Original Paper

Human NRDRB1, an Alternatively Spliced Isoform of NADP(H)-Dependent Retinol Dehydrogenase/Reductase Enhanced Enzymatic Activity of Benzil

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Key Words

NRDRB1 • Alternative splicing • Dicarboxyl reductase • Benzil • Metabolism of xenobiotics

Abstract

Aims: Human NRDRB1, a 226 amino acid alternatively spliced isoform of the NADP(H)-dependent retinol dehydrogenase/reductase (NRDR), lacks the complete coding region of exon 3, but preserves all the important functional motifs for NRDR catalytic activity. Nevertheless, its tissue distribution and physiological function remain to be elucidated. **Methods:** Expression of NRDRB1 and NRDR in cells and tissues was analyzed by semi-quantitative polymerase chain reaction (PCR) and western blot. NRDRB1 was expressed as a His₆ fusion protein and subjected to kinetics assays. **Results:** Recombinant NRDRB1 had 1.2 to 8.6 fold higher k_{cat}/K_m values than recombinant NRDR, depending on the substrate. NRDRB1 catalyzed the NADPH-dependent reduction of α -dicarboxyl compounds, such as isatin, 9,10-phenanthrenequinone, and especially benzil. The significantly high catalytic activity and the relatively high expression in human liver of NRDRB1 conferred cellular resistance to benzil-induced cell toxicity and over-expression of NRDRB1 in low expressing Ec109 cells significantly enhanced cell tolerance toward benzil. **Conclusions:** Based on its substrate specificity, catalytic activity and relatively high expression in human liver tissue, our results suggest that NRDRB1, an alternatively spliced isoform of NRDR *in vivo* functions better than NRDR as a dicarboxyl reductase for xenobiotics containing reactive carbonyls. Our study is the first reporting this phenomenon of the enzymes involved in biochemical reactions.

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Introduction

The short-chain dehydrogenase/reductase (SDR) superfamily comprises a large and diverse group of genes with members found in all organisms. Members of this family are mainly oligomeric enzymes with subunits of about 250-350 amino acid residues. SDR members share distinct sequence motifs comprising conserved nucleotide cofactor and active site residues [1-3], with a typical overall residue identity of 15-30%. Accordingly, SDR enzymes are mostly NAD(P)(H)-dependent dehydrogenases/reductases, acting on a heterogeneous set of substrates, including steroids, retinoids, prostaglandins, sugars and xenobiotics. NADP(H)-dependent retinol dehydrogenase/reductase (NRDR) encoded by the *DHRS4* gene is a member of SDR superfamily. We identified the NRDR protein in the cytosol of rabbit liver tissue and found it to harbor strong retinol oxidation and retinal reduction activity [4]. NRDR has subsequently been found to play a special role in the retinoic acid metabolism of animals, such as mouse, pig, and dog [5-7].

Human NRDR is encoded by the *DHRS4* gene within the *DHRS4* gene cluster along with its two immediately downstream homologous genes, *DHRS4L2* and *DHRS4L1* generated by gene duplication. The *DHRS4* gene contains 8 exons encoding two possible variants of human NRDR, containing 278 amino acids and 260 amino acids due to use of alternative translation initiation sites. In our previous study without N-terminal sequencing analysis of human NRDR, translation of human NRDR was assumed to start at the first initiation site, theoretically encoding a 29.5 kDa protein of 278 amino acids [8]. However, our recent studies (unpublished) provide indication that a second initiation site is likely to be the start of translation of the NRDR protein, leading to a 27.3 kDa mature protein with 260 amino acids. Various alternatively spliced variants of NRDR exist in normal human tissues and tumor cell lines [8-12]. Alternative splicing is a key phenomenon that contributes to the production of different mature transcripts from the same primary RNA sequence [13]. New high throughput sequencing technology has revealed that alternative splicing is evident in over 90% of human genes [14]. Currently, 30 differentially-spliced transcripts and concomitantly 30 different NRDR translation products are predicted by *NCBI*. However, only two isoforms of human NRDR have been detected, namely NRDRB1 [8] and NRDR A2 [12]. The 226-amino acid NRDRB1 lacking the complete coding region of exon 3 was originally identified in human cervical squamous carcinoma. At present, we found NRDRB1 to be predominantly expressed in human hepatocyte cells.

Although alternative splicing seems to be a widespread phenomenon in the human transcriptome, the *in vivo* functions of protein isoforms generated by alternative splicing remain poorly defined in the majority of cases. To date, little is known about the expression pattern and physiological role of NRDRB1. In this study, we generated prokaryotic and eukaryotic NRDRB1 and NRDR expression systems to determine protein biochemical activity and physiological function. Several steroids and xenobiotic α -dicarbonyl compounds, including benzil, were chosen as substrates in order to reveal the physiological function of NRDRB1 and give insight into the effect of alternative splicing on NRDR protein function.

Materials and Methods

Cell culture and human liver tissue samples

The following human cell lines were used: Hela cervical carcinoma cells, Ec109 esophageal carcinoma cells, HL7702 normal hepatocyte cells, HepG2 hepatocarcinoma cells, SKOV3 ovarian adenocarcinoma cells, A549 lung adenocarcinoma cells, HEK293 embryonic kidney cells and HS181.TES testis cells. All were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, at 37°C in a humidified atmosphere with 5% CO₂.

Histopathologically confirmed human normal liver tissues were obtained from four male patients, 35-45 years of age, hospitalized for cholecystitis and who underwent cholecystectomy at Shantou University

Table 1. Primers used in the present study

Primer Name	Sequence(5'-3')	Gene	Use
NRDRF	TCC ACC GAC GGG ATC GGC TT	NRDR	Semi qPCR
NRDRR	ATG CCA GCA CAA TCC TCT GGC		
NRDRB1F	TGG CCA CGA CTC TGG ACA TTA	NRDRB1	Semi qPCR
NRDRB1R	CCT TCT TAT CCG CAG GGT TTC		
ACBTF	TGA CGT GGA CAT CCG CAA AG	β -actin	Semi qPCR
ACBFR	CTG GAA GGT GGA CAG CGA GG		
pIVEXF	CATG <u>CCATGG</u> ATG GCC AGC TCC GGG ATG	NRDR/NRDRB1	RTS
pIVEXR	CCG <u>CTCGAG</u> TCA GAG GCG GGA CGG GGT TC		
Seq F	TAA TAC GAC TCA CTA TAG	NRDR/NRDRB1	Sequencing
Seq R	TTT CCA TTC GCC ATT CAG G		

Affiliated Hospital. The study protocol was approved by the Human Ethics Review Committee of Shantou University, and a signed consent form was obtained from each subject. Tissue samples were immediately frozen in liquid nitrogen and stored in a -80°C freezer until analysis.

Semi-quantitative PCR and western blot

The expression of NRDRB1 and NRDR in cells and tissues was analyzed by semi-quantitative polymerase chain reaction (PCR) and western blot analysis. Semi-quantitative PCR was used on mRNA isolated from 8 different cell lines and 4 different human liver tissue samples. PCR products were subjected to electrophoresis on a 1.2% agarose gel. Primers for specific amplifications are shown in Table 1. Rabbit anti-NRDR polyclonal antibody [9, 15] and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3,000, Pierce) were used in the western blot analysis. SDS-PAGE gels were stained with Coomassie Brilliant Blue to confirm the size of the targeted protein.

RNA extraction, RT-PCR and 3' and 5' RACE assay

Total RNA was extracted from the HL7702 cell line using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (1 μ g) from each sample was reverse-transcribed into cDNA using a 2-strand cDNA synthesis kit (Promega, Madison, WI). NRDR and NRDRB1 were cloned by PCR using two sets of primers: NRDRF and NRDRR, and NRDRB1F and NRDRB1R. Subsequently, for NRDRB1, 3'RACE and 5'RACE were performed to obtain the full-length cDNA using the protocol supplied by the manufacturer (Invitrogen, San Diego, CA), but slightly modified to use nested PCR. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel, and the corresponding PCR products were gel-purified and cloned into the pGEM-T vector (Promega). Positive clones were sequenced by the dideoxynucleotide method.

Expression and purification of NRDRB1 and NRDR in E. coli

NRDRB1 and NRDR cDNA were cloned into pDEST17 bacterial expression vectors (Gateway Expression system, Invitrogen) to express the proteins with His-tag sequences. The constructs were transformed into *E. coli* BL21-AI cells (Takara, Japan), and the open reading frame of the cDNA in the expression plasmid was verified by sequencing.

Overexpression of recombinant protein was induced at mid-log growth phase of BL21-AI using 0.2% L-arabinose at 25°C. The expressed NRDRB1 and NRDR protein was purified using affinity chromatography, as described previously [4]. The purified enzymes showed single protein bands on SDS-PAGE analysis. Typical yields for both proteins were 2-5 mg/L of culture, as measured by the BCA method.

Kinetic assays

The reductase activity of recombinant enzymes was determined at 25°C by measuring the rate of change in NADPH absorbance at 340 nm. The standard reaction mixture consisted of 0.1 M potassium phosphate, pH 6.5, 0.1 mM NADPH, 1 mM substrate, and enzyme in a total volume of 1.0 ml. The apparent K_m and k_{cat} values were determined over a range of eight substrate concentrations at a saturating concentration of coenzyme NADPH by fitting the initial velocities to the Michaelis-Menten equation, and are expressed as the means of three determinations.

Expression of NRDRB1 and NRDR proteins using the Wheat Germ in vitro Rapid Translation System (RTS)

Plasmids (pGEM-T Easy vector; Promega) encoding NRDR or NRDRB1 cDNA and primer set pIVEXF and pIVEXR were used to amplify the sequence of the NRDR and NRDRB1 regions. PCR conditions with Taq polymerase: initial denaturation for 3 min at 94°C, 25 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, elongation for 2 min at 72°C and a final elongation step of 8 min in 50 µl reaction mixture containing 25 µl 2×PCR mix, 1 µl of a 10 µmol/L solution of each primer, 4 µl DNA as template, and 19 µl distilled water. PCR products were subjected to electrophoresis on a 1.2% agarose gel and purified with the Gel Extraction Kit. Purified DNA was ligated into the pIVEX1.3 vector using the *Nco* I and *Xho* I restriction endonucleases (New England Biolabs, Frankfurt am Main, Germany). All DNA was verified by sequencing using the sequencing primer set SeqF and SeqR. Protein synthesis reactions were conducted following the manufacturer's protocol (Roche, Basel, Switzerland). Protein samples were subjected to western blotting using rabbit anti-human NRDR antibody as described above.

Transfection and over-expression of NRDRB1 and NRDR in HEK293 cells

HEK293 cells were inoculated into 100-mm Petri dishes (3×10^6 cells /dish) and transfected with 8 µg plasmid DNA, subcloned into the pcDNA3.0 mammalian expression vector using Polyfect transfection reagent (Qiagen, Hilden, Germany). Control cells were transfected with an equal amount of the parental vector alone. After 24 h post-transfection, cells were harvested with a rubber scraper, collected by low speed centrifugation, and then resuspended in hypotonic buffer (10 mM potassium phosphate buffer, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride), put on ice for 20 min, and finally homogenized using a Dounce homogenizer.

Catalytic activity of NRDRB1 and NRDR for benzil by HPLC

Benzil (2 mM) was reduced in the assay mixture containing each protein sample (10–20 µg) obtained above, 500 µM NADPH and 0.1 M potassium phosphate, pH 6.5, and incubation at 37°C for 4 h, then the reaction mixture was extracted with ethyl acetate, evaporated and purified by silica gel column chromatography [16]. All previous steps were done in the dark under minimal red light illumination. The products were analyzed using an HP1090 high-pressure liquid chromatography system (Hewlett-Packard). The stationary phase was a reverse-phase C18 column (Waters Symmetry, 4.6 mm×150 mm). The mobile phase was made up of 60% acetonitrile and 40% 0.1 M potassium phosphate at pH 6.5 (v/v). The flow rate was 1 ml/min, and analysis was simultaneously monitored at 254 nm.

Analysis of benzil-induced cell toxicity and detoxification for NRDRB1

Eight cell lines were plated into a 96-well plate at a density of 1×10^5 cells/well in medium containing 2% FBS and allowed to attach for 24 h. The following day, benzil was added for 24 h, then cell viability was evaluated by the trypan blue dye exclusion method, and expressed as % of control culture conditions as described previously [17]. To examine the relationship between cell viability and expression of NRDRB1, Ec109 cells were transfected with NRDR and NRDRB1. Transfected cells were maintained in medium containing 2% FBS for 24 h, then exposed to various concentrations of benzil for an additional 24 h. Cell viability was evaluated by trypan blue exclusion. Cell collection and preparation of the cell extracts were performed and probed for NRDRB1 and NRDR by western blotting.

Results

Functional analysis of human NRDRB1

According to the NCBI database, *DHRS4* is present in a wide range of living species from bacteria to humans. Ubiquitous expression in several tissues [18] and high conservation of NRDR in *Homo sapiens*, *Mus musculus* and *Xenopus laevis* suggest a basic metabolic role for NRDR. The typical features of enzymes of this family (Fig. 1), active site motifs and conserved amino acid residues follow the classical SDR pattern, and are present in NRDRB1 (containing the A84-K118 deletion from NRDR).

6×His-tagged NRDRB1 and NRDR could be clearly separated on the SDS-PAGE gel (Fig. 3a) [8]. NRDR and NRDRB1 showed an expected clear separation with the apparent

Fig. 1. Sequence alignment of human NRDRB1 and related SDR enzymes. An alignment comparing NRDRB1 (GI: 84688723) to the full-length proteins NRDR from human (GI: 32483357), mouse (NRDR_Mus) (GI: 13097510), *Xenopus laevis* (NRDR_Xenopus) (GI: 148222765), and *Arabidopsis thaliana* (NRDR_Xenopus) (GI: 18412959) was generated in the Clustal X program. For comparison, human Hep27 (GI: 5031737) and human DXCR (GI: 7705925) are included. The 34-amino acid sequence, from A84 to K118 deleted in NRDRB1, is shaded in gray. Boxing indicates the sequence motif for cofactor binding found in SDR enzymes. Asterisks above the sequence denote four essential residues that compose the catalytic tetrad characteristic of SDR proteins. The solid arrow points to the mature N-terminus as observed from sequence analysis of NRDRB1, also used for recombinant expression in *E. coli*. The dashed arrow indicates residues important for cofactor specificity. NAD(H) specificity is determined by an acidic residue, whereas NAD(P)(H)-dependent enzymes have no acidic residue at this position.

molecular weight on SDS-PAGE at 28 kDa and 25 kDa, respectively, in accordance with the theoretical mass of 27.5 kDa and 23.9 kDa.

Expression analysis of NRDRB1 in human cell lines and liver tissue

The expression of NRDR and NRDRB1 at both the mRNA and protein levels are shown in Fig. 2.

Unlike NRDR, which was ubiquitously expressed in all cell lines tested and human liver tissue, relatively high NRDRB1 expression was only detected in human liver tissue and human hepatic cells, even though there was also slight expression of NRDRB1 in both SKOV3 and Hela cell lines (Fig. 2a). Although NRDRB1 was never dramatically overexpressed when compared to the levels of NRDR, it was expressed at relatively high levels in human liver tissue and hepatic cells when compared with other cell lines tested in this study. Both the full-length NRDR and the NRDRB1 isoform were predominately expressed in human normal liver tissues (Fig. 2b). It should be noted that NRDRB1 mRNA was expressed at higher levels than NRDR mRNA in human liver tissues, though the protein expression of NRDRB1 was not dominant. This is not unexpected because the relation between mRNA and protein is not strictly linear, but has a more intrinsic and complex dependence [19].

Enzyme kinetics of NRDRB1 and NRDR

Kinetic constants for NRDRB1-mediated carbonyl reduction were performed by measuring the change in absorbance at 340 nm of eight substrate concentrations at a saturating concentration of coenzyme in the reaction. The kinetic constants of NRDRB1 and NRDR toward α -dicarbonyl compounds and 3-ketosteroids are shown in Table 2. We found that human NRDRB1 converts α -dicarbonyl compounds, such as benzil, 9,10-phenanthrenequinone and isatin, in an NADPH dependent manner (data not shown). NRDRB1 had a higher k_{cat} value and lower K_m value, and exhibited 1.2-fold (1-phenylisatin)

Fig. 2. Tissue distribution of human NRDRB1 and NRDR. mRNAs and proteins were detected by specific reverse transcription-polymerase chain reaction (RT-PCR) and western blotting followed by 12% SDS-PAGE. Agarose gel electrophoresis identifying NRDRB1 (amplified with primers NRDRB1F and NRDRB1R) and NRDR (amplified with primers NRDRF and NRDRR). As a loading control, β -actin primers were used. Cell and liver tissue extracts were subjected to western blotting using rabbit anti-human NRDR antibody. (a) Cells: 1, HL7702; 2, HepG2; 3, SKOV3; 4, Ec109; 5, HS181.TES; 6, HEK293; 7, A549; 8, Hela. Expression of NRDR and NRDRB1 mRNA and protein in four different liver tissue samples are shown in (b).

to 8.6-fold (benzil) higher catalytic efficiencies (k_{cat}/K_m) than NRDR towards the four substrates (Table 2). The k_{cat}/K_m value measured for 5 β -pregnane-3, 20-dione was in the same range for NRDR and NRDRB1. However, NRDR and NRDRB1 were not very active towards 5 β -androstan-17 β -ol-3-one in our study (Table 2). This is in contrast to a prior study that reported a K_m value in the low micromolar range [20].

NRDRB1 has a higher catalytic activity for benzil than NRDR

We expressed human NRDRB1 and NRDR both in HEK293 cells and in an *in vitro* translation system (RTS) and conducted HPLC analysis. Western blot analysis confirmed high-level over-expression of proteins in HEK293 cells versus low protein expression in the control cells transfected with the vector alone (Fig. 3b). NRDRB1 and NRDR were also expressed successfully by *in vitro* translation system (Fig. 3c). Both NRDR and NRDRB1 could reduce benzil to benzoin, but a lesser amount of benzoin was detected in the reaction products of NRDR than NRDRB1 (Fig. 4). In NRDRB1 reactions using benzil as a substrate, only benzoin could be detected. These results are consistent with our kinetics measurements using 340-nm spectrophotometric analysis. NRDRB1 has a higher catalytic activity than NRDR for benzil.

NRDRB1 is involved in benzil detoxification

The viability of cell lines decreased in a dose-dependent manner after treatment with benzil for 24 h over a benzil concentration range from 0 to 20 μM (Fig. 5a). Cell lines displayed a range of sensitivities. For example, at 5 μM benzil, viability was 96% for HL7702 cells but 33% for Ec109 cells, consistent with the high NRDR/NRDRB1 expression in HL7702 cells

Table 2. Kinetic constants of NRDRB1 and NRDR towards dicarbonyl compounds and steroids. The Reductase activities were assayed at pH 6.0, except for substrates shown with an asterisk, which were measured at pH 7.4. The standard errors of the values were less than 15%. K_m values are given in 10^{-6} M, k_{cat} values are given in min^{-1} and k_{cat}/K_m values are given in $\text{min}^{-1}\mu\text{M}^{-1}$. Values shown are the average of three to five experiments. The kinetic constants for the substrates were determined with 100 μM NADPH.

Substrate	NRDRB1			NRDR		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\mu\text{M}^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\mu\text{M}^{-1}$)
Benzil	1.8	3600	2045	7.1	1690	237
9,10-Phenanthrenequinone*	2.2	737	335	3.4	720	212
Isatin	475	3370	7.09	389	1520	4.00
1-Phenyisatin	18.8	2570	137	12.8	1500	117
Menadione	7.5	27.1	3.64	29.7	31.4	1.06
5 β -Pregnane-3,20-dione*	5.1	3.4	0.67	4.6	3.6	0.78
5 β -Androstan-17 β -ol-3-one*	127.8	11.8	0.09	117	12.6	0.11

Fig. 3. Purification of recombinant proteins and Western blotting analysis. (a) SDS-PAGE of purified recombinant NRDRB1 and NRDR. The amounts of the purified enzymes analyzed were 2 μg in 12% SDS-PAGE. Proteins were stained with 0.2% Coomassie Brilliant Blue R-250. Lanes: 1, NRDRB1; 2, NRDR. (b) HEK293 cells were transfected with expression plasmids encoding human NRDRB1 and NRDR. Transfection of empty vector was used as a negative control. After 24 h, cell lysates were prepared and probed for NRDRB1 and NRDR by western blotting. Lanes: 1, negative control; 2, NRDRB1; 3, NRDR. (c) Western blotting analysis of His₆-tagged proteins in RTS. Proteins synthesized in the cell-free protein expression system were analyzed for NRDRB1 and NRDR by western blotting. Lanes: 1, negative control; 2, NRDR; 3, NRDRB1.

and low NRDR/NRDRB1 expression in Ec109 cells. We tested whether NRDRB1 could confer resistance to benzil-induced toxicity by over-expressing NRDRB1 in Ec109 cells. The viability of the control cells decreased in a dose-dependent manner after treatment with benzil for 24 h, but less benzil cytotoxicity was observed in the transfected cells (Fig. 5b). These results indicate that NRDRB1 can detoxify benzil *in vivo*.

Fig. 4. HPLC analysis of catalytic activity of NRDRB1 and NRDR for benzil. (a) Wavelength-scan of standards by UV/VIS spectrophotometry. (b) Standards for benzil, benzoin and hydrobenzoin. Retention times are 12.32 min, 5.48 min, and 3.84 min, respectively. (c) HPLC analysis of recombinant NRDRB1 and NRDR towards benzil. HEK293 cells were transfected with expression vector harboring the cDNA for NRDRB1 or NRDR, or the vector alone. Benzil reductase activity of control plasmid, NRDRB1, and NRDR overexpressed in HEK293 cells is shown in (1), (2), and (3), respectively. (4)-(6) indicate the benzil reductase activity of the negative control, NRDRB1, and NRDR expressed by *in vitro* translation system.

Discussion

The expression pattern of NRDRB1 characterized in this report indicates that, similar to NRDR, NRDRB1, an alternatively spliced isoform of NRDR, exists not only in human cervical squamous carcinoma and Hela cell line, but also in human liver tissue and several other cell lines, *e.g.* the HL7702, HepG2, and SKOV3 cell lines. In addition, NRDRB1 was predominantly expressed in human liver tissue.

Although there is a 34-amino acid deletion in NRDRB1 due to alternative splicing, the common TGxxxGxG sequence motif for binding of coenzyme and the catalytic YxxxK sequence motif shared by the SDR superfamily are preserved (Fig. 1). Homologues of NRDR from humans and other animals have been shown to be active on an overlapping spectrum of carbonyl substrates [4, 5, 21-25], including steroids, retinoids, prostaglandins, sugars, and xenobiotics. The missing amino acid fragment in NRDRB1 is encoded by the third exon and does not belong to any known functional region. Thus we predicted that the deletion should not affect the catalytic character of the enzyme.

Enzymatic assay unequivocally indicates that both NRDR and NRDRB1 exhibit high catalytic activity on xenobiotic α -dicarbonyl compounds, consistent with previous findings [20, 25]. However, NRDRB1 shows significantly higher activity towards these substrates

Fig. 5. NRDRB1 is involved in benzil-induced detoxification. (a) Benzil-induced cytotoxicity of different cells. Cells were cultured for 24 h with various concentrations of benzil. Cell viability is expressed as % of control culture conditions that were treated with vehicle (dimethyl sulfoxide). (b) Effects of NRDRB1 overexpression on benzil-induced cytotoxicity of Ec109 cells. Cells were transfected with the expression vector encoding NRDR (cross), NRDRB1 (closed circles), or empty vector (open circles), and treated for 24 h with various concentrations of benzil. Ec109 cells transfected with empty vector or expression plasmids coding for human NRDRB1 and NRDR were harvested after cultured 24 h post-transfection. Western blot of whole cell lysates is shown in the upper right of (b).

compared with NRDR. The region lost in NRDRB1 is located outside the functional motif of the protein, and we predict the fragment lost in NRDRB1 shortens the distance between the coenzyme binding site and the substrate binding site, facilitating the interaction of the two active sites. According to the enzyme kinetics of NRDRB1 and NRDR, the K_m and k_{cat} values for 5 β -pregnane-3, 20-dione metabolism are comparable to that of AKR1C1, which is considered a very efficient enzyme for reduction of 3-ketosteroids [26]. Therefore, in addition to AKR1C1, NRDR and NRDRB1 may also physiologically act as 3-ketosteroid reductase that convert 5 β -pregnane-3, 20-dione to 5 β -pregnan-3 β -ol-20-one, and may be involved in regulating the concentrations of active 3 β -hydroxysteroids in humans. Among substrates tested, catalytic efficiency of NRDRB1 towards benzil is the highest. To exclude artifactual loss of enzyme activity during purification, we also expressed human NRDRB1 and NRDR both in HEK293 cells, and in an *in vitro* translation system. These results similarly demonstrated a high capacity to reduce benzil to benzoin, but had no effect on further reduction to hydrobenzoin, suggesting that NRDR and its isoform NRDRB1 are only

involved in the first reduction step of benzil. Quantitative enzymatic assays demonstrated that NRDRB1 exhibits much higher benzil reductive activity than NRDR. The high reactivity to benzil of NRDRB1 is significant from a pharmacological point of view, because benzil is one of the most widely used environmental diketones. It is used as a photosensitizer, organic synthesis intermediate, and adhesive, as well as a pesticide and especially in food packaging printing inks [27]. *In vivo*, benzil is not only a metabolite of the antiplatelet drug diltiazem [28], but is also a specific competitive reversible inhibitor of human carboxylesterases [29].

Addition of benzil to different human cell lines revealed dose-dependent cytotoxicity. HL7702, HepG2, SKOV3, and Hela cells, all expressing NRDRB1, exhibit higher survival in the presence of benzil than the other four cell lines without detectable NRDRB1 expression. Over-expression of NRDRB1 in Ec109 cells enhances cell survival as well. Thus NRDRB1 confers resistance to benzil-induced cytotoxicity, as would be predicted from our results showing NRDRB1 has higher benzil reductive activity than its full-length enzyme. In terms of pharmacology, NRDRB1 may play a role in detoxifying benzil, as well as reactive α -dicarbonyl compounds that are ingested.

Reactive α -dicarbonyl compounds are generated during the course of metabolic processes, through oxidative stress in a variety of biological systems, or are present in dietary constituents [30]. When two carbonyl groups are adjacent on a carbon chain, the reactivity of each carbonyl group tends to be elevated, and those compounds with α -dicarbonyl groups are cytotoxic, *e.g.* causing mitotic chromosome loss [31], or are prone to conversion into advanced glycation end-products [32]. The enzymatic detoxification of reactive α -dicarbonyl compounds in eukaryotes is achieved by several members of the aldoketo reductase (AKR) and SDR families, including aldehyde reductase, aldose reductases, dihydrodiol dehydrogenase, DCXR, and sepiapterin reductase [33]. In our present study, the k_{cat}/K_m value for benzil is over 600-fold higher than that of sepiapterin reductase, which has been known as the most efficient mammalian benzil reductase [34]. We also find that α -dicarbonyl compounds with aromatic rings are also excellent substrates for human NRDR, especially NRDRB1.

Alternative splicing is one of the most important mechanisms for generating a large number of mRNA and protein isoforms from the surprisingly small number of human genes. Unlike promoter activity, which primarily regulates the amount of transcripts, alternative splicing changes the structure of the transcripts and their encoded proteins, and can result in altered binding properties, intracellular localization, enzymatic activity, protein stability, and posttranslational modifications. The magnitude of the effects range from a complete loss of function or acquisition of a new function, to very subtle modulations, which are observed in the majority of cases reported [35-38]. In our present study, NRDRB1 and NRDR share similar substrate specificity. However, deletion of the amino acid segment encoded by exon 3 to form NRDRB1 enhances catalytic activity towards some substrates, especially α -dicarbonyl compounds with aromatic rings. To the best of our knowledge, our study is the first to describe this phenomenon of the enzymes involved in biochemical reactions.

The *DHRS4* gene cluster is located in a highly variable region [39], suspected to contain clues for the genomic evolution in man. Only one copy of the *DHRS4* is found in mice, while the duplicated *DHRS4L2* gene is found in primates, and a second *DHRS4L1* duplication is present only in humans. Alternative splicing of *DHRS4* becomes more complex as the complexity of its gene structure evolves, generating multiple protein isoforms that enable organisms to adjust to more complex environments and protect themselves from xenobiotic damage.

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