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CBR1 is a predominant doxorubicin reductase in the human liver*

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non-standard abbreviations: CBR1, carbonyl reductase 1; DOX, doxorubicin; DOX-OL, doxorubicinol; AKR, aldo-keto reductases; AUC, area under the curve; hydroxy-PP, 4-amino-1-tert-butyl-3-(2-hydroxyphenyl)pyrazolo[3,4-d]pyrimidine; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylene diamine tetraacetic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; IC_{50} , half maximal inhibitory concentration; K_i , enzyme inhibition dissociation constant; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF-membrane, polyvinylidene fluoride-membrane; BSA, bovine serum albumin; PCR, polymerase chain reaction; V_{max} ,maximum reaction velocity

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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). Since 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 inter-individual differences in their activities. Human tissues express at least two DOX-reducing enzymes. High-clearance organs (kidney, liver, and the gastro-intestinal tract) express an enzyme with an apparent K_m of ~140 μM. Out of 6 enzymes found to reduce DOX, K_m values in this range are exhibited by CBR1 and AKR1C3. CBR1 is expressed in these three organs at higher levels than AKR1C3, while 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 for 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-fold and 22-fold, respectively, but showed no association with *CBR1* gene variants found in these samples. Instead, the inter-individual 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.

Introduction

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 inter- and intra-patient differences (Frost et al., 2002; Palle et al., 2006), which may be important both for the individual anti-tumor response and for side effects of DOX such as cardiotoxicity. Individual plasma concentrations and 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 inter-individual 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 due to the saturation of biliary excretion (Wihlm et al., 1997). However, the DOX-OL/DOX quotient is very variable (range 0 to 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 which 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 anti-tumor 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 CBR1 expressed as a mouse transgene (Forrest et al., 2000). Furthermore, catalytic activity was demonstrated for AKR1A1 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, are known. Therefore, we investigated the kinetics of DOX reduction of 7 cytosolic aldo-keto reductases and of 2 carbonyl reductases, since 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. Since liver achieves highest DOX concentrations of all 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

Doxorubicin and daunorubicin were purchased from Pfizer (Karlsruhe, Germany). Doxorubicinol was kindly provided by Dr. A. Andersen, Clinical Pharmacology Section, The Norwegian Radium Hospital Oslo, Norway. Stock solutions were prepared with double distilled water and aliquots 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. (Tanaka et al., 2005) by M. Perscheid and Prof. Nubbemeyer in the Department of Organic Chemistry, Johannes Gutenberg University of Mainz, Germany. The structure was confirmed by ^{1}H and ^{13}C NMR and by mass spectrometry. Acetonitrile was purchased from VWR (Darmstadt, Germany), NADPH tetrasodium salt from Sigma-Aldrich (Taufkirchen, Germany). All other substances were purchased from Applichem (Darmstadt, Germany).

Biological material

CBR1, CBR3, AKR1B1, 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), AKR1C1, AKR1C2, AKR1C3, AKR1C4 expressed and purified in the Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, USA (Burczynski et al., 1998). GST-tagged AKR1A1 was purchased from Abnova (Taiwan).

Myocardial biopsies were obtained by Dr. G. Reinerth from patients undergoing aorto-coronary bypass grafting at the Department of Cardiothoracic and Vascular Surgery, Johannes-Gutenberg-University of Mainz, Germany. Samples of kidney, liver, muscle, colon, stomach and lung used to determine the kinetics of DOX reduction were kindly provided by

S. Schäfer and Prof. S. Biesterfeld from the Department of Pathology, University of 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 in Berlin, Germany, as described (Wolbold et al., 2003). Normal liver tissue surrounding primary liver tumours or liver metastases was resected and used to prepare cytosols. Written informed consent was obtained from all donors. Removal and usage of all tissue samples was approved by the responsible ethics committees.

Preparation of cytosolic fractions

Human small intestine cytosol was purchased from Biopredic (Rennes, France) and liver cytosol pooled from 22 individuals from Gentest BD Biosciences (Heidelberg, Germany). All other cytosols were prepared from tissue samples homogenization in an ultra turrax in 10 mM HEPES, pH 7.4, 0.15 M potassium chloride, 1 mM sodium-EDTA, 1 mM DTT, 0.2 mM pefablock SC, followed by 30 min centrifugation at 16000g. The resulting supernatants were centrifuged for 45 min at 100000g. Final supernatants were frozen at -80 °C until use.

Doxorubicin reductase assay

Sixty µg cytosol or 5 µg recombinant protein were incubated with different concentrations of DOX (end concentration: 1, 10, 25, 50, 100, and 250 µM) in Tris/HCl (final concentration 30 mM, pH 7.4). After pre-incubation of 6 min at 37^{o}C, the reaction was started by adding NADPH to a final concentration of 2 mM and a final volume of 100 µl. 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^{o}C, the reaction was stopped by adding 100 µl ice-cold acetonitrile with daunorubicin as internal standard. The samples were

centrifuged for 5 min, the supernatant was diluted in the mobile phase up to 10-fold (depending on the doxorubicin concentration) and chromatographed. The assays involving the inhibitor hydroxy-PP (dissolved in 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 µM.

Anthracycline HPLC measurements

Reversed-phase chromatography was carried out on a Merck column LiChroCART 125-4, LiCrospher 100 RP-8, 5µm, protected by a LiChroCART 4-4, LiCrospher 100CN 5µm 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 fluorimetrically 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 intra- and inter-day variation coefficients were <10% for all three substances. The kinetic parameters of DOX reduction were calculated using Sigma Plot (Systat). The calculations of the IC_{50} and K_i in the experiments with hydroxy-PP were performed by nonlinear regression analysis using Graphpad PRISM.

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 µl (100 mM Tris, pH 7.4 and 0.5 mM NADPH, 25 °C). The reaction was started by the addition of 10 µg enzyme. Menadione was measured at a concentration of 120 µM, the reaction mixture contained 1 % ethanol. Phenanthrenequinone was measured at a concentration of 36 µM, containing 10 % DMSO.

SDS/PAGE and Western Blot of CBR1

SDS/PAGE of 2 µg human liver cytosol was carried out in 12% polyacrylamide resolving gels (Laemmli, 1970). Each gel contained a standard curve consisting of 25, 50, 75, 100 ng of purified CBR1. Gels were transferred electrophoretically onto a PVDF-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% BSA. The blots were incubated overnight with the CBR1 (1:20000, Ab4148, Abcam, Cambridge, UK) and GAPDH (1:5000, Sc-32233, Santa Cruz, USA) antibodies in Buffer A containing 1% BSA at 4°C. After washing, bound antibodies were allowed to react for 1 h at room temperature with horse-raddish peroxidase secondary antibodies (1:20000, anti goat IgG Ab7132, Abcam, Cambridge, UK; 1:20000, anti mouse IgG A9044, Sigma-Aldrich, Taufkirchen, Germany). The antibody complexes were detected by enhanced chemiluminescence (ECL, GE Healthcare, Uppsala, Germany) and visualized by exposure to hyperfilm (GE Healthcare, Uppsala, Germany). The relative quantities of Western blot bands were analyzed densitometrically with the software Clarity One (Bio-Rad, München, Germany).

Quantitative real-time PCR

The mRNA copy numbers of AKR and CBR genes were evaluated via quantitative real-time PCR on a BioRad ICycler (München, Germany) using a standard protocol. Taqman probes labelled with 6-carboxyfluorescein (FAM; 5'reporter) and MGB-NFQ (3'quencher) were purchased from Applied Biosystems (Darmstadt, Germany). Normalized cDNA from different human tissues (Human MTC Panels I and II) were purchased from BD Biosciences (Heidelberg, Germany). 2.5ng cDNA were used with the TaqMan Universal PCR master mix (Applied Biosystems, Darmstadt, Germany). Serial dilutions (10 to 1,000,000 copies) of

plasmids containing target portions of the genes to be analyzed were used to create calibration curves for the individual genes. All 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 3730, Darmstadt, Germany). Sequences of primers used for PCR amplification and sequencing are provided in Supplementary Material. Results were visually inspected with GAP4.

Statistics

Statistical calculations were performed with SPSS. K_m and V_{max} values were calculated with Michaelis-Menten equation using SigmaPlot.

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Results

DOX reduction by cytosols from different human organs

The reduction of DOX to DOX-OL was detected in cytosols from all 7 human organs investigated (Table 1). There appear to be at least two distinct doxorubicin reductases, with the apparent K_m values of ~140 and ~240 μM. The highest V_{max} and clearance values were observed in cytosols from livers and kidneys, followed by the gastro-intestinal tract tissues stomach and colon, all of which exhibit higher affinity towards DOX. Similarly to lung and skeletal muscle, heart cytosols express a lower affinity reductase.

DOX reduction by aldo-keto reductases and carbonyl reductases

Nine purified aldo-keto reductases and carbonyl reductases 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^{-1} mg^{-1}) production of DOX-OL at 100 μM DOX (Table 2). Moderate conversion (V_{max} values 1.1 - 2.8 nmol min^{-1} mg^{-1}; Fig. 1 and Table 2) was observed with AKR1A1, AKR1B10 and AKR1C4. K_m values for these 3 enzymes were >240 μM. AKR1B1 showed no saturation up to 250 μM DOX. In consequence, neither V_{max} nor K_m value could be calculated for AKR1B1, but its specific activity at 100 μM 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 aldo-keto reductases and carbonyl reductases

The mRNA expression level was determined for the 9 cytosolic aldo-keto reductases and carbonyl reductases via real-time PCR (Fig. 2 and supplementary material). According to the

maximal transcript expression in any organ, the enzymes may be divided into 3 groups: CBR3, AKR1B10, AKR1C2, and AKR1C3 express <5,000, whereas CBR1, AKR1A1, AKR1B1, and AKR1C1, between 5,000 and 200,000 transcripts/ng cDNA. AKR1C4 shows the highest expression, with 1,400,000 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 and supplementary 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 firstly on the particularly high expression of CBR1 and AKR1C3 mRNA in these organs (Fig. 2). Secondly 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 in comparison to 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 µM, data not shown) was similar to 0.8 µM reported by Tanaka and colleagues (Tanaka et al., 2005). Unexpectedly, besides CBR1, hydroxy-PP inhibited also AKR1C3. The K_i-values were 1.4 µM for AKR1C3, 10.5 µM for CBR1, and 9.9 µM 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).

To achieve additional differentiation between CBR1 and AKR1C3, the reduction of doxorubicin to doxorubicinol 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 μM indomethacin and it was identical with that observed with the purified CBR1. At 50μM, 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 doxorubicin is reduced predominantly by CBR1.

Inter-individual 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), due to 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; Krook et al., 1993b; Wermuth et al., 1993). Western blot analysis of all 3 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 to 2907 pmol/(min*mg)) (Fig. 5). Three years after the initial kinetic determination, the velocity of DOX reduction was replicated in all samples at a single DOX concentration of 250 μM. 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

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were reduced by 50% (data not shown). When CBR1 protein expression was compared to DOX reduction, the correlation was statistically significant ($P<0.05$) in 6 out of 9 individual Western blots, with correlation coefficients (r) for these 6 blots between 0.65 and 0.97 (Fig. 4B). There was no statistically significant correlation within the entire set, most likely reflecting the considerable inter-experimental 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'-UTR) and 2 kb sequence upstream of exon 1 were sequenced in DNA samples corresponding to 57 of the liver cytosols investigated above. A total of 11 polymorphisms were detected, including a novel indel variant (Table 3). The variant comprises 31 bp (chr21:36,364,127-36,364,157; hg18 of the human genome assembly) of the promoter-associated CpG island. This variant, found only in one heterozygous and one homozygous sample was therefore 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 in comparison to other samples. Rs9024 is located within the CBR1 polyadenylation site, whereas the remaining 9 gene variants are silent 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 nine most common haplotypes (Table 3) accounting for 82% of all 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 intravenous DOX bolus, liver achieves highest doxorubicin concentrations of all 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 gastro-intestinal tract represented by stomach and colon. Of the known NADPH-dependent, cytosolic carbonyl reductases (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, 6 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: firstly, the K_m of DOX reduction is almost identical for CBR1 and human liver cytosol. Secondly, CBR1 is prominently expressed in the liver. Thirdly, 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. Lastly 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 μM) 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 μM), which is much lower than K_i values found for CBR1 (10.5 μM) and for human liver cytosol (9.9 μM). If AKR1C3 was 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 none or very low hepatic CBR1 expression. However, CBR1 protein was found expressed in all human liver samples investigated. The indispensability of CBR1 expression and function is in agreement with the paucity of non-synonymous *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, inter-enzyme 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 µM, 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 HPLC. Furthermore, the accuracy of the K_m estimation may have suffered from a much smaller DOX concentration range (up to 100 µM) (Lovless et al., 1978). While Km values in this range may seem irrelevant, considering the typical range of plasma DOX concentration (0.1 µM – 1µM). However, it should be taken into account that intracellular DOX concentrations are many fold higher, due to 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 µM. 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). The

particular sensitivity of the heart to DOX-induced toxicity may be therefore 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 µM). AKR1C4 is expressed exclusively in the liver, whereas the AKR1B1 has a non-saturated 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 µM) as well as with the expression pattern of this enzyme. This is also in agreement with Minotti and colleagues, who proposed AKR1A1 to be the human cardiac DOX reductase based on inhibition studies in human heart cytosol (Mordente et al., 2003), although the apparent K_m value of this reaction (79 µM) 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 µM may be explained by the low activity of AKR1A1 combined with the simultaneous expression of CBR1, which has a higher affinity towards DOX.

The daunorubicin-metabolizing enzymes AKR1C1 (O'Connor et al., 1999) and AKR1C2 (Ohara et al., 1995) exhibited very low to none activity towards 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, while DOX reduction was not detectable with CBR3, the same protein batch reduced 9,10-phenanthrenequinone, although not menadione (H.J. Martin, unpublished observations). Taken together, with the very low expression of CBR3 transcripts (supplementary 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. Statistically significant correlation has been found between DOX reduction and CBR1 expression in 6 out of 9 individual Western blots. The variability in the expression and activity of CBR1 is in agreement with the recent report of inter-individual differences in the hepatic metabolism of the CBR1 substrate menadione (Covarrubias et al., 2006). The inter-individual 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, but 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 non-genetic factors would be consistent with the intra-individual differences in DOX pharmacokinetics observed in 1/3 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, gastro-intestinal tract) and with

the catalytic activity of CBR towards 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 to reduce its side-effects. While 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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Footnotes

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Legends for figures

Figure 1. Kinetics of DOX reduction to DOX-OL for recombinant enzyme preparations of CBR1, AKR1A1, AKR1B1, AKR1B109, AKR1C3, and AKR1C4. 5 µg of the recombinant proteins were incubated with different concentrations of DOX (end concentration: 1, 10, 25, 50, 100, and 250 µM). After 30 min the reaction was stopped and the amount of produced DOX-OL was determined by HPLC.

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

Figure 3. The effect of hydroxy-PP on the DOX reduction to DOX-OL by CBR1, AKR1C3 and by liver cytosol. Sixty µg cytosol or 5 µg recombinant protein were incubated with 250µM 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.

Figure 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 µM). After 30 min the reaction was stopped and the amount of DOX-OL was determined by HPLC.

Figure 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 µM). DOX-OL was detected by HPLC.

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Tables

Table 1. Kinetic parameters K_m (µM), V_{max} (pmol/(min*mg cytosolic protein) and V_{max}/K_m (µL/(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 ± SEM.

	K_m ± SEM (µM)	**V_{max} ± SEM (pmol/(mg*min))**	**V_{max}/ K_m (µL/(mg*min))**	**No. of samples**
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

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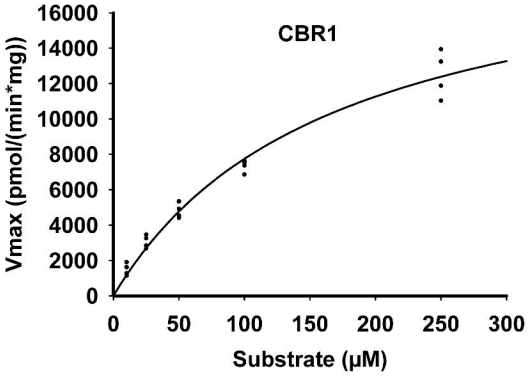
Table 2. Kinetic parameters K_m (µM), V_{max} (nmol/(min*mg)), V_{max}/K_m, k_{cat} (min-1) and catalytic efficiency (k_{cat}/K_m, min*mg) for DOX reduction to DOX-OL by the specified purified aldo-keto reductases and carbonyl reductases. n.d. = not determined

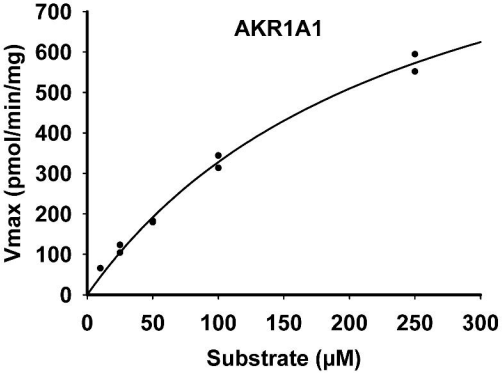
	K_m (µM)	**V_{max} (nmol/(mg*min))**	**V_{max}/K_m (mL/(mg*min))**	**k_{cat} (min^{-1})**	**k_{cat}/K_m (1/(min*mM))**
AKR1A1	247	1.1	0.004	0.04	0.168
AKR1B1			Non-saturated kinetics		
AKR1B10	311	2.8	0.009	0.10	0.324
AKR1C3	129	183.5	1.422	6.75	52.510
AKR1C4	281	1.2	0.004	0.04	0.158
CBR1	167	20.6	0.123	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.

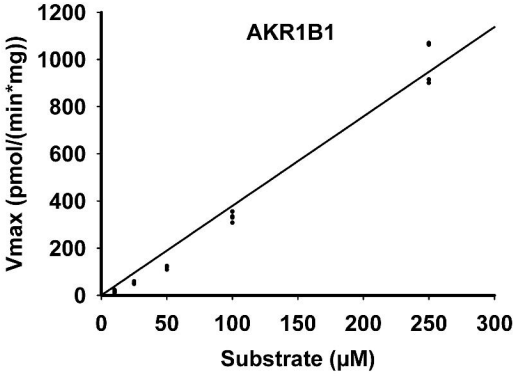
Table 3. *CBR1* gene variants and the resulting 9 most frequent haplotypes determined by sequencing of 57 DNA samples. Bonferroni-corrected significance level for compliance with Hardy-Weinberg equilibrium (X^2 test) was set at <0.005. Fields in grey mark differences in comparison with the haplotype 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
X^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

Figure 1


CBR1
Vmax (pmol/(min*mg))
16000
14000
12000
10000
8000
6000
4000
2000
0
0
50
100
150
200
250
300
Substrate (μM)


AKR1A1
Vmax (pmol/min/mg)
700
600
500
400
300
200
100
0
0
50
100
150
200
250
300
Substrate (μM)


AKR1B1
Vmax (pmol/(min*mg))
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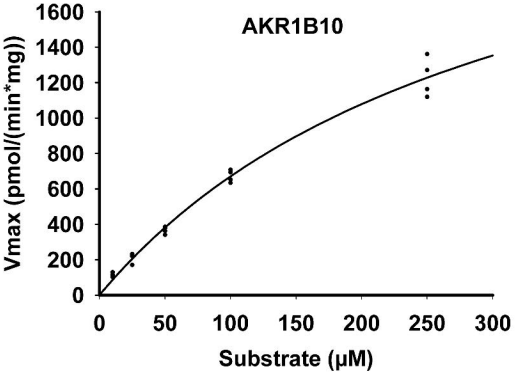
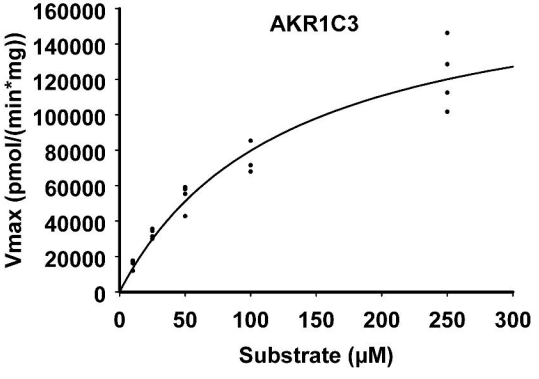
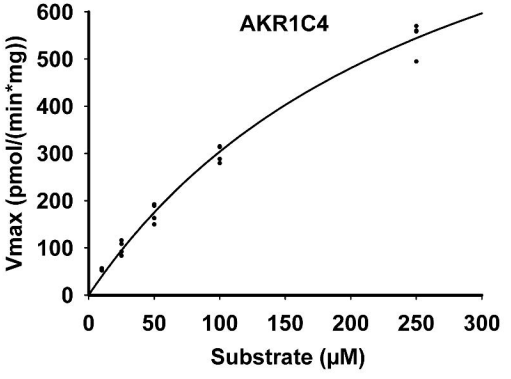
1000
800
600
400
200
0
0
50
100
150
200
250
300
Substrate (μM)


AKR1B10
Vmax (pmol/(min*mg))
1600
1400
1200
1000
800
600
400
200
0
0
50
100
150
200
250
300
Substrate (μM)


AKR1C3
Vmax (pmol/(min*mg))
160000
140000
120000
100000
80000
60000
40000
20000
0
0
50
100
150
200
250
300
Substrate (μM)


AKR1C4
Vmax (pmol/(min*mg))
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500
400
300
200
100
0
0
50
100
150
200
250
300
Substrate (μM)

Figure 2

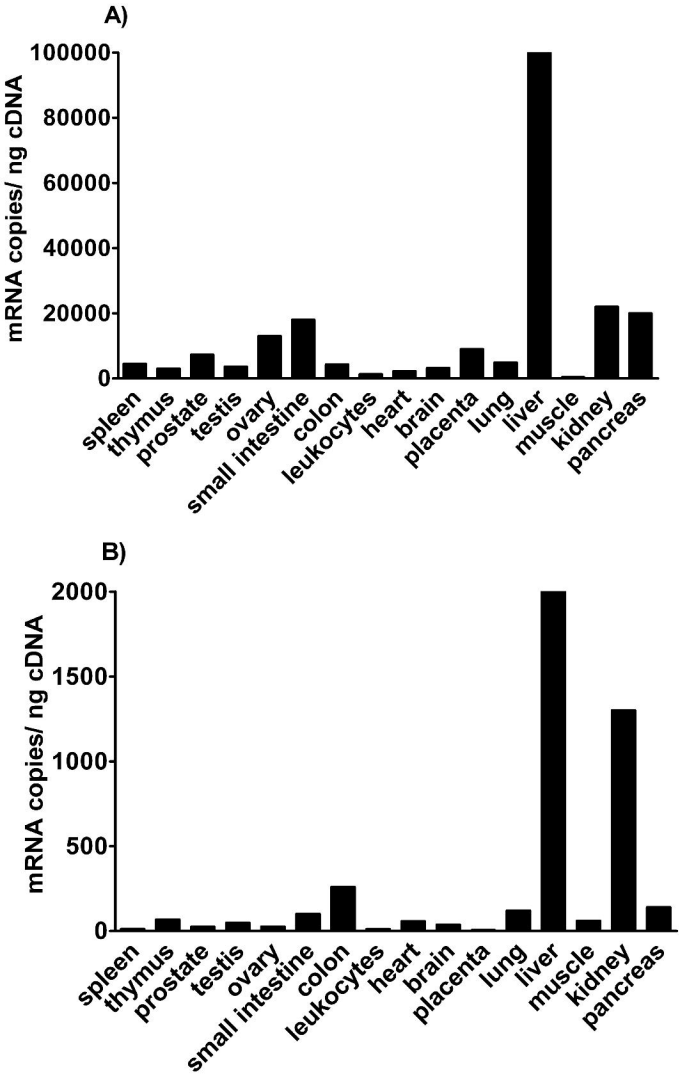

A)
mRNA copies/ ng cDNA
100000
80000
60000
40000
20000
0
spleen
thymus
prostate
testis
ovary
small intestine
colon
leukocytes
heart
brain
placenta
lung
liver
muscle
kidney
pancreas
B)
mRNA copies/ ng cDNA
2000
1500
1000
500
0
spleen
thymus
prostate
testis
ovary
small intestine
colon
leukocytes
heart
brain
placenta
lung
liver
muscle
kidney
pancreas

Figure 3

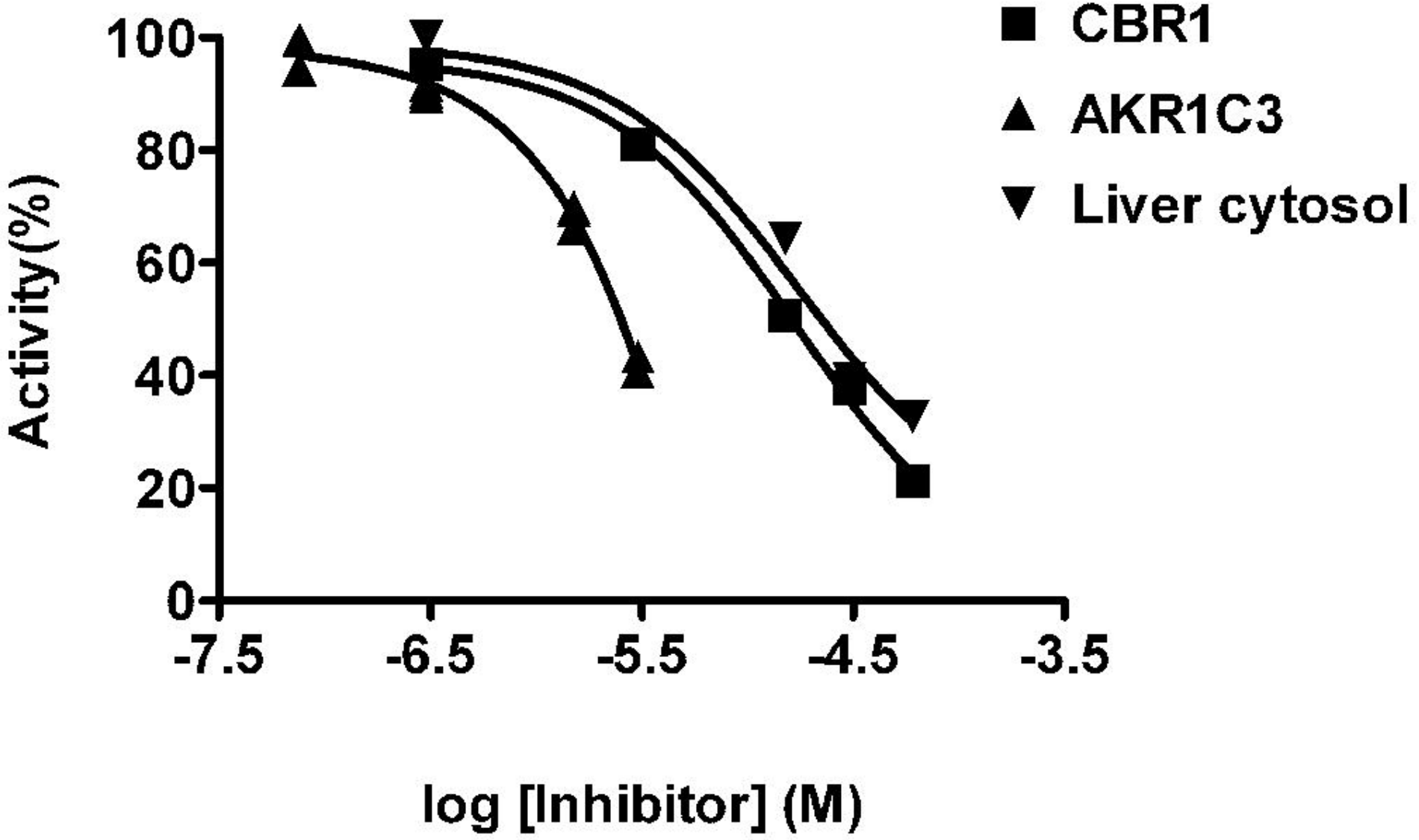
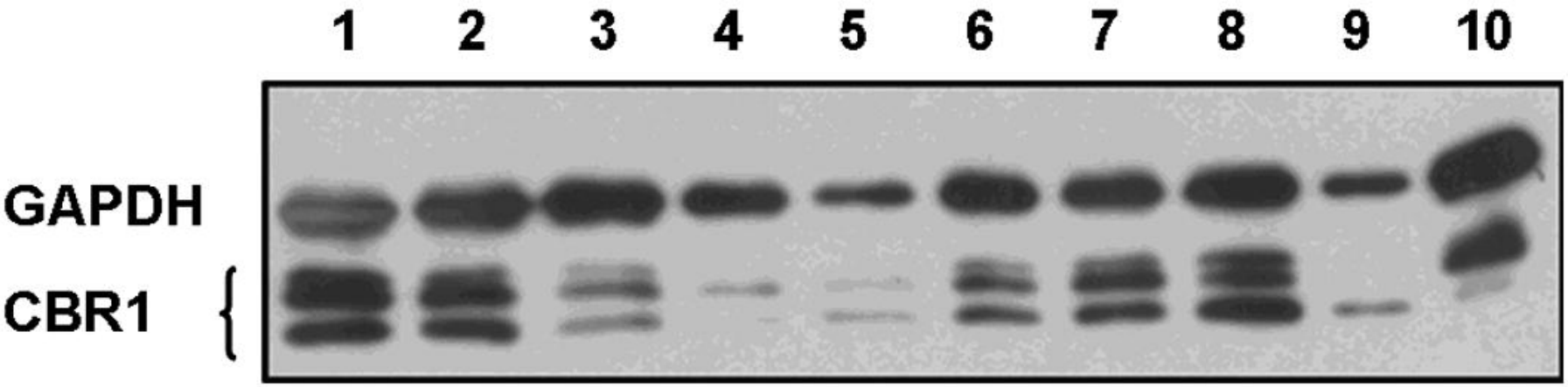

CBR1
AKR1C3
Liver cytosol
Activity(%)
100
80
60
40
20
0
-7.5
-6.5
-5.5
-4.5
-3.5
log [Inhibitor] (M)

Figure 4

A)


1
2
3
4
5
6
7
8
9
10
GAPDH
CBR1

B)

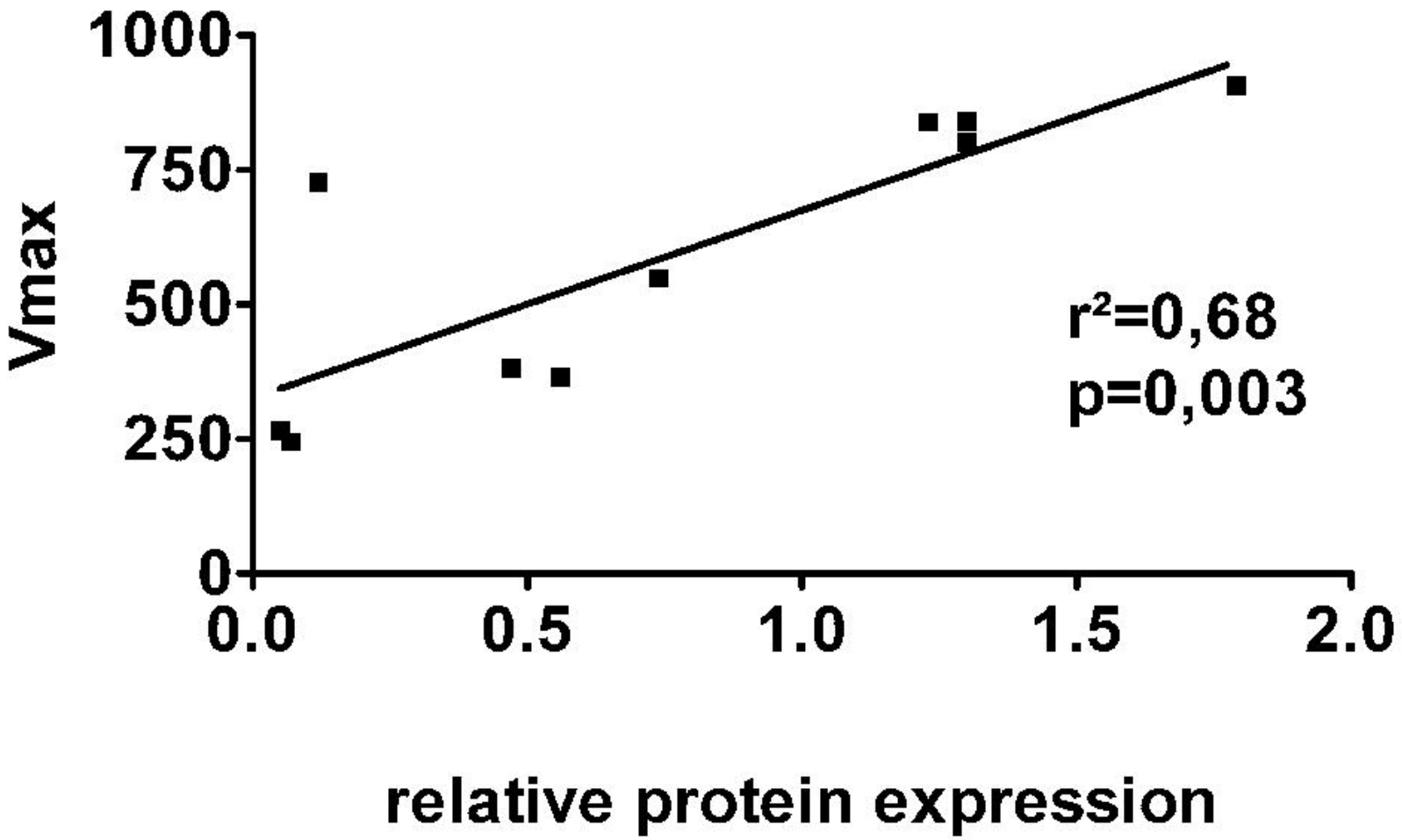
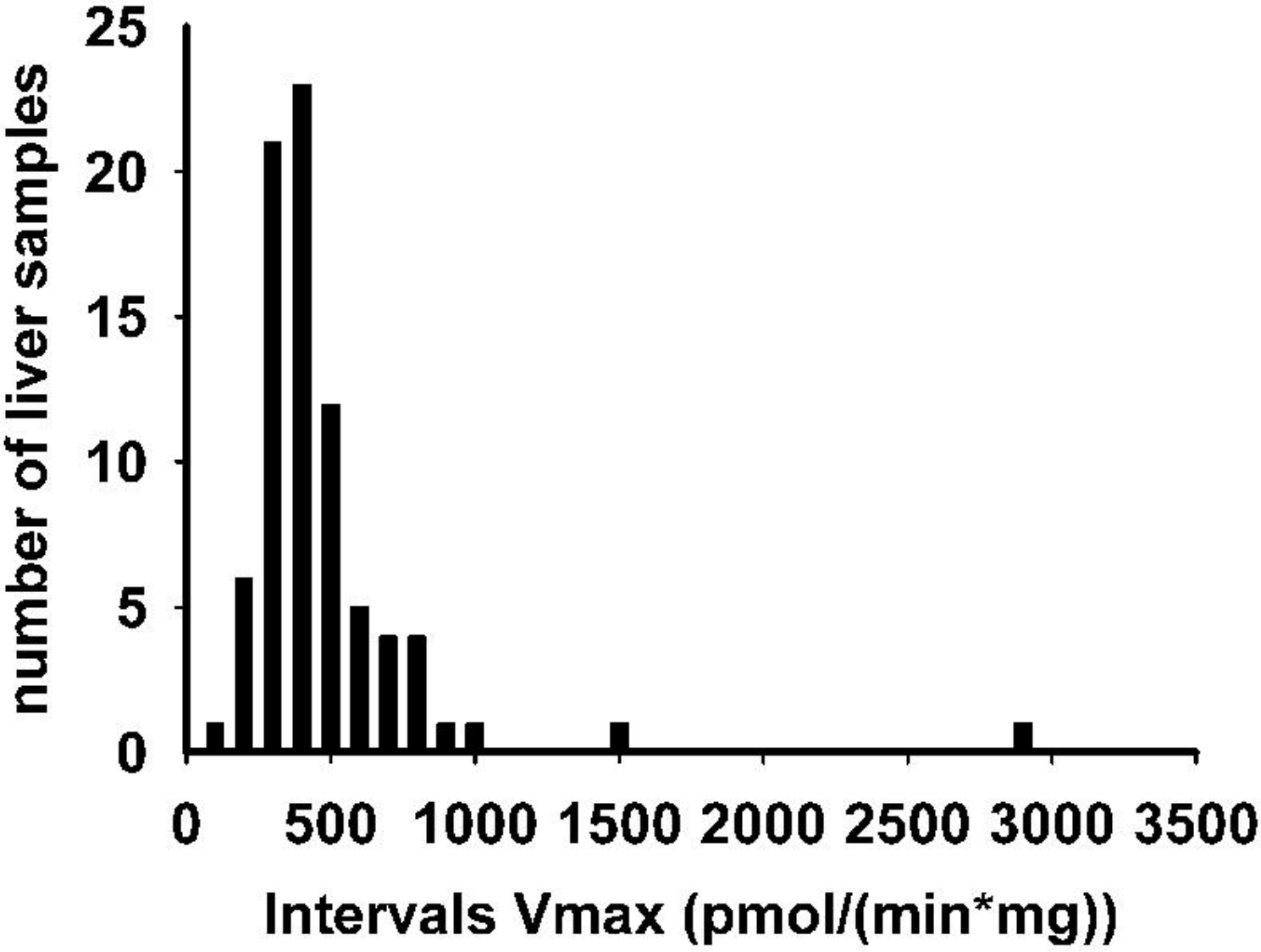

1000
750
500
250
0
Vmax
0.0
0.5
1.0
1.5
2.0
r²=0,68
p=0,003
relative protein expression

Figure 5


number of liver samples
25
20
15
10
5
0
0
500
1000
1500
2000
2500
3000
3500
Intervals Vmax (pmol/(min*mg))