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# Carbonyl Reductase 1 Is a Predominant Doxorubicin Reductase in the Human Liver[S]

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**ABSTRACT:**

**A first step in the enzymatic disposition of the antineoplastic drug doxorubicin (DOX) is the reduction to doxorubicinol (DOX-OL). Because DOX-OL is less antineoplastic but more cardiotoxic than the parent compound, the individual rate of this reaction may affect the antitumor effect and the risk of DOX-induced heart failure. Using purified enzymes and human tissues we determined enzymes generating DOX-OL and interindividual differences in their activities. Human tissues express at least two DOX-reducing enzymes. High-clearance organs (kidney, liver, and the gastrointestinal tract) express an enzyme with an apparent $K_m$ of ~140 $\mu$M. Of six enzymes found to reduce DOX, $K_m$ values in this range are exhibited by carbonyl reductase 1 (CBR1) and aldo-keto reductase (AKR) 1C3. CBR1 is expressed in these three organs at higher levels than AKR1C3, whereas AKR1C3 has higher catalytic efficiency. However, inhibition constants for DOX reduction with 4-amino-1-*tert*-butyl-3-(2-hydroxyphenyl)pyrazolo[3,4-d]pyrimidine (an inhibitor that can discriminate between CBR1 and AKR1C3) were identical for CBR1 and human liver cytosol, but not for AKR1C3. These results suggest that CBR1 is a predominant hepatic DOX reductase. In cytosols from 80 human livers, the expression level of CBR1 and the activity of DOX reduction varied >70- and 22-fold, respectively, but showed no association with *CBR1* gene variants found in these samples. Instead, the interindividual differences in CBR1 expression and activity may be mediated by environmental factors acting via recently identified xenobiotic response elements in the CBR1 promoter. The variability in the CBR1 expression may affect outcomes of therapies with DOX, as well as with other CBR1 substrates.**

The anthracycline doxorubicin (DOX) belongs to the most successful chemotherapeutic drugs (Minotti et al., 2004). This contrasts with the incomplete understanding of its pharmacodynamics (Gewirtz, 1999) and pharmacokinetics. The latter exhibits large interpatient and intrapatient differences (Frost et al., 2002; Palle et al., 2006), which may be important both for the individual antitumor response and for side effects of DOX such as cardiotoxicity. Individual plasma concentrations and area under the curve (AUC) values of DOX differ up to 10-fold (Frost et al., 2002 and references therein). High plasma DOX correlates with remission in children with acute myeloid leukemia (Palle et al., 2006) and in adult patients with acute nonlymphocytic leukemia (Preisler et al., 1984). On the other hand, high DOX plasma concentrations may lead to cardiotoxicity (Minotti et al., 2004). The reasons for the interindividual variability in DOX pharmacokinetics are unknown. Their understanding could improve outcomes of DOX therapies by individual dosage adjustment based on therapeutic drug monitoring.

Approximately 50% DOX is removed from the body unchanged (Joerger et al., 2005). The other 50% undergoes metabolism, chiefly via two-electron reduction to the C13-alcohol metabolite doxorubicinol (DOX-OL). Further metabolites, mainly aglycones of DOX and DOX-OL, are detected at much lower concentrations than DOX-OL (Joerger et al., 2005). The AUC of DOX-OL amounts on average to approximately 50% of DOX AUC but may reach 400% at high DOX doses, probably because of the saturation of biliary excretion (Wihlm et al., 1997). However, the DOX-OL/DOX quotient is very variable

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**ABBREVIATIONS:** DOX, doxorubicin; AUC, area under the curve; DOX-OL, doxorubicinol; CBR1, carbonyl reductase 1; AKR, aldo-keto reductase; hydroxy-PP, 4-amino-1-*tert*-butyl-3-(2-hydroxyphenyl)pyrazolo[3,4-d]pyrimidine; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; HPLC, high-performance liquid chromatography.

(range 0–0.81) in dose-normalized patients. Only a minority of DOX-OL variability can be explained by the individual DOX levels (Frost et al., 2002).

Variability in the generation of DOX-OL may be clinically important. DOX-OL is more cardiotoxic than DOX (Olson et al., 1988), which is supported by four lines of evidence. First, cardiomyopathy correlates with the accumulation of DOX-OL in the heart (Olson and Mushlin, 1990; Cusack et al., 1993; Stewart et al., 1993; Sacco et al., 2003). Second, anthracyclines that form less alcohol metabolite are less cardiotoxic (Menna et al., 2007). Third, an overexpression of a DOX reductase in mouse heart led to DOX-OL accumulation and accelerated cardiomyopathy (Forrest et al., 2000). Conversely, knockouts heterozygous for the cardiac DOX reductase were less sensitive to anthracyclines (Olson et al., 2003). On the other hand, anthracycline alcohol metabolites are less active in topoisomerase inhibition (Ferrazzi et al., 1991) and tumor cell killing (Dorr et al., 1991). A reduced antitumor activity can produce tumor resistance. Taken together, the individual variation in the conversion of DOX to DOX-OL may affect both the antitumor effects and the risk of cardiotoxicity.

Despite the importance of DOX reduction, the underlying enzymes and their tissue distribution are poorly characterized. Reduction of DOX to DOX-OL is predominantly catalyzed in the cytosol (Minotti et al., 2004). Thus far, DOX reduction has been shown for a recombinant human carbonyl reductase 1 (CBR1) expressed as a mouse transgene (Forrest et al., 2000). Furthermore, catalytic activity was shown for aldo-keto reductase (AKR) 1A1 and AKR1B1 at a single DOX concentration of 1 mM (O'Connor et al., 1999) and excluded for AKR1C2 (Takahashi et al., 2008). However, neither detailed kinetics nor the individual importance of these enzymes in the reduction of DOX to DOX-OL, nor the existence of additional ones, is known. Therefore, we investigated the kinetics of DOX reduction of seven cytosolic AKRs and of two CBRs because these enzyme families catalyze cytosolic carbonyl reduction (Rosemond and Walsh, 2004). Besides conflicting results obtained in heart cytosols (Mordente et al., 2003; Salvatorelli et al., 2006), kinetic data of DOX reduction in human tissues are available only for a single sample of liver and kidney (Lovless et al., 1978). Therefore, DOX reduction was also investigated in a panel of 10 human tissues. Because liver achieves the highest DOX concentrations of all the human organs studied thus far (Lee et al., 1980) and our preliminary data suggested it as the most important organ in the enzymatic DOX disposition, we concentrated on the identification of the hepatic DOX reductase. Our data identify CBR1 as a predominant hepatic DOX-reducing enzyme and reveal a substantial variability in its activity. This variability may reflect the impact of environmental induction rather than the individual status of *CBR1* gene variants and may contribute to the pharmacokinetic variability of DOX and DOX-OL. In contrast, DOX reduction in the heart is mediated by a distinct enzyme, most likely by AKR1A1.

## Materials and Methods

**Chemicals.** DOX and daunorubicin were purchased from Pfizer (Karlsruhe, Germany). DOX-OL was provided by Dr. A. Andersen (Clinical Pharmacology Section, The Norwegian Radium Hospital, Oslo, Norway). Stock solutions were prepared with double-distilled water, and aliquots were stored at −80°C. The inhibitor (4-amino-1-*tert*-butyl-3-(2-hydroxyphenyl)pyrazolo[3,4-d]pyrimidine), henceforth referred to as hydroxy-PP, was synthesized according to Tanaka et al. (2005) by M. Perscheid and Prof. Nubbemeyer (Department of Organic Chemistry, Johannes Gutenberg University, Mainz, Germany). The structure was confirmed by $^{1}H$ and $^{13}C$ NMR and by mass spectrometry. Acetonitrile was purchased from VWR (Darmstadt, Germany), and NADPH tetrasodium salt was from Sigma-Aldrich (Taufkirchen, Germany). All other substances were purchased from Applichem (Darmstadt, Germany).

**Biological Material.** CBR1, CBR3, AKR1B1, and AKR1B10, expressed as histidine-tagged proteins, were purified in the Institute of Toxicology and Pharmacology for Natural Scientists (Kiel, Germany) (Doorn et al., 2004; Martin et al., 2006), and AKR1C1, AKR1C2, AKR1C3, and AKR1C4 were expressed and purified in the Department of Pharmacology, University of Pennsylvania School of Medicine (Philadelphia, PA) (Burczynski et al., 1998). Glutathione *S*-transferase–tagged AKR1A1 was purchased from Abnova (Taipei, Taiwan).

Myocardial biopsies were obtained by Dr. G. Reinerth from patients undergoing aortocoronary bypass grafting at the Department of Cardiothoracic and Vascular Surgery, Johannes-Gutenberg-University (Mainz, Germany). Samples of kidney, liver, muscle, colon, stomach, and lung used to determine the kinetics of DOX reduction were provided by S. Schäfer and Prof. S. Biesterfeld from the Department of Pathology, University of Mainz (Mainz, Germany). Eighty liver samples for the determination of levels of CBR1 expression were collected from patients of European Caucasian descent during surgical interventions conducted at the Department of Surgery, Campus Virchow, University Medical Centre Charité, Humboldt University (Berlin, Germany) as described (Wolbold et al., 2003). Normal liver tissue surrounding primary liver tumors or liver metastases was resected and used to prepare cytosols. Written informed consent was obtained from all the donors. Removal and usage of all the tissue samples were approved by the responsible ethics committees.

**Preparation of Cytosolic Fractions.** Human small intestine cytosol was purchased from Biopredic (Rennes, France), and liver cytosol was pooled from 22 individuals from Gentest BD Biosciences (Heidelberg, Germany). All the other cytosols were prepared from tissue sample homogenization in an Ultra-Turrax (IKA Works, Inc., Wilmington, NC) in 10 mM HEPES, pH 7.4, 0.15 M potassium chloride, 1 mM sodium-EDTA, 1 mM DTT, 0.2 mM Pefablock SC (Roche Diagnostics, Mannheim, Germany), followed by 30-min centrifugation at 16,000*g*. The resulting supernatants were centrifuged for 45 min at 100,000*g*. Final supernatants were frozen at −80°C until use.

**DOX Reductase Assay.** Sixty micrograms of cytosol or 5 $\mu$g of recombinant protein was incubated with different concentrations of DOX (end concentration: 1, 10, 25, 50, 100, and 250 $\mu$M) in Tris/HCl (final concentration 30 mM, pH 7.4). After preincubation of 6 min at 37°C, the reaction was started by adding NADPH to a final concentration of 2 mM and a final volume of 100 $\mu$l. The protein concentration and incubation time were within the linear part of the appropriate reaction velocity curves (data not shown). After 30 min at 37°C, the reaction was stopped by adding 100 $\mu$l of ice-cold acetonitrile with daunorubicin as internal standard. The samples were centrifuged for 5 min, and the supernatant was diluted in the mobile phase up to 10-fold (depending on the DOX concentration) and chromatographed. The assays involving the inhibitor hydroxy-PP [dissolved in dimethyl sulfoxide (DMSO), final DMSO concentration 1%] and indomethacin (dissolved in ethanol, final ethanol concentration 1%) were performed in the same way at a single DOX concentration of 250 $\mu$M.

**Anthracycline High-Performance Liquid Chromatography Measurements.** Reversed-phase chromatography was carried out on a Merck column LiChroCART 125-4, LiCrospher 100 RP-8, 5 $\mu$m, protected by a LiChroCART 4-4, LiCrospher 100CN 5-$\mu$m guard column (Merck, Darmstadt, Germany). Isocratic elution was performed with freshly prepared filtered mobile phase consisting of 80:20 (v/v) mixture of 25 mM ammonium acetate buffer, pH 4.0/acetonitrile adjusted with acetic acid. The elution rate was 1.5 ml/min. Anthracyclines were detected fluorometrically with excitation at 480 nm and emission at 595 nm. Retention times (min) were 5.4 for DOX-OL, 9.5 for DOX, and 25.8 for daunorubicin, respectively. The intraday and interday variation coefficients were <10% for all three substances. The kinetic parameters of DOX reduction were calculated using SigmaPlot (Systat Software, Inc., Point Richmond, CA). The calculations of the $IC_{50}$ and $K_i$ in the experiments with hydroxy-PP were performed by nonlinear regression analysis using GraphPad PRISM (GraphPad Software Inc., San Diego, CA).

The enzymatic activity for menadione and 9,10-phenanthrenequinone was determined by measuring the decrease in absorbance at 340 nm in a total volume of 800 $\mu$l (100 mM Tris, pH 7.4 and 0.5 mM NADPH, 25°C). The reaction was started by the addition of 10 $\mu$g of enzyme. Menadione was measured at a concentration of 120 $\mu$M, and the reaction mixture contained 1% ethanol. Phenanthrenequinone was measured at a concentration of 36 $\mu$M, containing 10% DMSO.

**SDS/Polyacrylamide Gel Electrophoresis and Western Blot of CBR1.** SDS/polyacrylamide gel electrophoresis of 2 $\mu$g of human liver cytosol was

TABLE 1

*Kinetic parameters $K_m$ ($\mu M$), $V_{max}$ [pmol/(min · mg cytosolic protein)], and $V_{max}/K_m$ [$\mu$l/(mg cytosolic protein · min)] for DOX reduction to DOX-OL determined in the specified human tissues*

$V_{max}$ and $K_m$ data are presented as mean values ± S.E.M.

| | $K_m$ ± S.E.M. | $V_{max}$ ± S.E.M. | $V_{max}/K_m$ | No. of samples |
|---|---|---|---|---|
| | *$\mu M$* | *pmol/(mg · min)* | *$\mu$l/(mg · min)* | |
| Liver | 163 ± 21 | 337 ± 61 | 2.1 | 6 |
| Stomach | 132 ± 16 | 84 ± 18 | 0.6 | 6 |
| Colon | 140 ± 9 | 50 ± 12 | 0.4 | 4 |
| Kidney | 134 ± 18 | 127 ± 35 | 0.9 | 5 |
| Heart | 239 ± 18 | 56 ± 4 | 0.2 | 10 |
| Skeletal muscle | 244 ± 54 | 76 ± 8 | 0.3 | 2 |
| Lung | 231 ± 20 | 56 ± 15 | 0.2 | 4 |

TABLE 2

*Kinetic parameters $K_m$ ($\mu M$), $V_{max}$ [nmol/(min · mg)], $V_{max}/K_m$, $k_{cat}$ ($min^{-1}$), and catalytic efficiency ($k_{cat}/K_m$, min · mg) for DOX reduction to DOX-OL by the specified purified aldo-keto reductases and carbonyl reductases*

| | $K_m$ | $V_{max}$ | $V_{max}/K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|---|---|---|---|---|---|
| | *$\mu M$* | *nmol/(mg · min)* | *ml/(mg · min)* | *$min^{-1}$* | *l/(min · mM)* |
| AKR1A1 | 247 | 1.1 | 0.004 | 0.04 | 0.168 |
| AKR1B1 | | | Nonsaturated kinetics | | |
| AKR1B10 | 311 | 2.8 | 0.009 | 0.10 | 0.324 |
| AKR1C3 | 129 | 183.5 | 1.422 | 6.75 | 52.510 |
| AKR1C4 | 281 | 1.2 | 0.004 | 0.04 | 0.158 |
| CBR1 | 167 | 20.6 | 0.123 | 0.62 | 3.724 |
| AKR1C1 | N.D. | <0.02 | N.D. | N.D. | N.D. |
| AKR1C2 | N.D. | <0.02 | N.D. | N.D. | N.D. |
| CBR3 | N.D. | <0.02 | N.D. | N.D. | N.D. |

N.D., not determined.

carried out in 12% polyacrylamide resolving gels (Laemmli, 1970). Each gel contained a standard curve consisting of 25, 50, 75, and 100 ng of purified CBR1. Gels were transferred electrophoretically onto a polyvinylidene difluoride membrane using a Bio-Rad Mini TransBlot apparatus (Bio-Rad, München, Germany). Protein binding sites were blocked for 1 h in 10 mM Tris/154 mM NaCl/0.005% (v/v) Tween 20, pH 7.4 (buffer A), which contained 3% bovine serum albumin. The blots were incubated overnight with the CBR1 (1:20,000, Ab4148, Abcam, Cambridge, UK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, Sc-32233, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies in buffer A containing 1% bovine serum albumin at 4°C. After washing, bound antibodies were allowed to react for 1 h at room temperature with horseradish peroxidase secondary antibodies (1:20,000, anti-goat IgG Ab7132, Abcam; 1:20,000, anti-mouse IgG A9044, Sigma-Aldrich). The antibody complexes were detected by enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden) and visualized by exposure to hyperfilm (GE Healthcare). The relative quantities of Western blot bands were analyzed densitometrically with the software Clarity One (Bio-Rad).

**Quantitative Real-Time Polymerase Chain Reaction.** The mRNA copy numbers of AKR and CBR genes were evaluated via quantitative real-time polymerase chain reaction (PCR) on a Bio-Rad ICycler using a standard protocol. Taqman probes labeled with 6-carboxyfluorescein (5′reporter) and minor groove binder/nonfluorescent quencher (3′quencher) were purchased from Applied Biosystems (Darmstadt, Germany). Normalized cDNA from different human tissues (human multiple tissue cDNA panels I and II) were purchased from BD Biosciences (Heidelberg, Germany), and 2.5 ng of cDNA was used with the TaqMan Universal PCR master mix (Applied Biosystems). Serial dilutions (10 to 1 million copies) of plasmids containing target portions of the genes to be analyzed were used to create calibration curves for the individual genes. All the assays were done in triplicate.

**Sequencing.** Sequencing of PCR-amplified *CBR1* exons and of the 5′ regulatory region was performed using PCR primers and dye terminator chemistry (Applied Biosystems). Sequences of primers used for PCR amplification and sequencing are provided in the Supplemental Material. Results were visually inspected with Genome Assembly Program 4 (http://staden.sourceforge.net).

**Statistics.** Statistical calculations were performed with SPSS (SPSS Inc., Chicago, IL). $K_m$ and $V_{max}$ values were calculated with Michaelis-Menten equation using SigmaPlot (Systat Software, Inc.)

## Results

**DOX Reduction by Cytosols from Different Human Organs.** The reduction of DOX to DOX-OL was detected in cytosols from all seven human organs investigated (Table 1). There appear to be at least two distinct DOX reductases, with apparent $K_m$ values of ~140 and ~240 $\mu$M. The highest $V_{max}$ and clearance values were observed in cytosols from livers and kidneys, followed by the gastrointestinal tract tissues stomach and colon, all of which exhibit higher affinity toward DOX. Similarly to lung and skeletal muscle, heart cytosols express a lower affinity reductase.

**DOX Reduction by AKRs and CBRs.** Nine purified AKRs and CBRs were tested for their ability to reduce DOX to DOX-OL. No DOX reduction was detected in the absence of any enzyme and NADPH. AKR1C1, AKR1C2, and CBR3 showed no or very little (<0.02 nmol/min/mg) production of DOX-OL at 100 $\mu$M DOX (Table 2). Moderate conversion ($V_{max}$ values 1.1–2.8 nmol/min/mg) (Fig. 1; Table 2) was observed with AKR1A1, AKR1B10, and AKR1C4. $K_m$ values for these three enzymes were >240 $\mu$M. AKR1B1 showed no saturation up to 250 $\mu$M DOX. In consequence, neither $V_{max}$ nor $K_m$ value could be calculated for AKR1B1, but its specific activity at 100 $\mu$M DOX was similar to that of AKR1A1 (data not shown). The highest $V_{max}$ values, the lowest $K_m$ values, and hence the highest intrinsic clearance values were found in CBR1 and AKR1C3 (Table 2). Likewise, AKR1C3 and CBR1 had the highest turnover number ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$) values (Table 2).

**Tissue Expression of Transcripts Encoding AKRs and CBRs.** The mRNA expression level was determined for the nine cytosolic AKRs and CBRs via real-time PCR (Fig. 2; Supplemental Material). According to the maximal transcript expression in any organ, the enzymes may be divided into three groups: CBR3, AKR1B10, AKR1C2, and AKR1C3 express <5000, whereas CBR1, AKR1A1, AKR1B1, and AKR1C1 express between 5000 and 200,000 transcripts/ng cDNA. AKR1C4 shows the highest expression, with 1.4 million transcripts/ng cDNA detected exclusively in the liver. Prominent expression in organs with the highest DOX-reducing activity (i.e., liver and kidney) (Table 1) was found for CBR1, AKR1A1, and AKR1C3 (Fig. 2; Supplemental Material).

**Measurements with the Inhibitor Hydroxy-PP.** The above experiments pointed to CBR1 and AKR1C3 as candidates for the high affinity DOX reductase detected in the cytosol of the liver and kidney. This was based first on the particularly high expression of CBR1 and AKR1C3 mRNA in these organs (Fig. 2). Second, CBR1 and AKR1C3 showed $K_m$ values very similar to those found in human liver and kidney cytosols (compare Tables 1 and 2). However, whereas AKR1C3 exhibited almost 12-fold higher molecular clearance (Table 2), its hepatic expression level, at least on the mRNA level, was 50-fold lower compared with CBR1. To further differentiate between the contributions of CBR1 and AKR1C3 to the hepatic DOX reduction, we applied to these enzymes and to a human liver cytosol pooled from 22 individuals hydroxy-PP, recently described as a specific inhibitor of CBR1 (Tanaka et al., 2005). The $IC_{50}$ value for menadione metabolism by CBR1 (1.3 $\mu$M, data not shown) was similar to 0.8 $\mu$M reported by Tanaka et al. (2005). Unexpectedly, besides CBR1, hydroxy-PP inhibited also AKR1C3. The $K_i$ values were 1.4 $\mu$M for AKR1C3, 10.5 $\mu$M for CBR1, and 9.9 $\mu$M for human liver cytosol, respectively. The inhibition curves of CBR1 and human liver cytosol were nearly congruent, consistent with CBR1 being the main hepatic DOX reductase (Fig. 3).

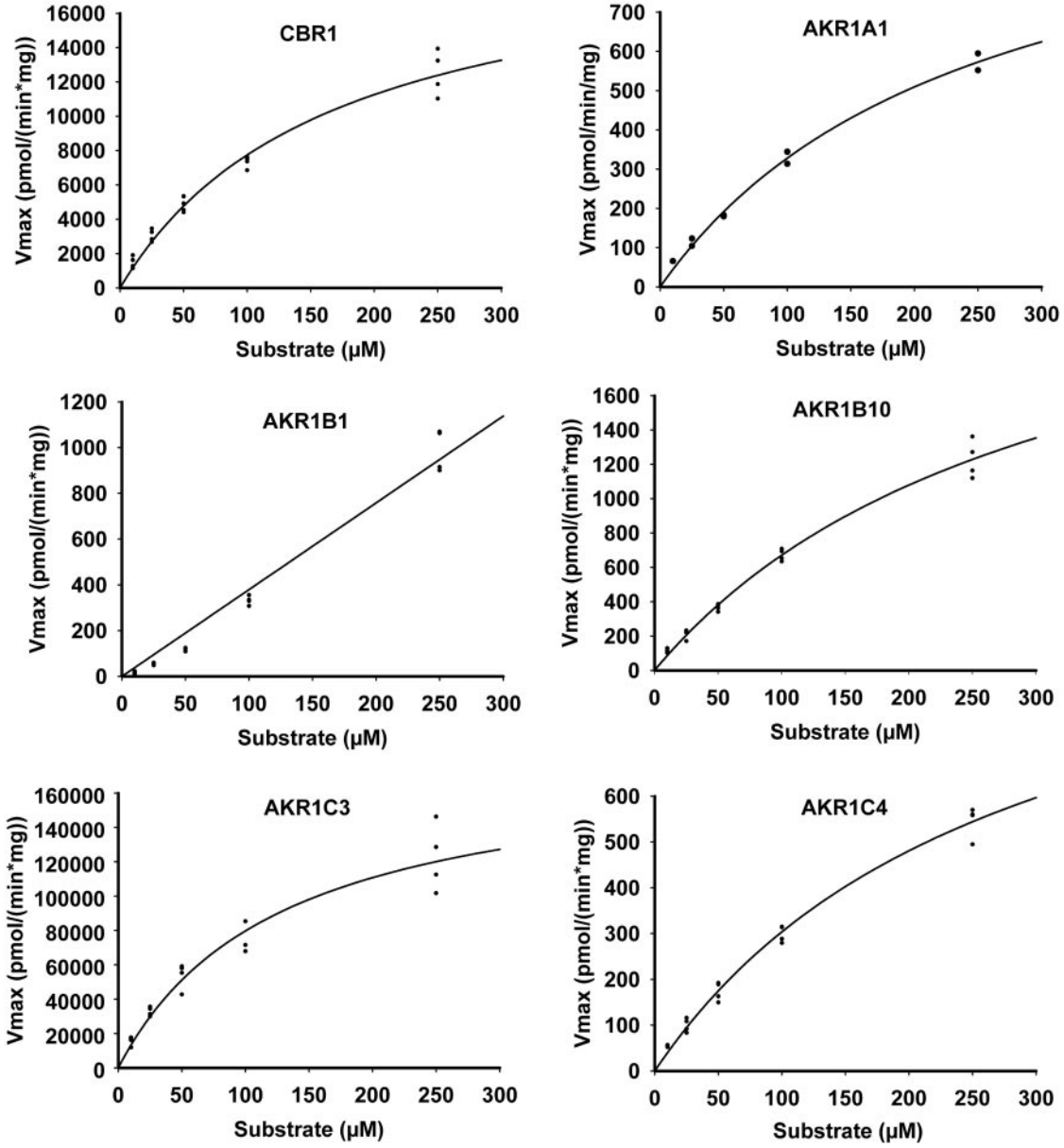


FIG. 1. Kinetics of DOX reduction to DOX-OL for recombinant enzyme preparations of CBR1, AKR1A1, AKR1B1, AKR1B109, AKR1C3, and AKR1C4. Five micrograms of the recombinant proteins was incubated with different concentrations of DOX (end concentration: 1, 10, 25, 50, 100, and 250 $\mu$M). After 30 min the reaction was stopped, and the amount of produced DOX-OL was determined by HPLC.

To achieve additional differentiation between CBR1 and AKR1C3, the reduction of DOX to DOX-OL by the same pooled liver cytosol was investigated in the presence of the specific inhibitor of AKR1C3, indomethacin (Byrns et al., 2008). The inhibition of the reaction was $<$10% at 50 $\mu$M indomethacin, and it was identical with that observed with the purified CBR1. At 50 $\mu$M, indomethacin inhibits the reduction of various AKR1C3 substrates by $>$80% (Byrns et al., 2008). This result is in agreement with the hydroxyl-PP inhibition experiment shown in Fig. 3 and supports the above conclusion that DOX is reduced predominantly by CBR1.

**Interindividual Variability in the Expression of CBR1 and in DOX Reduction among 80 Human Liver Biopsies.** CBR1 shows a triple band in Western blot (Fig. 4A) as a result of the binding of 2-oxocarbonyl acids such as pyruvate and 2-oxoglutarate to lysine 239. This modification reflects the metabolic state of the cell and has no apparent effect on CBR1 activity (Krook et al., 1993a,b; Wermuth et al., 1993). Western blot analysis of all three bands taken together revealed a large variability in CBR1 expression among cytosols from 80 human liver biopsies. CBR1 protein was detected in every liver, indicating the absence of frequent null alleles. There was a 320-fold difference in CBR1 expression between the highest and lowest sample, but this was reduced to 70-fold when the highest sample was removed. The largest difference in CBR1 expression between two samples in an individual Western blot was 62-fold.

The $V_{max}$ of DOX reduction measured in the same cytosols showed a 22-fold variation [mean value 554, range 131-2907 pmol/(min · mg)] (Fig. 5). Three years after the initial kinetic determination, the velocity of DOX reduction was replicated in all the samples at a single DOX concentration of 250 $\mu$M. The correlation ($r^2$) between the $V_{250}$ values from both measurements was 0.91 ($P < 0.001$), although the $V_{250}$ values from the second measurement were reduced by 50% (data not shown). When CBR1 protein expression was compared with DOX

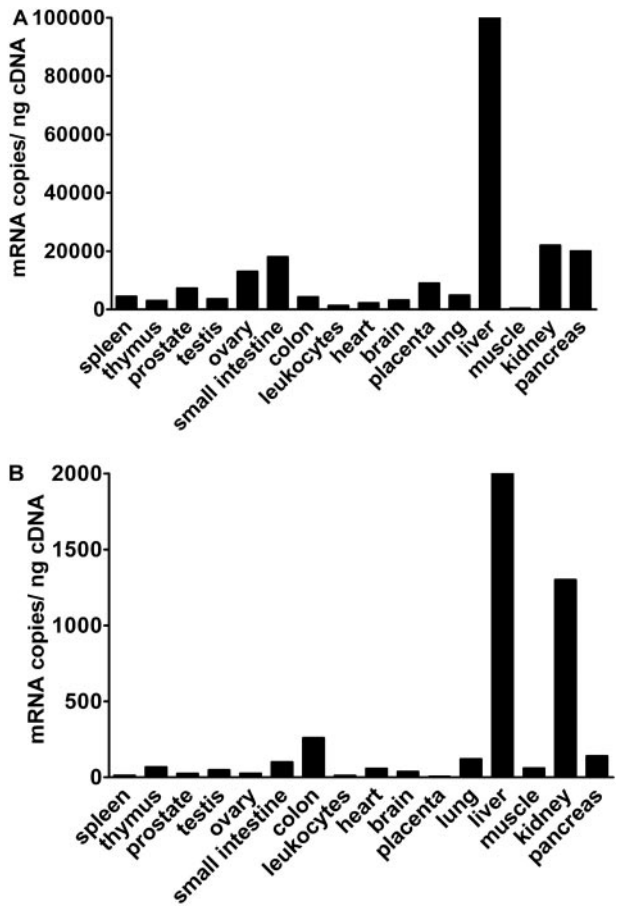


FIG. 2. mRNA expression of CBR1 (A) and AKR1C3 (B) in different human tissues.

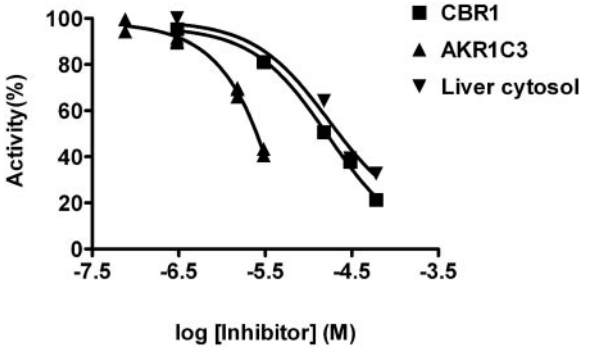


FIG. 3. The effect of hydroxy-PP on the DOX reduction to DOX-OL by CBR1, AKR1C3, and liver cytosol. Sixty micrograms of cytosol or 5 $\mu$g of recombinant protein was incubated with 250 $\mu$M DOX and with different concentrations of hydroxy-PP (an inhibitor of CBR1 and AKR1C3). After 30 min the reaction was stopped, and the amount of produced DOX-OL was determined by HPLC.

reduction, the correlation was statistically significant ($P < 0.05$) in six of nine individual Western blots, with correlation coefficients ($r$) for these six blots between 0.65 and 0.97 (Fig. 4B). There was no statistically significant correlation within the entire set, most likely reflecting the considerable interexperimental variability of Western blot. There was no evidence of protein polymorphisms affecting DOX affinity when considering $K_m$ values (data not shown).

***CBR1* Gene Variability and Its Effect on DOX to DOX-OL Reduction in Liver Biopsies.** The *CBR1* exons (including the 5′- and 3′-untranslated region) and 2-kilobase sequence upstream of exon 1

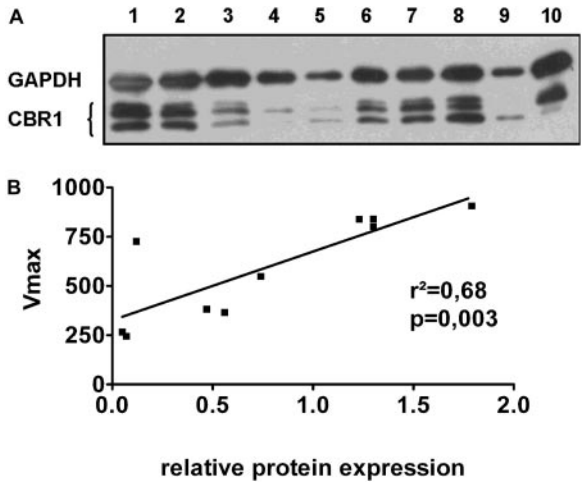


FIG. 4. A, Western blot of 10 different human hepatic cytosols probed with CBR1- and GAPDH-specific antibodies. B, correlation between the GAPDH-normalized CBR1 expression obtained from A with the corresponding $V_{max}$ values of DOX reduction to DOX-OL. The hepatic cytosols were incubated with different concentrations of DOX (end concentrations: 1, 10, 25, 50, 100, and 250 $\mu$M). After 30 min the reaction was stopped, and the amount of DOX-OL was determined by HPLC.

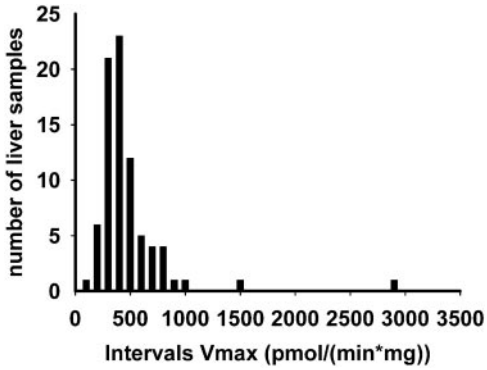


FIG. 5. Distribution of $V_{max}$ values of DOX reduction to DOX-OL in 80 liver samples. The samples were incubated with DOX (end concentrations: 1, 10, 25, 50, 100, and 250 $\mu$M). DOX-OL was detected by HPLC.

were sequenced in DNA samples corresponding to 57 of the liver cytosols investigated above. Eleven polymorphisms were detected, including a novel indel variant (Table 3). The variant comprises 31 base pairs (chr21:36,364,127–36,364,157; hg18 of the human genome assembly) of the promoter-associated CpG island. Therefore, this variant, found only in one heterozygous and one homozygous sample, was excluded from further haplotype and association calculations. Nevertheless, the $V_{max}$ values of DOX reduction in these two samples [655 and 243 pmol/(min · mg), respectively] were inconspicuous compared with other samples. Rs9024 is located within the CBR1 polyadenylation site, whereas the remaining nine gene variants are silent single-nucleotide polymorphisms (SNPs) within the CBR1 protein coding region. No SNP showed association with $V_{max}$ of DOX reduction or with CBR1 protein expression. A similar result (Kruskal-Wallis test $>0.05$) was obtained when SNPs were converted into haplotypes, with the nine most common haplotypes (Table 3) accounting for 82% of all the haplotypes in this sample set.

## Discussion

We report that DOX reduction in human tissues is catalyzed by at least two different enzymes and identify CBR1 as a predominant hepatic DOX reductase. Following an i.v. DOX bolus, liver achieves

TABLE 3

*CBR1 gene variants and the resulting 9 most frequent haplotypes determined by sequencing of 57 DNA samples*

Bonferroni-corrected significance level for compliance with Hardy-Weinberg equilibrium ($\chi^2$ test) was set at <0.005. Bold print indicates differences in comparison with the haplotype H1.

| SNP No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| rs No. | N.A. | 2239859 | 1005696 | 1005695 | 2835265 | 2835266 | 20572 | 2230192 | 9024 | 998384 | 998383 |
| B (= minor) allele freq. | 0.03 | 0.44 | 0.49 | 0.43 | 0.18 | 0.03 | 0.18 | 0.01 | 0.21 | 0.35 | 0.35 |
| Genotypes AA, AB, BB | 55, 1, 1 | 24, 16, 17 | 18, 22, 17 | 19, 27, 11 | 41, 11, 5 | 54, 3, 0 | 41, 11, 5 | 56, 1, 0 | 38, 14, 5 | 25, 24, 8 | 25, 24, 8 |
| $\chi^2$ | <0.001 | 0.005 | 0.23 | 0.97 | 0.03 | 0.98 | 0.03 | 1 | 0.14 | 0.85 | 0.85 |
| Haplotypes (frequency) | | | | | | | | | | | |
| H1 (25.4%) | | T | T | G | C | G | C | G | G | G | C |
| H2 (22.3%) | | **G** | **G** | **C** | C | G | C | G | G | **C** | **G** |
| H3 (13.5%) | | T | T | G | **T** | G | **T** | G | **A** | G | C |
| H4 (5.1%) | | **G** | **G** | **C** | C | G | C | G | G | G | C |
| H5 (4.8%) | | T | **G** | **C** | C | G | C | G | G | **C** | **G** |
| H6 (3.1%) | | **G** | **G** | G | C | G | C | G | G | G | C |
| H7 (2.8%) | | T | **G** | G | C | G | C | G | G | G | C |
| H8 (2.6%) | | **G** | **G** | **C** | C | **A** | C | G | G | G | C |
| H9 (2.5%) | | **G** | T | G | C | G | C | G | G | G | C |

N.A., not available.

the highest DOX concentrations of all the organs studied thus far (Lee et al., 1980). Considered together with its size and the high DOX intrinsic clearance by the hepatic cytosol (Table 1), liver appears to be the most important organ in DOX metabolism, followed by kidney and the gastrointestinal tract, represented by stomach and colon. Of the known NADPH-dependent, cytosolic CBRs (Rosemond and Walsh, 2004), all but one (xylulose reductase) were investigated in the present study in the form of recombinant enzymes as candidates for the hepatic DOX reductase. Altogether, six enzymes (CBR1, AKR1A1, AKR1B1, AKR1B10, AKR1C3, and AKR1C4) were capable of DOX reduction. The conclusion that CBR1 is a predominant DOX reductase in the human liver is based on several lines of evidence: first, the $K_m$ of DOX reduction is almost identical for CBR1 and human liver cytosol. Second, CBR1 is prominently expressed in the liver. Third, the expression of CBR1 correlates with the $V_{max}$ of DOX reduction in a majority of Western blot analyses of a large set of liver biopsies. Last and most importantly, the inhibition constants determined using the CBR1 inhibitor hydroxy-PP are identical for CBR1 and human liver cytosol.

Admittedly, two of these characteristics (hepatic expression and $K_m$ value ~140 $\mu$M) also apply to AKR1C3. The crucial argument against AKR1C3 being the principal hepatic DOX reductase is its $K_i$ value determined with hydroxy-PP (1.4 $\mu$M), which is much lower than $K_i$ values found for CBR1 (10.5 $\mu$M) and for human liver cytosol (9.9 $\mu$M). If AKR1C3 were a major hepatic DOX-reducing enzyme, the $K_i$ of human liver cytosol would be expected to be similarly low. The minor role of AKR1C3 in the hepatic DOX reduction is also consistent with the relatively low expression level of AKR1C3 transcripts in human organs. AKR1C3 could play a role in DOX reduction in individuals with no or very low hepatic CBR1 expression. However, CBR1 protein was found expressed in all the human liver samples investigated. The indispensability of CBR1 expression and function is in agreement with the paucity of nonsynonymous *CBR1* gene variants detected in the present study and with the lethal phenotype of *CBR1* deletion in the mouse (Olson et al., 2003). On the other hand, it should be cautioned that the activity of AKR1C3 may have been underestimated. Indeed, interenzyme comparisons such as the one presented in our work may be confounded by enzyme tagging (Bains et al., 2008), as well as by purification, reconstitution, and reaction conditions.

Previous measurements (Lovless et al., 1978) of the hepatic and renal DOX reduction resulted in similar $V_{max}$ values, whereas the $K_m$ values were even higher (275 and 539 $\mu$M, respectively). However, these $K_m$ (Lovless et al., 1978) values were determined in single samples of each organ using thin-layer chromatography, which is a less precise technique than high-performance liquid chromatography (HPLC). Furthermore, the accuracy of the $K_m$ estimation may have suffered from a much smaller DOX concentration range (up to 100 $\mu$M) (Lovless et al., 1978), whereas $K_m$ values in this range may seem irrelevant, considering the typical range of plasma DOX concentration (0.1–1 $\mu$M). However, it should be taken into account that intracellular DOX concentrations are many -fold higher as a result of extensive accumulation (Minotti et al., 2004).

Heart, skeletal muscle, and lung express one or several other DOX reductases, with an apparent $K_m$ value of ~240 $\mu$M. Interestingly, the heart exhibits the third-lowest $V_{max}$ of DOX reduction of 10 human organs investigated. This is in agreement with the observation that DOX-OL levels in the heart are not higher than in other organs (Stewart et al., 1993). Therefore, particular sensitivity of the heart to DOX-induced toxicity may be related not to a particularly high formation or accumulation of DOX-OL but rather to higher sensitivity to DOX-OL. Regarding the molecular identity of the DOX-reducing enzyme in the heart, AKR1B10 unlikely plays a role, as judged from the low level of transcripts and from the much higher apparent $K_m$ value (311 $\mu$M). AKR1C4 is expressed exclusively in the liver, whereas the AKR1B1 has nonsaturated kinetics. This leaves AKR1A1 as the best candidate for the principal cardiac DOX reductase and is indeed in excellent agreement with the apparent $K_m$ (247 $\mu$M), as well as with the expression pattern of this enzyme. This is also in agreement with Mordente et al. (2003), who proposed AKR1A1 to be the human cardiac DOX reductase based on inhibition studies in human heart cytosol, although the apparent $K_m$ value of this reaction (79 $\mu$M) was 3-fold lower than in our hands (Salvatorelli et al., 2006). Besides heart, AKR1A1 is also prominently expressed in the liver. The absence from the liver of a DOX-reducing activity with an apparent $K_m$ of 247 $\mu$M may be explained by the low activity of AKR1A1 combined with the simultaneous expression of CBR1, which has a higher affinity toward DOX.

The daunorubicin-metabolizing enzymes AKR1C1 (O'Connor et al., 1999) and AKR1C2 (Ohara et al., 1995) exhibited very low to no activity toward DOX, indicating a high stereospecificity of anthracycline reduction. Likewise, no DOX reduction was catalyzed by CBR3. The substrate spectrum and the physiological importance of this enzyme are poorly characterized and partly controversial. Thus, although DOX reduction was not detectable with CBR3, the same protein batch reduced 9,10-phenanthrenequinone but not menadione (H. J. Martin, unpublished observations). Taken together, with the very low expression of CBR3 transcripts (Supplemental Material),

these results argue against any major role of this isozyme in drug disposition.

The hepatic CBR1 expression level exhibits a substantial variability, which is paralleled by the variability in the individual activity of DOX reduction. A statistically significant correlation has been found between DOX reduction and CBR1 expression in six of nine individual Western blots. The variability in the expression and activity of CBR1 is in agreement with the recent report of interindividual differences in the hepatic metabolism of the CBR1 substrate menadione (Covarrubias et al., 2006). The interindividual differences in CBR1 expression and activity may contribute to the variability of DOX and DOX-OL reduction in cancer patients. As already stated, the variable pharmacokinetics of DOX and DOX-OL affects both the individual risk of cardiotoxicity and also the tumor response. An appraisal of the clinical importance of CBR1 variability would benefit from the availability of surrogate markers of its activity. The variability in the expression and activity of many drug-metabolizing enzymes is partly determined by the individual genetic background. However, in an attempt restricted to the protein-coding region of CBR1 and its proximal promoter sequences, we failed to detect frequent germline gene variants naturally occurring in the human population associating with CBR1 expression or DOX reductase activity. The responsible gene variants may be instead located in proteins regulating CBR1 expression. Alternatively, the CBR1 expression variability may reflect the individual induction status by xenobiotics. The human *CBR1* promoter undergoes induction by xenobiotics, which is mediated by the aryl hydrocarbon receptor (Lakhman et al., 2007). Modulation of CBR1 expression by nongenetic factors would be consistent with the intraindividual differences in DOX pharmacokinetics observed in one third of patients treated with DOX at an interval of 4 weeks (Palle et al., 2006). Inducibility of CBR1 by environmental factors would be consistent with the prominent expression of CBR1 in organs involved in drug disposition (liver, kidney, gastrointestinal tract) and with the catalytic activity of CBR toward numerous xenobiotics (Rosemond and Walsh, 2004; Hoffmann and Maser, 2007).

Pharmacological modulation of enzymes metabolizing oncological drugs is being explored as a strategy to improve outcomes of cancer therapies (Scripture et al., 2005). Through reduction of DOX pharmacokinetic variability, CBR1 inhibition could improve the therapeutic response to DOX and reduce its side effects. Although this strategy remains valid, it should be emphasized that the recently developed inhibitor of CBR1, hydroxy-PP (Tanaka et al., 2005), may be less specific than originally assumed, as evidenced by its inhibition of AKR1C3. Finally, the original target of hydroxy-PP, CBR1, appears to play no major role in DOX reduction in the human heart, as judged from the kinetic measurements of cardiac cytosol.

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