acid) and solvent B (methanol). The gradient elution program was set as follows: 0-5 min, 15% solvent B; 5-25 min, 15% to 60% solvent B; 25-25.1 min, 60% to100% solvent B; 25.1-30 min, 15% solvent B. The wavelength was set at 305 nm. The quantitative analysis of N1-deoxyquinocetone, N1-deoxy mequindox, and N1-deoxycyadox were performed according to the peak area. Kinetic parameters for the biotransformation of N1-deoxyquinocetone, N1-deoxy mequindox, and N1-deoxycyadox were calculated by fitting data into the Michaelis-Menten equation, v= Vmax [S] / (Km+ [S]) by GraphPad Prsim 5 software.

**Site-directed Mutagenesis and Enzyme Activity Assay**

*SsAOX1* cDNA in the pQE-30 expression vector was mutagenized using the QuickChange II site-directed mutagenesis kit (Stratagene). The mutagenesis was performed according to the manufacturer’s suggested protocol. Primers were designed using the online tool provided by Stratagene and listed in Supplemental Table 2. All mutations were verified by sequencing (Invitrogen, Guangzhou, China).

After expression, all of the mutants were tested for activity towards benzaldehyde by the method of Enrico Garattini (Schumann et al., 2009). Briefly, the enzyme assays
were performed at 30 °C in Tris buffer (50 mM, 1 mM EDTA, pH 7.4) in a final volume of 0.5 mL. The total enzyme concentration was 30 nM for wild-type SsAOX1 or variants. The enzyme activity was spectrophotometrically monitored at 600 nm with 100 mM 2, 6-dichlorphenolindophenol as the electron acceptor. The activity of SsAOX1 G1019T towards the QdNOs was evaluated as described in the Materials and Methods section.
Results

cDNA Cloning of Aldehyde Oxidase from Pig Livers

Through RT-PCR using degenerate primers based on the sequence similarity of mammalian AOX1, three PCR products were obtained and assembled to produce the full-length porcine AOX1 (data not shown). The cloned porcine AOX1 was named SsAOX1 (GenBank accession No: JX977168). The ORF of SsAOX1 is 4017 bp, which encodes a 1338-residue polypeptide. The deduced protein has a molecular mass of 147710 Da and exhibited an 87% amino acid sequence identity with human and bovine AOX1 (data not shown). Genomic analysis showed that SsAOX1 is located on chromosome 15 in the genome of Sus scrofa and bears 35 exons and 34 introns (Fig. 2A).

Motif prediction and amino acid sequence alignment showed that SsAOX1 is a typical aldehyde oxidase, containing two non-identical 2Fe-2S centers in the N-terminus, an FAD-binding region in the intermediate region, and a molybdenum cofactor and substrate pocket in the C-terminus (Fig. 2A). The alignment of SsAOX1 with other mammalian AOX1 showed that these motifs are very conserved among mammalian AOX 1(Fig. 2B).
Recombinant Expression and Characterization of SsAOX1 in E. coli

A His-tagged SsAOX1 protein of approximately 150 kDa was expressed (Fig. 3). Similar with other reported AOs (Kurosaki et al., 2004), a major band with a molecular mass of 150 kDa and two minor degraded bands of approximately 130 kDa and 80 kDa were detected by SDS-PAGE (Fig. 3A) and confirmed by western blot analysis using an antibody against AOX1 (Santa Cruz Biotechnology, CA, USA) (Fig. 3B).

CD is a useful technique for the structural study of proteins. We investigated the secondary structures of SsAOX1 by CD. As shown in Fig. 4A, the CD spectra of SsAOX1 exhibited the characteristic signature of an $\alpha$ helix, with minima at 222 and 208 nm. The spectrum corresponds to the SsAOX1 protein with secondary structures of 32.7% $\alpha$-helix, 17.6% $\beta$-sheet, 16.9% $\beta$-turn, and approximately 54% other.

Because AO was reported to function as a homodimer, the native formation of SsAOX1 was analyzed by native PAGE. As shown in Fig. 4B, the SsAOX1 in native sample buffer ran at approximately 300 kDa, whereas the boiled SsAOX1 in denaturation buffer ran at approximately 150 kDa, which indicates the purified SsAOX1 formed a functional homodimer under native conditions.
SsAOX1 Catalyzed N-oxide Reduction of Quinoxaline 1,4-di-N-oxides at the N1 position

To determine whether the recombinant expressed and purified SsAOX1 could catalyze the N-oxide reduction of QdNOs, three representative QdNOs, quinocetone, mequindox and cyadox (Fig. 1), were incubated with SsAOX1, and the metabolites were detected by HPLC. After the reaction, only the N1-deoxidized products of the three tested drugs were clearly detected, whereas no N4-deoxidized products were detected (Fig. 5 and Supplemental Fig.4). Furthermore, the N1-deoxidization was inhibited by an AO inhibitor (chlorpromazine) and abolished by boiling the proteins (data not shown), indicating that the N1-deoxidization was catalyzed by AO, and the N-O bond at N1 position might be more resistant than that of N4 position to reduction by AO. Subsequently, the enzyme kinetic parameters were determined. The results showed that SsAOX1 had a highest activity to quinocetone, followed by mequindox and cyadox, with the $k_{cat}/K_m$ values of 1.94±0.04, 1.27±0.15 and 0.43±0.09 min$^{-1}$ μM$^{-1}$, respectively (Table 1). However, SsAOX1 had lowest substrate affinity to quinocetone, followed by cyadox and mequindox, with the $K_m$ values of 4.36±0.56, 3.16±0.48 and 2.96 ±0.51 μM, respectively (Table 1).
Substitution of Glycine 1019 with Threonine Endows SsAOX1 with N-oxide Reductive Activity at the N4 position of Quinoxaline 1,4-di-N-oxides

To further elucidate the catalytic mechanism of SsAOX1 towards the QdNOs, five mutants with amino acid at position 1019, which is one of the active sites lying closest to substrate in substrate binding pocket (Dastmalchi, 2005), were generated and analyzed. Because the reduction of AO by the oxidation of its substrate (e.g., benzaldehyde and 4,6-dihydroxypyrimidine) is a vital step for the subsequent reduction of N-O bond (Sugihara et al., 1996), the activity of mutants towards its standard substrate, benzaldehyde, was analyzed. The G1019T mutant exhibited a six-fold increase in benzaldehyde oxidation activity, whereas other 4 mutants displayed a negligible effect (Table 2). Therefore, the activity of the G1019T mutant towards the QdNOs was further analyzed. Interestingly, the N1, N4 double deoxidized metabolites were detected with all of the three tested QdNOs drugs, whereas no detectable single N4 deoxidized metabolites were detected (Fig. 6 and supplemental Fig. 5).

To further confirm the N4 deoxidizing activity of mutant G1019T, the N1 deoxidized metabolites of the QdNOs (1-deoxyquinocetone, 1-deoxymequindox and 1-deoxycyadox) were incubated with SsAOX1 G1019T and compared with wild type SsAOX1 in the same reaction system. After the reaction, obvious N1, N4 dideoxidized metabolites were detected in the reaction with mutant G1019T (Fig. 7A, B and C), whereas no or small amount of N1, N4 dideoxidized metabolites were detected with wild type SsAOX1 (Fig. 7D, E and F), indicating that G1019T endowed SsAOX1 with the N4 deoxidizing activity towards the QdNOs. However, it is not
clear whether the N-4 reduction requires the N-1 reduction or not. It is possible
SsAOX1 G1019T can reduce N4 before N1 reduction, but the reduction of N1 is quite
easier than that of N4 (Shen et al., 2010; Shan et al., 2012; Zheng et al., 2011) (Fig. 5).
Therefore, the 4-deoxy products by SsAOX1 G1019T may not easy to be detected.
Discussion

The QdNOs consist of a group of quinoxaline derivates, which are widely used as human drugs and animal feed additives. However, the genotoxicity and reproductive toxicity of QdNOs has been reported recently (Ihsan et al., 2011; Ihsan et al., 2013). The N-oxide groups of QdNOs are the main functional moiety responsible for their activity and toxicity (Carta et al., 2005). Thus, understanding the metabolic mechanism of the N-oxide groups is important for the application and administration of QdNOs. In this study, the N-oxide reduction enzyme for QdNOs in swine, SsAOX1, was identified and characterized. SsAOX1 catalyzed the N-oxide reduction at the N1 position, and the substitution of glycine 1019 with threonine endows the enzyme with the N-oxide reductive activity at the N4 position.

AOs are capable of transforming a wide array of substrates. Along with the presence of significant levels of enzymatic activity in the cytosolic fraction, AOs have attracted increasing interest in the drug metabolism and drug discovery fields (Garattini et al., 2008; Garattini and Terao, 2011; Garattini and Terao, 2013; Pryde et al., 2010). AOs can function not only as oxidases but also as reductases. The oxidizing substrate varies from aldehyde-containing compounds to various types of aromatic azo-and oxo-heterocyclic compounds and iminium-containing compounds (Garattini and Terao, 2011; Garattini and Terao, 2012). The reduction substrate includes N-oxides, sulfoxides, nitro-compounds, and heterocycles (Kitamura and Tatsumi, 1984; Tatsumi et al., 1982; Tatsumi et al., 1983). In this study, we cloned
and characterized the AO isoform in swine for N-oxide reduction of three widely used QdNOs (quinocetone, mequindox and cyadox) in animal feed additives. The N-O bonds were shown to be not only the activity group of the QdNOs but also the toxicity group (Carta et al., 2005). Hence, understanding the metabolic pathways of N-oxide reduction of the QdNOs is important for their application and administration.

Previously, we have shown that cyadox could be metabolized to 1-deoxycyadox and 4-deoxycyadox in pig liver extracts (Zheng et al., 2011). Using the inhibitor approach, we showed that the metabolism might be catalyzed by AO and XO (Zheng et al., 2011). However, the SsAOX1 could only metabolize cyadox to 1-deoxycyadox (Fig. 5C). Because the number of AOs can range from 1-4 in vertebrates (Garattini et al., 2009; Kurosaki et al., 2013), we propose that there is another AO or XO responsible for the N-oxide reduction at the N4 position of the QdNOs, quinocetone, mequindox and cyadox.

Interestingly, SsAOX1 primarily catalyzed the N-oxide reduction at the N1 position of the three tested QdNOs (Fig. 5). This result is in agreement with the metabolism profile of quinocetone and mequindox. Shen et al. showed that 1-deoxyquinocetone was abundant in swine urine, but 4-deoxyquinocetoone was not (Shen et al., 2010). Qi et al. showed that 1-deoxymequindox was more abundant than 4-deoxymequindox in chicken (Shan et al., 2012). We propose that the high abundance of the N-1 reduced metabolites may be partially due to the activity of AO and the structure of these drugs. It was reported that N1 reduction can happen either by enzymatic or nonenzymatic
catalysis (Zheng et al., 2011), but not for N4 reduction, which indicate that the N-O bond at N1 position is not as stable as N4 position. The difference between the N-O bonds at the N1 position and the N4 position is that the N-O bond at the N1 position is closer proximity to the electron-withdrawing group at the C2 position, but not for N-O bound at N4 position (Fig. 1). Based on the structure of three QdNOs, the in vivo and in vitro metabolism data, we propose that the electron groups adjacent to the N-O bond may influence its stability. Therefore, it would be helpful to clarify this by changing the group at C-2 and C-3 position. Several researchers have attempted to modify the molecular structure of drugs to prevent them from being metabolized by AO (Linton et al., 2011; Pryde et al., 2012). For N-oxide reduction, our results may contribute this approach, by suggestion modification the N-O bond adjacent groups. However, through site-directed mutagenesis, we found that the substitution of glycine 1019 with threonine at the substrate binding pocket endows SsAOX1 with the N-oxide reductive activity at N4 position of the QdNOs (Fig. 6, 7A, 7B and 7C). There is a hydroxyl group at the side chain of threonine, whereas glycine does not contain that group. The amino acid at 1019 was reported to be one of the most important active sites, which located in close proximity to the substrate (Coelho et al., 2012; Dastmalchi, 2005; Enroth et al., 2000). Because threonine has a nucleophilic side chain, whereas glycine has not, we hypothesize that the nucleophilic side chain of amino acid at 1019 may increase the interaction between AO and N-O bonds.
In conclusion, this study identified the enzyme (SsAOX1) for N-oxide reduction of QdNOs in swine. SsAOX1 catalyzed the N-oxide reduction at the N1 position of the QdNOs, and its activity might be influenced by the composition of the C2 group of its substrate. Furthermore, the substitution of glycine 1019 with threonine endows SsAOX1 with N-oxide reductive activity at the N4 position of the QdNOs. However, the mechanism of N-oxide reduction of QdNOs is not clear enough so far. To best understand it, the substitution of groups at C2 and C3 positions would be helpful and it may be interesting in further research.
Authorship Contribution

Participated in research design: Yiqun Deng, Peiqiang Mu and Ming Zheng.

Conducted experiments: Peiqiang Mu, Xianqing Tang, Yufan Wang, Kaixin Wu, Ming Xu, Ming Zheng, Yuanming Zhen.

Performed data analysis: Qingmei Chen, Yiqun Deng, Peiqiang Mu, Lijuan Wang, Ming Zheng.

Wrote or contributed to the writing of the manuscript: Yiqun Deng, Peiqiang Mu and Ming Zhen.
References


Takekawa K, Sugihara K, Kitamura S and Ohta S (2001) Enzymatic and
non-enzymatic reduction of brucine N-oxide by aldehyde oxidase and catalase.

*Xenobiotica* 31(11): 769-782.


Footnote

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Peiqiang Mu and Ming Zheng contributed equally to this work.
Figure Legends

**Fig. 1.** Molecular structures of the quinoxaline 1,4-di-N-oxides used in this study

**Fig. 2.** The gene exon-intron structure, predicted motifs of SsAOX1 and their conservation among mammalian AOX1s. (A) The location exon-intron structure of SsAOX1 and the predicted motifs of its deduced amino acid sequences. (B) The alignment of SsAOX1 with other mammalian AOX1. Identical amino acids among all of the aligned sequences are marked with an asterisk, and the conserved amino acids are marked with one or two dots. The underline sequence with solid line, dashed line and dashed line with two dots indicates the [2Fe-2S] redox center I (FesSI), [2Fe-2S] redox center II (FesSII) and FAD binding domain (FAD) respectively. The box showed part of the molybdenum cofactor and substrate-binding domain (MoCo). The accession number of *Sus scrofa* (Ss) AOX1, *Bos taurus* (Bt) AOX1, *Homo sapiens* (Hs)AOX1, *Mus musculus* (Mm)AOX1, *Pongo abelii* (Pa) AOX1, *Oryctolagus cuniculus* (Oc) AOX1 and *Rattus norvegicus* AOX1 is AGC31499, NP_788841, NP_001150, NP_033806, NP_001125740, NP_001075459 and NP_062236 respectively.

**Fig. 3.** Recombinant expression and purification of SsAOX1 in *E.coli*. (A) SDS-PAGE analysis of the expression of SsAOX1 in *E.coli* M15. M1 indicates the SDS-PAGE protein standards. Lanes 1-5 indicates the non-induced cells, IPTG
induced cells, the soluble cell extracts, cell debris and purified SsAOX1, respectively.

(B) Western blot analysis of the expression and purification of SsAOX1. M2 indicates
the biotin-labeled protein standards. Lanes 1-5 indicates the same sample as (A).

**Fig. 4.** Structure characterization of SsAOX1. (A) The circular dichroism spectra of
SsAOX1. (B) Analysis of SsAOX1 on native PAGE. M: Native protein molecular
standards; D indicates SsAOX1 in denatured buffer and boiled at 100 °C for 10 min;
ND indicates SsAOX1 in native buffer.

**Fig. 5.** The HPLC chromatographs of SsAOX1 metabolized quinocetone, mequindox
and cyadox. The arrows indicate the peaks of quinocetone, mequindox, cyadox and
their N-oxide reduced metabolites. Quinocetone, 1-deoxyquinocetone,
4-deoxyquinocetone and 1,4-dideoxyquinocetone were eluted at 20.6, 22.0, 21.3 and
22.6 min respectively. Mequindox, 1-deoxymequindox, 4-deoxymequindox and
1,4-dideoxymequindox were eluted at 15.2, 20.1, 17.9 and 21.2 min respectively.
Cyadox, 1-deoxycyadox, 4-deoxycyadox and 1,4-dideoxycyadox were eluted at 14.9,
17.1, 17.9 and 18.7 min, respectively.

**Fig. 6.** The HPLC chromatographs of SsAOX1 G1019T metabolized quinocetone (A),
mequindox (B) and cyadox (C). The arrows indicate the peaks of quinocetone,
mequindox, cyadox and their N-oxide reduced metabolites.
Fig. 7. The HPLC chromatographs of SsAOX1 or SsAOX1 G1019T metabolized N1-reduced QdNOs. (A) SsAOX1 G1019T metabolized 1-deoxyquinocetone. (B) SsAOX1 metabolized G1019T 1-deoxymequindox. (C) SsAOX1 G1019T metabolized 1-deoxycyadox. (D) SsAOX1 metabolized 1-deoxyquinocetone. (E) SsAOX1 metabolized 1-deoxymequindox. (F) SsAOX1 metabolized 1-deoxycyadox.

The arrows indicate the peaks of quinocetone, mequindox, cyadox and their N-oxide reduced metabolites.
### Tables

**TABLE 1** Enzymatic kinetic parameters of SsAOX1 towards quinocetone, mequindox, and cyadox

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Quinocetone</th>
<th>Mequindox</th>
<th>Cyadox</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ [nmol/min·mg protein]</td>
<td>2882.52±11.32</td>
<td>1276.45±62.13</td>
<td>463.5±20.0</td>
</tr>
<tr>
<td>$K_m$ [$\mu$M]</td>
<td>4.36±0.56</td>
<td>2.96±0.51</td>
<td>3.16±0.48</td>
</tr>
<tr>
<td>$k_{cat}$ [min$^{-1}$]$^a$</td>
<td>8.47±0.03</td>
<td>3.75±0.16</td>
<td>1.36±0.06</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ [min$^{-1}$µM$^{-1}$]</td>
<td>1.94±0.04</td>
<td>1.27±0.15</td>
<td>0.43±0.09</td>
</tr>
</tbody>
</table>

The values represent the mean±S.D. of three duplicate experiments.

$^a$ $k_{cat}$ was calculated from Mr 147, 000
TABLE 2 Enzymatic kinetic parameters of SsAOX1 and variants towards benzaldehyde.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Kinetic parameters for benzaldehyde&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ [nmol/min·mg protein]</td>
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<tr>
<td>Wild Type</td>
<td>19380±1750.50</td>
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<tr>
<td>G1019T</td>
<td>24270±2283.65</td>
</tr>
<tr>
<td>G1019A</td>
<td>22290±2497.53</td>
</tr>
<tr>
<td>G1019D</td>
<td>9120±1194.26</td>
</tr>
<tr>
<td>G1019E</td>
<td>11160±1357.81</td>
</tr>
<tr>
<td>G1019K</td>
<td>10230±950.15</td>
</tr>
</tbody>
</table>

The values represent the mean±S.D. of three duplicate experiments.

<sup>a</sup> $k_{\text{cat}}$ was calculated from Mr 147, 000.
Figure 1

Mequindox

Cyadox

Quinocetone

1-deoxymequindox

1-deoxyCyadox

1-deoxyquinocetone

4-deoxymequindox

4-deoxyCyadox

4-deoxyquinocetone

1,4-dideoxymequindox

1,4-dideoxyCyadox

1,4-dideoxyquinocetone
Figure 3
Figure 4

A

[Graph showing UV absorbance spectra with wavelength in nm on the x-axis and absorbance (ε) in 10^3 deg.cm^2.dmol^-1 on the y-axis.]

B

[Image showing native PAGE and Western blot results. Left: Native PAGE with markers and bands indicating homodimer and monomer. Right: Western blot with Anti AOX1 antibody showing bands for homodimer and monomer.]

7.5% Native PAGE

Anti AOX1

Homodimer

Monomer
Figure 5

A

![Graph A with peaks for (1-deoxyquinocetone) at 22.0 min, Quinocetone at 20.6 min, and 1-deoxymequindox at 20.1 min.]

B

![Graph B with peaks for (1-deoxymequindox) at 20.1 min, Mequindox at 15.2 min.]

C

![Graph C with peaks for Cyadox at 14.9 min, and 1-deoxycyadox at 17.1 min.]

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Figure 7

(A) (1,4-dideoxyquinocetone) 22.6 min
(1-deoxyquinocetone) 22.0 min

(B) (1,4-dideoxymequindox) 21.2 min
(1-deoxymequindox) 20.1 min

(C) (1,4-dideoxyquinocetone) 22.6 min
(1-deoxyquinocetone) 22.0 min

(D) (1-deoxyquinocetone) 22.0 min

(E) (1-deoxymequindox) 21.2 min
(1,4-dideoxymequindox) 21.2 min

(F) (1-deoxycyadox) 17.1 min
(1,4-dideoxyquinocetone) 18.7 min
N-oxide reduction of quinoxaline-1,4-dioxides catalyzed by porcine aldehyde oxidase SsAOX1

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Supplemental Table 1 Degenerate primers used for *SsAOX1* cloning

<table>
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<tr>
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<tr>
<td>R1</td>
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</tr>
<tr>
<td>F2</td>
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</tr>
<tr>
<td>R2</td>
<td>GCTGTGTGGATGGAAGGTGTG</td>
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<tr>
<td>F3</td>
<td>ATGGCAGAATCTTGGCCCT</td>
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<tr>
<td>R3</td>
<td>TCATATGGGTACACTCCAAGGA</td>
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Supplemental Table 2  Primers used for SsAOX1 mutagenesis

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<td>F</td>
<td>GTATCCTGTGGCAGGACCTTTTCACTTGCTATGGTTC</td>
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<tr>
<td></td>
<td>R</td>
<td>GACCGATCAGCAAGTGAAAAGGTGCCGACAGGATAC</td>
</tr>
<tr>
<td>G1019A</td>
<td>F</td>
<td>GTATCCTGTGGGACCTTTTCACTTGCTATGGGTC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GACCGATCAGCAAGTGAAAAGGTGCCGACAGGATAC</td>
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<tr>
<td>G1019D</td>
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<td>GTATCCTGTGGGACCTTTTCACTTGCTATGGGTC</td>
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<td>GACCGATCAGCAAGTGAAAAGGTGCCGACAGGATAC</td>
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<tr>
<td>G1019K</td>
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<td></td>
<td>R</td>
<td>GACCGATCAGCAAGTGAAAAGGTGCCGACAGGATAC</td>
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**Supplemental Fig. 1.** HPLC graphs of standard quinocetone (A), 1-deoxyquinocetone (B), 4-deoxyquinocetone (C) and 1,4-dideoxyquinocetone (D).

**Supplemental Fig. 2.** HPLC graphs of standard mequindox (A), 1-deoxymequindox (B), 4-deoxymequindox (C) and 1,4-dideoxymequindox (D)
Supplemental Fig. 3. HPLC graphs of standard cyadox (A), 1-deoxycyadox (B), 4-deoxycyadox (C) and 1,4-dideoxycyadox (D)

Supplemental Fig. 4. HPLC graphs of controls of SsAOX1 activity. (A) Negative control without substrate. (B) Boiled SsAOX1 with quinocetone. (C) Boiled SsAOX1 with mequindox. (D) Boiled SsAOX1 with cyadox.
**Supplemental Fig. 5.** HPLC graphs of controls of SsAOX1G1019T activity. (A) Negative control without substrate. (B) Boiled SsAOX1G1019T with quinocetone. (C) Boiled SsAOX1G1019T with mequindox. (D) Boiled SsAOX1G1019T with cyadox.