DMD Fast Forward. Published on December 28, 2005 as doi:10.1124/dmd.105.007971

Purification and characterization of AKR1B10 from human liver: role in carbonyl reduction of xenobiotics

Hans-Jörg Martin, Ursula Breyer-Pfaff, Vladimir Wsol, Simone Venz, Simone Block, and Edmund Maser

Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, D-24105 Kiel (H.-J.M, S.B., E.M.); Department of Pharmacology and Toxicology, University of Tuebingen, D-72074 Tuebingen (U.B.-P.); Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovskeho 1203, CZ-50005 Hradec Kralove (V.W.); Ernst-Moritz-Arndt-University Greifswald, Laboratory for Functional Genomics, D-17489 Greifswald (S.V.)

Running Title: Characterization of Aldo-Keto Reductase 1B10

Address correspondence to: Edmund Maser, Institute of Toxicology and Pharmacology for Natural Scientists, D-24105 Kiel, Brunswikerstr. 10, Germany; phone, +49 431 597-3540; fax, +49 431 597-3558; e-mail, maser@toxi.uni-kiel.de

Number of text pages:	17
Number of tables:	4
Number of figures:	4
Number of references:	27

Number of words in	
Abstract:	248
Introduction:	467
Discussion:	1259

Abbreviations: AKR, aldo-keto reductase; EDTA, ethylenediaminetetraacetic acid, disodium salt; FPLC, Fast Protein Liquid Chromatography; HT, hydroxytryptamine; ESI-MS/MS, Electro-Spray-Ionization-Tandem-Mass-Spectrometry; NNAL, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol; NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; PCR, polymerase chain reaction; SDR, short-chain dehydrogenase/reductase.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Members of the aldo-keto reductase (AKR) superfamily have a broad substrate specificity in catalyzing the reduction of carbonyl-group containing xenobiotics. In the present investigation, a member of the aldose reductase subfamily, AKR1B10, was purified from human liver cytosol. This is the first time AKR1B10 has been purified in its native form. AKR1B10 showed a molecular mass of 35 kDa upon gel filtration and SDS-PAGE. Kinetic parameters for the NADPH-dependent reduction of the antiemetic 5-HT_3 receptor antagonist dolasetron, the anti-tumor drugs daunorubicin and oracin, and the carcinogen 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) to the corresponding alcohols have been determined by HPLC. K_m-values ranged between 0.06 mM for dolasetron and 1.1 mM for daunorubicin. Enzymatic efficiencies calculated as k_{cat}/K_m were more than 100 $mM^{-1}\,min^{-1}$ for dolasetron and 1.3, 0.43 and 0.47 $mM^{-1}\,min^{-1}$ for daunorubicin, oracin and NNK, respectively. Thus, AKR1B10 is one of the most significant reductases in the activation of dolasetron. In addition to its reducing activity, AKR1B10 catalyzed the $NADP^+$-dependent oxidation of the secondary alcohol (*S*)-1-indanol to 1-indanone with high enzymatic efficiency (k_{cat}/K_m = 112 $mM^{-1}\,min^{-1}$). The gene encoding AKR1B10 was cloned from a human liver cDNA library and the recombinant enzyme was purified. Kinetic studies revealed lower activity of the recombinant compared to the native form. Immunoblot studies indicated large interindividual variations in the expression of AKR1B10 in human liver. Since carbonyl reduction of xenobiotics often leads to their inactivation, AKR1B10 may play a role in the occurrence of chemoresistance of tumors towards carbonyl-group bearing cytostatic drugs.

The redox reaction interconverting ketones and alcohols is mainly catalyzed by enzymes belonging to two superfamilies, the aldo-keto reductases (AKR) and short-chain dehydrogenases/reductases (SDR). Currently, more than 3000 SDRs are known while 121 AKRs are listed in the AKR-homepage (http://www.med.upenn.edu/akr/), 13 of which are occurring in man (Bohren et al., 1989; Hyndman et al., 2003; Penning, 2004). Despite their overall structural similarity, AKR enzymes show considerable diversity in terms of substrate specificity, acting on numerous aliphatic and aromatic aldehydes and ketones, steroids, prostaglandins, sugars and xenobiotics. AKRs can be considered as enzymes participating in phase I drug metabolism and accelerating the elimination of potential toxicants. Carbonyl group reduction provides the hydroxy group necessary for conjugation reactions and the resulting hydrophilic molecules can be excreted via bile and/or urine.

AKR1B10 belongs to the aldose reductases (AKR1B subfamily) and was discovered as an enzyme being overexpressed in human liver cancers and in the search of aldose reductase homologues (Jez et al., 1997; Cao et al., 1998; Scuric et al., 1998; Hyndman and Flynn, 1998). It shares 70 % identity with human aldose reductase AKR1B1. Expression levels are high in human small intestine and adrenal gland and lower in all other tissues tested so far (Cao et al., 1998; Hyndman and Flynn, 1998). Recent observations suggest that its physiological substrates are retinal isomers (Crosas et al. 2003). In previous studies, we purified oxidoreductases from human liver cytosol catalyzing redox reactions of various substrates (Breyer-Pfaff and Nill, 2000; Atalla et al., 2000). Based on this procedure we could now purify AKR1B10 in an active state and identify its nature by ESI-MS/MS analysis. To investigate the role of this comparatively new member of the AKR family in the carbonyl reduction of xenobiotics, we overexpressed AKR1B10 in *E. coli* and used both, the native and recombinant enzymes, to characterize enzymatic properties and assess kinetic parameters. As AKR1B10 is overexpressed in lung cancer (Hyndman and Flynn, 1998; Penning, 2005;), we checked the purified enzyme for its ability to accept NNK as substrate. There is strong evidence that NNK plays a major role in the causation of lung cancer in smokers (Hecht, 1998). In addition, the two anti-cancer drugs daunorubicin, which is among others used in the treatment of lung cancer, and oracin, a potential cytostatic drug for oral use which is

at present in phase II of clinical trials, were tested for carbonyl reduction. All of the above-mentioned compounds were converted by AKR1B10 to the corresponding alcohols. We also present data for carbonyl reduction of the opioid receptor antagonist naltrexone and the antiemetic 5-HT_3 receptor antagonist dolasetron which are efficiently reduced *in vivo* (Gonzalez and Brogden, 1988; Dow and Berg, 1995). While the reduction of naltrexone proceeded slowly, AKR1B10 was among the most efficient reductases of dolasetron characterized so far (Breyer-Pfaff and Nill, 2004).

Materials and Methods

The following columns and materials used for protein isolation were purchased from Pharmacia (Freiburg, Germany): Superdex™ 75 column, 1 ml HiTrap™ Chelating column, Sephadex G-100, Q Sepharose, CM Sepharose, Polybuffer Exchanger PBE 94, and Polybuffer 74. Hydroxyapatite Bio-Gel HTP was from Bio-Rad, a 50-ml ultrafiltration chamber and filters YM10 from Amicon, (*S*)-(+)- and (*R*)-(–)-1-indanol, glyceraldehyde, NADPH and glucose-6-phosphate from Sigma-Aldrich. The molecular weight standards were from Sigma-Aldrich and Fermentas. NADP-tetrasodium salt was purchased from AppliChem (Darmstadt, Germany), glucose-6-phosphate dehydrogenase from yeast, grade I, ca. 350 U/mg, from Roche Diagnostics (Mannheim, Germany). NNK was supplied from Campro Scientific (Emmerich, Germany). Naltrexone hydrochloride was kindly provided by DuPont Pharma (Bad Homburg, Germany) and dolasetron mesilate hydrate by Hoechst Marion Roussel (Bad Soden, Germany). Reduced dolasetron was prepared according to Dow and Berg (1995) as described (Breyer-Pfaff and Nill 2004). Oracin and dihydrooracin were gifts from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Daunorubicin was from Rhône-Poulenc, Cologne, Germany, reduced daunorubicin was obtained from Pharmacia-Farmitalia Carlo Erba (Milan, Italy). Pfu-polymerase and T4 ligase were purchased from Fermentas and restriction enzymes from New England Biolabs. Bacterial strains used in this study and the pQE-60 vector were from Qiagen (Hilden, Germany). The human liver cDNA library Uni-ZAp ®XR was from Stratagene. Primers were prepared by MWG (Ebersberg, Germany). Ampicillin and kanamycin were from AGS (Heidelberg, Germany).

Enzyme isolation and purification. Human liver samples were provided by the Department of Surgery, University of Tuebingen. They were either excess normal tissue removed on partial hepatectomy for liver metastases or materials excluded from transplantation for medical reasons. The samples were stored at -80°C for 0.5 to 7 years. Homogenates were prepared with 4 volumes of homogenization buffer (250 mM sucrose, 20 mM Tris-HCl, 5 mM EDTA, adjusted to pH 7.4 at 37°C) and cytosol was obtained by differential centrifugation. The enzyme isolation was a modification

(Breyer-Pfaff and Nill, 2000) of the method of Hara et al. (1990) ;. Cytosol from 5-7 g of liver was concentrated to half its volume by ultrafiltration and fractionated on a 2.5 × 100 cm column with Sephadex G-100 using 25 mM Tris-HCl pH 7.4. Fractions testing positive for oxidoreductase activity were concentrated with the medium being changed to buffer A (400 ml 6.25 mM Tris + 0.625 mM EDTA adjusted to pH 8.0 with HCl and 100 ml glycerol, with 2-mercaptoethanol added to 5 mM before use). In all further purification steps, 2-mercaptoethanol was added to buffers to a concentration of 5 mM prior to use. The elution rate was 1 ml/min except on chromatofocusing, and fractions of 5 ml or 2.5 ml were tested for enzymatic activity, then combined, concentrated and stored in buffer A at -80°C.

Three enzymatically active peaks were obtained on elution of a 2.5 × 10 cm column of Q Sepharose with a NaCl gradient in buffer A: 0 M (0-100 min), linear gradient to 0.1 M (100-340 min), linear gradient to 0.5 M (340-440 min). The second peak (Q2) collected from 200 to 320 ml exhibited (*S*)-1-indanol oxidase activity. It was applied to a 1 × 13 cm column of CM Sepharose in 10 mM sodium phosphate + 0.5 mM EDTA adjusted to pH 6.5. After 20 min of isocratic elution, a linear gradient was started that attained 0.1 M NaCl within 60 min. Peaks with (*S*)-1-indanol oxidase activity were eluted at 35 – 80 ml (Q2-CM1) and 80 – 165 ml (Q2-CM2). From the latter peak, AKR1C2 and carbonyl reductase form I and II were isolated (Breyer-Pfaff and Nill 2000). The Q2-CM1 concentrate was purified on a 1.1 × 9.5 cm column of Bio-Gel HTP equilibrated with 10 mM Tris-HCl pH 7.5. Linear gradients were run in which the Tris buffer was replaced by 0.2 M sodium phosphate pH 6.5. The concentration of phosphate was 0.05 M after 60 min, 0.1 M after 90 min, held at 0.1 M for 30 min and increased to 0.2 M within 60 min. AKR1B10 activity monitored by oxidation of (*S*)-1-indanol was eluted at 63-105 ml. Final purification was achieved by chromatofocusing on a 0.9 × 56 cm column of Polybuffer Exchanger PBE 94 equilibrated with 25 mM imidazole-HCl pH 7.4 and 5 mM 2-mercaptoethanol. The column was run with Polybuffer 74/water (1 : 7, v/v) adjusted to pH 5.0 with HCl. Fractions of 4 ml/30 min were collected, the enzyme being eluted at pH 7.1 in fractions 28-32 (108-128 ml).

Nano-Liquid-Chromatography-Electro-Spray-Ionization-Tandem-Mass-Spectrometry (Nano-LC-ESI-MS/MS). The purified protein was subjected to SDS-PAGE and stained with Colloidal Coomassie Brilliant Blue according to the manufacturers' instructions (GE Healthcare, Freiburg, Germany). For identification by mass spectrometry the protein was excised from the gel stained with Colloidal Coomassie Brilliant Blue, washed (2×100µl 50 mM NH_4HCO_3/ 50% (v/v) MeOH, 30 min, 37°C) and dehydrated with 100% acetonitrile for 15 min. After removal of acetonitrile, trypsin solution (10 ng/µl trypsin in 20 mM ammonium bicarbonate) was added until the gel pieces stopped swelling and digestion was allowed to proceed for 16 to 18 hours at 37°C. For peptide extraction, gel pieces were subsequently covered with 15 µl 1% (v/v) formic acid and incubated in an ultrasonic bath for 20 min. A second peptide extraction was done with 20 µl 50% (v/v) acetonitrile. Both peptide-containing supernatants were transferred into micro vials for mass spectrometric analysis. High Pressure Liquid Chromatography separation was performed on an Ultimate™ system (LC Packings, Amsterdam, Netherlands). The system was coupled via a nanoLC inlet (New Objective, Woburn, MA, USA) to the Q-TOF mass spectrometer (Q-Star Pulsar i, Applied Biosystems, Foster City, CA, USA) equipped with a nano-electrospray source (Protana, Odense, Denmark). Peptide chromatography and tandem mass spectrometry were performed according to Eymann et al.(Eymann et al., 2004). The resulting MS/MS data were analysed with the Bioanalyst™ Software (Applied Biosystems, Foster City, CA, USA) and the integrated Mascot script. For database searches the Mascot search engine (Matrix Science Ltd, London, UK) was used with a human sequence database. Proteins were regarded as positively identified when a mowse score of 53 corresponding at least to a p-value of 0.05 was obtained.

Gel permeation chromatography. Gel filtration was performed on an Amersham Pharmacia ÄKTA™ FPLC system using a Pharmacia Superdex™ 75 column. The column was equilibrated with a buffer containing 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.2 mM 2-mercaptoethanol, and 10% glycerol. Elution of 12 µg of native AKR1B10 was detected by recording the absorption at 280 nm. The standard curve was generated by using the low molecular weight calibration kit from Amersham-Pharmacia.

Cloning and expression of AKR1B10 in *E. coli*. The coding sequence of *AKR1B10* (NCBI Reference Sequence accession number: NM_020299) was amplified from a human liver cDNA-library (Stratagene) by PCR using primers of the following sequence: forward primer, ATGGCCACGTTTGTGGAG; reverse primer, CCTCAATATTCTGCATCGAAGG. PCR was performed with *Pfu*-Polymerase in 30 cycles with an annealing temperature of 52°C. In a second PCR reaction, *BgI* II restriction sites were added using appropriate primers and the resulting construct was ligated into the pQE-70 (Qiagen) vector such that the protein was translated in frame from the vector's translation start site resulting in a protein containing a C-terminal His-tag. For expression of AKR1B10 protein, SG13009 cells (Qiagen) were transformed with the expression plasmid and a 200 ml culture containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) was grown to OD_{600} = 0.6 at 37 °C. Expression was induced by supplementing the culture medium with 1 mM isopropyl-1-thio-galactopyranoside. Cells were harvested after 3 hours by centrifugation (6000 *g*, 10 min) and resuspended in 20 ml of Tris buffer (20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 10 mM imidazole) before lysation in a French-pressure™ cell. The resulting suspension was centrifuged for 1 hour at 100000 *g* at 4°C and the supernatant either stored at –80°C or directly used for Ni-affinity chromatography.

Purification of recombinant AKR1B10. Purification of AKR1B10 was achieved by FPLC (ÄKTA™-Purifier, Amersham-Pharmacia) on a 1 ml Ni^{2+} HisTrap™-FF column. A linear gradient of 10 mM – 500 mM imidazole in 20 mM NaH_2PO_4, 100 mM NaCl, 10% glycerol, pH 7.4 was used. AKR1B10 was eluted from the column at an imidazole concentration of about 200 mM. Protein-containing fractions were analyzed by SDS-PAGE. Samples containing pure AKR1B10 were pooled and 5× concentrated by ultrafiltration, thereby exchanging the elution buffer with 10 mM Tris·HCl, pH 8.0, 0.5 mM EDTA, 20% glycerol, 5 mM 2-mercaptoethanol. Purified protein was stored in this buffer at –80°C until further use.

Immunoblotting. Antisera against full length AKR1B10 were prepared by Eurogentec, Belgium, by using recombinant protein produced in our lab. Electroblotting was performed in a semidry blotting system. Proteins were transferred on a nitrocellulose membrane, and antigen-antibody complexes were visualized by chemoluminescence (ECL plus detection kit, Amersham-Biosciences). Primary antibodies were diluted 1/1000; the secondary antibody (peroxidase-conjugated swine anti-rabbit immunoglobulin, DakoCytomation, Denmark) was used in a 1/10000 dilution.

Enzymatic tests. Oxidoreductase activities in gel filtration eluates were monitored by HPLC measurement of (*Z*)-10-hydroxynortriptyline production from (*Z*)-10-oxonortriptyline (Breyer-Pfaff and Nill 2000). Alternatively, naltrexone reduction by aliquots of the protein fractions was assayed spectrophotometrically. In further purification steps, NADPH generation with (*S*)-1-indanol as substrate served for activity testing. In a total volume of 0.6 ml, samples were incubated at 37°C with 100 mM Tris-HCl pH 7.4, 8 mM $MgCl_2$, 25 mM KCl and either 0.1 mM NADPH or 0.25 mM $NADP^+$. The decrease or increase of absorption at 339 nm in the presence of 0.1 mM naltrexone or 0.5 mM (*S*)-1-indanol, respectively, was registered for 5 - 10 min. Under the same conditions, (*R*)-1-indanol oxidation and DL-glyceraldehyde reduction were measured.

Enzyme kinetics. Dolasetron reduction kinetics were determined by incubating 0.01 - 0.3 mM substrate with 1 µg of purified AKR1B10 in 0.36 ml of the above buffer containing 0.2 mM $NADP^+$, 2 mM glucose-6-phosphate and 1 U/ml glucose-6-phosphate dehydrogenase for NADPH generation. After 20 min at 37°C, the reaction was stopped by adding 40 µl of 2 M perchloric acid and cooling on ice. Reduced dolasetron was analysed in the supernatant by HPLC (Breyer-Pfaff and Nill, 2004). In analogous fashion, naltrexone reduction was checked in a few samples. NNK (0.05 - 1.5 mM), oracin (0.02 - 0.75 mM) and daunorubicin (0.15 - 2 mM) reduction kinetics were assayed with 2.72, 1.36 and 0.68 µg of purified AKR1B10, respectively, in a total volume of 0.1 ml in 0.1 M sodium phosphate buffer, pH 7.4 and an NADPH-generating system (final concentrations: $NADP^+$, 3 mM; glucose 6-phosphate, 20 mM; glucose-6-phosphate dehydrogenase, 1.8 units / 0.1 ml; $MgCl_2$, 10 mM). Incubation times were 60, 30 and 10 min, respectively. Increase of product concentration was linear during the

given periods of time. Reactions were stopped by the addition of 40 µl 30% ammonia and cooling on ice. The mixtures were extracted twice with 300 µl of ethylacetate (thrice in the case of daunorubicin) and the combined organic phases were evaporated under vacuum. The residue was dissolved in the respective mobile phase and analyzed by HPLC as described (Wsol et al., 1996; Breyer-Pfaff et al., 2004). For daunorubicinol detection, a modified method of Fogli et al. (Fogli et al., 1999) was used (mobile phase: 50 mM sodium phosphate-acetonitrile (75:25), pH 4.0; flow rate: 1.5 ml/min). (*S*)-1-Indanol (0.25 - 4.0 mM) oxidation kinetics was assayed spectrophotometrically as described above. For the determination of kinetic constants, the results were analyzed by least-squares nonlinear fitting of a Michaelis-Menten hyperbola or the Hill equation (GraphPad Prism™, GraphPad Software, Inc.).

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

Results

In the search for enzymes participating in the reduction of xenobiotic ketones, a purification scheme was developed which led to the simultaneous purification of the three aldo-keto reductases AKR1C1, -1C2, -1C4 and the short-chain dehydrogenase carbonyl reductase (Breyer-Pfaff and Nill, 2000). Monitoring (*S*)-1-indanol oxidation activity led to the isolation of an additional enzyme which we could now unequivocally identify as AKR1B10 by ESI-MS/MS analysis (Table 1). The purified protein proved to be homogeneous on SDS-PAGE. The estimated molecular mass corresponded to that of the primary structure deduced from the cDNA sequence and was the same as that of AKR1C1 and AKR1C2 (34 - 36 kDa, Fig. 1A). A molecular mass of 35 kDa for AKR1B10 was also found by size-exclusion chromatography (Fig. 2).

The coding sequence of human AKR1B10 was obtained by PCR from a human liver cDNA-library. After ligation into an expression vector, the protein was overexpressed in *E. coli* cells and purified in an active state by metal chelate chromatography (Fig. 1B). The purified recombinant enzyme was soluble and could be concentrated to at least 16 mg/ml without precipitation.

Western blot of human liver cytosol probed with antibodies raised against recombinant AKR1B10 showed the presence of the enzyme in only two out of five livers (Fig. 3). This indicates interindividual variation in the expression of AKR1B10 in human tissues.

The native and recombinant enzyme preparations displayed different kinetic properties, as shown for example for indanol oxidation (Fig. 4C). In all cases the catalytic efficiency of the native enzyme was higher than that of its recombinant counterpart (Table 3). Incubation of AKR1B10 with an NADPH-regenerating system and the substrates listed in Tables 2 and 3 yielded the corresponding alcohols which were detected and quantified by HPLC. In the case of 1-indanol oxidation, the linear increase in absorbance at 339 nm was monitored, corresponding to the conversion of the cofactor $NADP^+$ to NADPH. AKR1B10 rapidly oxidized (*S*)-1-indanol, while (*R*)-1-indanol was oxidized at a 10-fold lower rate (Table 2). All data obtained were in accordance with Michaelis-Menten kinetics with the

exception of those for daunorubicin. Carbonyl reduction of daunorubicin was better fitted by the Hill-equation with a Hill-coefficient of 1.5 (Fig. 4). With oracin some substrate inhibition was observed. K_m values were in the micromolar range for dolasetron and in the low millimolar range for the other compounds tested (Table 3). k_{cat}/K_m values were highest for dolasetron and indanol (>100 mM^{-1} min^{-1}) and much lower for NNK, daunorubicin and oracin (around 1 mM^{-1} min^{-1}, Table 3).

Experiments with common oxidoreductase inhibitors revealed a low potential of barbital and some antiinflammatory drugs and the most potent inhibition with lithocholic acid (Table 4).

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

A modification of the original purification scheme of Hara et al. (1990) consisting of several chromatographic steps allowed for the simultaneous isolation of the three aldo-keto reductases AKR1C1, -1C2, -1C4 and the short-chain dehydrogenase/reductase carbonyl reductase from human liver cytosol (Breyer-Pfaff and Nill, 2000). An additional fraction could be isolated irregularly and shown to contain a protein with (*S*)-1-indanol oxidizing activity. This protein was now identified by ESI-MS/MS as the aldo-keto reductase AKR1B10. This is the first time that this relatively new member of the aldo-keto reductase superfamily has been purified from human tissue in its native state. AKR1B10 mRNA levels were reported to be high in small intestine, colon and adrenal gland, but only moderate amounts were found in liver (Cao et al., 1998; Hyndman and Flynn, 1998). This is in accordance with the occasional failure to detect a peak of enzymatic activity when cytosol of different livers was subjected to the purification procedure. Interindividual variability in AKR1B10 expression was also shown in the present study by Western blot analysis, where no AKR1B10 protein was detected in three out of five human livers tested. To obtain a larger enzyme quantity, we cloned AKR1B10 in bacteria and isolated the recombinant protein by metal chelate chromatography. Native and recombinant enzyme were not identical regarding their kinetic parameters. Catalytic efficiencies of the native enzyme were 1.6 to 20 times higher compared to the recombinant form depending on the substrate. At the moment we cannot tell if this loss of activity (Table 3) is due to adverse effects of the additional amino acids derived from the cloning procedure, sub-optimal purification conditions or a lack of posttranslational modifications.

In order to assess the importance of AKR1B10 in the detoxification of xenobiotics, we determined the kinetic parameters of several carbonyl-group containing compounds. Recently, it was shown that AKR1B10 is a very efficient retinal reductase (Crosas et al., 2003). Published data indicate that the preferred substrates for this enzyme are aldehydes (Cao et al., 1998; Crosas et al., 2003), but both the native and the recombinant enzyme reduced the carbonyl group of various ketones (Table 3). In

addition to its well-documented function as a reductase, AKR1B10 oxidized indanol in the presence of $NADP^+$ *in vitro*. The reaction rate with (*S*)-1-indanol was similar to that of AKR1C2 and AKR1C4 and about half that of AKR1C1 (Breyer-Pfaff and Nill, 2000). AKR1B10 exhibited an about 10-fold lower activity towards (*R*)-1-indanol (Table 2). The opiate receptor antagonist naltrexone was reduced by AKR1B10 very slowly as it was also found for AKR1C1 and AKR1C2 (Breyer-Pfaff and Nill, 2004).

In contrast, AKR1B10 is one of the most active reductases towards dolasetron, a $5\text{-}HT_3$ receptor antagonist used as an antiemetic drug which is efficiently reduced to its corresponding alcohol *in vivo*. The k_{cat}/K_m value exceeded 100 mM^{-1} min^{-1} in three independent kinetic experiments (Table 3). The enzymatic efficiency is at least as high as that of AKR1C1 and superior to that of AKR1C2 in dolasetron reduction (Breyer-Pfaff and Nill, 2004). Reduced dolasetron is the active metabolite with a half-life ($t_{½}$) of 7-8 hours, whereas a $t_{½}$ of 0.13-0.24 hours is observed for the parent carbonyl compound (Gan, 2005 and references cited therein). Carbonyl reductase was held responsible for this rapid elimination, because conversion to the alcohol also took place in whole blood (Dow and Berg, 1995). In view of its widespread tissue distribution (Wirth and Wermuth, 1992), carbonyl reductase may in fact have a role, but its k_{cat}/K_m value in dolasetron reduction is only about one-third that of AKR1B10 and AKR1C1 (Breyer-Pfaff and Nill, 2004). Participation of AKR1B10 in first-pass reduction of dolasetron may be of importance if the enzyme is highly expressed in human small intestine as reported by Cao et al. (1998) and as reflected by its original name "human small intestine reductase" (Hyndman and Flynn, 1998). While the reduction of dolasetron is the prerequisite for its binding to the receptor and the resulting antiemetic effect, the reduction of carbonyl groups in cytostatic drugs often leads to their inactivation. We found that the anthracycline derivative daunorubicin and the novel anticancer drug oracin, which is currently in phase II of clinical trial, both were substrates for AKR1B10 albeit both were reduced with much lower enzymatic efficiency than dolasetron (Table 3). Surprisingly, daunorubicin reduction kinetics showed no Michaelis-Menten hyperbola but a sigmoidal pattern which was fitted by the Hill-equation with a Hill-coefficient of n = 1.5. Hill-coefficients >1 indicate a cooperative mode of action which is typical for multimeric enzymes. Nonetheless, size

exclusion chromatography with the native enzyme revealed the expected molecular mass of the monomer (found, 35 kDa; calculated, 36 kDa; Fig. 2). Although some multimeric aldo-keto reductases are known, most members of this superfamily are monomers. Daunorubicin is mainly reduced in the cytosol by carbonyl reductase (k_{cat}/K_m = 64 mM^{-1} min^{-1}) and to a lesser extent by AKR1C2 and AKR1A1 (previously designated as dihydrodiol dehydrogenase 2, DD2, and aldehyde reductase, ALR, respectively, in Ohara et al. (1995). In comparison, the catalytic efficiencies of AKR1B10 for daunorubicin (k_{cat}/K_m = 1.3 mM^{-1} min^{-1}) and oracin (k_{cat}/K_m = 0.43 mM^{-1} min^{-1}) are low. As detailed above, AKR1B10 expression is low or absent in normal liver. On the other hand, it was easily detectable in all five hepatocellular carcinoma tissues tested, while mRNA levels in non-tumor samples were minimal (Scuric et al., 1998). Analogously, no or low AKR1B10 expression was found in human lung (Cao et al., 1998; Hyndman and Flynn, 1998), but recently, Fukumoto et al. reported on the overexpression of AKR1B10 in smokers' non-small cell lung carcinomas and suggested it as a diagnostic marker (Fukumoto et al., 2005; Penning, 2005). As daunorubicin is used in the treatment of this disease, its carbonyl reduction (i.e. deactivation) by AKR1B10 may become a critical determinant in chemotherapy (Soldan et al., 1999; Ax et al., 2000; Lee et al., 2001).

The overexpression of AKR1B10 in squamous cell carcinoma of the lung raises the question if inhibition of this enzyme might be beneficial for patients, as the physiological role of AKR1B10 is not yet fully understood. Our results show that, on the one hand, AKR1B10 may contribute to drug resistance in cancer chemotherapy. On the other hand, one of the most potent lung carcinogens in tobacco smoke, NNK, is also reduced to the corresponding alcohol NNAL by this enzyme. This reduction constitutes the first step in the major detoxification pathway of NNK in humans and can be followed by conjugation with glucuronic acid. In human lung and liver cytosol, mostly aldo-keto reductases of the AKR1C family and carbonyl reductase of the SDR superfamily contribute to the reduction of NNK (Atalla et al., 2000; Breyer-Pfaff et al., 2004). In cancer tissues, where AKR1B10 may obviously be overexpressed manifold, the contribution of individual enzymes to NNK reduction has yet to be determined.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

In conclusion, we purified AKR1B10 from human liver in an active state and cloned it from a cDNA library. The enzyme proved to be an efficient reductase of the antiemetic pro-drug dolasetron to the corresponding alcohol, a high-affinity 5-HT_3 receptor blocker. Two anti-cancer drugs, daunorubicin and oracin, and the tobacco-specific carcinogen NNK were found to be substrates of the enzyme although with low catalytic efficiency. In non-cancerous human lung tissue AKR1B10 mRNA is rarely found and expression levels of AKR1B10 in human liver are low and show high interindividual variability as revealed by Western blot analysis. Since AKR1B10 is overexpressed in human non-small cell lung carcinoma and human hepatocellular carcinoma, it may become an important factor in drug resistance against carbonyl-group bearing anti-tumor drugs in these tissues.

References

Atalla A., Breyer-Pfaff U., and Maser E. (2000) Purification and characterization of oxidoreductases-catalyzing carbonyl reduction of the tobacco-specific nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in human liver cytosol. *Xenobiotica* **30:**755-769.

Ax W., Soldan M., Koch L., and Maser E. (2000) Development of daunorubicin resistance in tumour cells by induction of carbonyl reduction. *Biochem. Pharmacol.* **59:**293-300.

Bohren K. M., Bullock B., Wermuth B., and Gabbay K. H. (1989) The aldo-keto reductase superfamily. cDNAs and deduced amino acid sequences of human aldehyde and aldose reductases. *J. Biol. Chem.* **264**:9547-9551.

Breyer-Pfaff U., and Nill K. (2000) High-affinity stereoselective reduction of the enantiomers of ketotifen and of ketonic nortriptyline metabolites by aldo-keto reductases from human liver. *Biochem. Pharmacol.* **59:**249-260.

Breyer-Pfaff U., and Nill K. (2004) Carbonyl reduction of naltrexone and dolasetron by oxidoreductases isolated from human liver cytosol. *J. Pharm. Pharmacol.* **56:**1601-6.

Breyer-Pfaff U., Martin H .J., Ernst M., and Maser E. (2004) Enantioselectivity of carbonyl reduction of 4-methylnitrosamino-1-(3-pyridyl)-1-butanone by tissue fractions from human and rat and by enzymes isolated from human liver. *Drug. Metab. Dispos.* **32:**915-922.

Cao D., Fan S. T., and Chung S. S. (1998) Identification and characterization of a novel human aldose reductase-like gene. *J. Biol. Chem.* **273:**11429-11435.

Crosas B., Hyndman D. J., Gallego O., Martras S., Pares X., Flynn T. G., and Farres J. (2003) Human aldose reductase and human small intestine aldose reductase are efficient retinal reductases: consequences for retinoid metabolism. *Biochem. J.* **373:**973-979.

Dow J., and Berg C. (1995) Stereoselectivity of the carbonyl reduction of dolasetron in rats, dogs, and humans. *Chirality* **7:**342-348.

Eymann C., Dreisbach A., Albrecht D., Bernhardt J., Becher D., Gentner S., Tam le T., Buttner K., Buurman G., Scharf C., Venz S., Völker U., Hecker M. (2004) A comprehensive proteome map of growing Bacillus subtilis cells. *Proteomics* **4:**2849-76.

Fogli S., Danesi R., Innocenti F., Di Paolo A., Bocci G., Barbara C., and Del Tacca M. (1999) An improved HPLC method for therapeutic drug monitoring of daunorubicin, idarubicin, doxorubicin, epirubicin, and their 13-dihydro metabolites in human plasma. *Ther. Drug. Monit.* **1:**367-375.

Fukumoto S., Yamauchi N., Moriguchi H., Hippo Y., Watanabe A., Shibahara J., Taniguchi H., Ishikawa S., Ito H., Yamamoto S., Iwanari H., Hironaka M., Ishikawa Y., Niki T., Sohara Y., Kodama T., Nishimura M., Fukayama M., Dosaka-Akita H., and Aburatani H. (2005) Overexpression of the aldo-keto reductase family protein AKR1B10 is highly correlated with smokers' non-small cell lung carcinomas. *Clin. Cancer Res.* **11:**1776-1785

Gan T. J. (2005) Selective serotonin 5-HT3 receptor antagonists for postoperative nausea and vomiting: are they all the same? *CNS Drugs* **19:**225-238.

Gonzalez J .P., and Brogden R. N. (1988) Naltrexone. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the management of opioid dependence. *Drugs* **35:**192-213.

Hara A., Taniguchi H., Nakayama T., and Sawada H. (1990) Purification and properties of multiple forms of dihydrodiol dehydrogenase from human liver. *J. Biochem.* (Tokyo) **108:**250-254.

Hecht S. S. (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem. Res. Toxicol.* **11:**559-603.

Hyndman D. J., and Flynn T. G. (1998) Sequence and expression levels in human tissues of a new member of the aldo-keto reductase family. *Biochim. Biophys. Acta* **1399:**198-202.

Hyndman D., Bauman D. R., Heredia V. V., and Penning T. M. (2003) The aldo-keto reductase superfamily homepage. *Chem. Biol. Interact.* **143-144:**621-631.

Jez J. M., Flynn T. G., and Penning T. M. (1997) A new nomenclature for the aldo-keto reductase superfamily. *Biochem. Pharmacol.* **54:**639-647.

Lee K .W., Ko B .C., Jiang Z., Cao D., and Chung S. S. (2001) Overexpression of aldose reductase in liver cancers may contribute to drug resistance. *Anticancer Drugs* **12:**129-132.

Ohara H., Miyabe Y., Deyashiki Y., Matsuura K., and Hara A. (1995) Reduction of drug ketones by dihydrodiol dehydrogenases, carbonyl reductase and aldehyde reductase of human liver. *Biochem. Pharmacol.* **50:**221-227.

Penning T. M. (2004) Introduction and overview of the aldo-keto reductase superfamily, In: Penning T. M., and Petrash J. M., editors. *Aldo-keto reductases and toxicant metabolism,* American Chemical Society; p. 3–21, Washington (D.C).

Penning T. M. (2005) AKR1B10: a new diagnostic marker of non-small cell lung carcinoma in smokers. *Clin. Cancer Res.* **11:**1687-1690.

Scuric Z., Stain S. C., Anderson W. F., and Hwang J. J. (1998) New member of aldose reductase family proteins overexpressed in human hepatocellular carcinoma. *Hepatology* **27:**943-950.

Soldan M., Ax W., Plebuch M., Koch L., and Maser E. (1999) Cytostatic drug resistance. Role of phase-I daunorubicin metabolism in cancer cells. *Adv. Exp. Med. Biol.* **463:**529-538.

Wirth H., and Wermuth B. (1992) Immunohistochemical localization of carbonyl reductase in human tissues. *J. Histochem. Cytochem.* **40:**1857-1863.

Wsol V., Kvasnickova E., Szotakova B., and Hais I. M. (1996) High-performance liquid chromatographic assay for the separation and characterization of metabolites of the potential cytostatic drug oracine. *J. Chromatogr. B. Biomed. Appl.* **681:**169-175.

Footnotes

This work was supported by grants from the Deutsche Forschungsgemeinschaft (BR 478/14-1; MA 1704/3-2; MA 1704/3-3) and the Alexander von Humboldt-Foundation.

Figure Legends

Figure 1: SDS-PAGE of purified aldo-keto reductases (Coomassie-stain). **A**: The purification of aldo-keto reductases AKR1B10, AKR1C1 and AKR1C2 from human liver cytosol yielded single products with molecular masses of approximately 35 kDa. **B**: Recombinant AKR1B10 was obtained by overexpression in *E. coli*. Cells were disrupted in a French-pressure™ cell and soluble proteins were subjected to Ni-affinity chromatography (see methods). Lane assignments are as follows: M, marker proteins; 1, soluble protein fraction; 2, flowthrough fraction; 3, wash fraction; 4; proteins eluted with low (ca. 10 – 60 mM) imidazole concentrations; 5, AKR1B10 eluted with ca. 200 mM imidazole. The molecular mass of the recombinant protein (including His-tag) is 37.8 kDa in comparison to 36.0 kDa calculated from the primary structure of the native enzyme.

Figure 2: Gel filtration of 12 µg of native AKR1B10. The molecular mass (on the logarithmic scale) was plotted against the corresponding K_{av} value for each protein standard derived from its elution pattern (measured by the absorption at 280 nm). The standard proteins (ribonuclease A, 13.7 kDa; chymotrypsinogen A, 25 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa) are represented by unfilled circles. The K_{av} of AKR1B10 (black diamond) was located on the calibration curve and the molecular mass was calculated as 35 kDa.

Figure 3: Western blot of human liver cytosol and *E. coli* cells overexpressing AKR1B10. An amount of 20 µg of cytosolic proteins, prepared from 5 different human liver samples (lanes 1-5), and soluble proteins from *E. coli* cells overexpressing AKR1B10 (lane 6) were loaded. Only in two out of five cytosolic fractions a signal at 36 kDa is detectable (lanes 1 and 5).

Figure 4: Kinetics of oracin, daunorubicin and dolasetron carbonyl reduction and (*S*)-1-indanol oxidation by native AKR1B10. Reduced reaction products were quantified by HPLC or in the case of

(*S*)-1-indanol oxidation the increase in absorbance at 339 nm was measured. Individual data points or means of four independent experiments ± SD are shown. **A**: Reduction of oracin by AKR1B10 displays Michaelis-Menten kinetics with substrate inhibition. **B:** Kinetic data for daunorubicin reduction by AKR1B10 are significantly (P = 0.0013) better fitted by the Hill-equation than by the Michaelis-Menten hyperbola. The inset shows the deviation from linearity when the same data are graphed in a Hanes-Woolf plot. **C**: Oxidation of (*S*)-1-indanol by native AKR1B10 (unfilled circles) and recombinant AKR1B10 expressed in *E. coli* (filled squares). **D**: Reduction of dolasetron by AKR1B10.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Identification of AKR1B10 (Swiss-Prot entry name = AK1BA_HUMAN) by ESI-MSMS

A, Protein data [a]; **B**, Peptide data [b]; **C**, Sequence coverage [c].

Swiss-Prot Release 17.02.2005; cut off score > 26 with p-value < 0.05. Search parameters: MS/MS ion search, enzyme : trypsin, variable modifications : oxidation (M), peptide mass tolerance: ± 200 ppm, fragment mass tolerance: ± 0.6 Da, max missed cleavages: 1, number of queries: 335.

A

accession no.	accession code	MW [Da]	score	title_text	pI	sequence coverage [%]	peptides sequenced
O60218	AK1BA	35998	1016	Aldo-keto reductase family 1 member B10	7,63	59	17

B

start - end	observed mass	Mr (expt)	Mr (calc)	Δ	missed cleavage sites	sequence	ion score
13 - 22	551.35	1100.68	1100.61	0.08	0	MPIVGLGTWK	61
34 - 41	432.77	863.52	863.45	0.07	0	VAIDAGYR	35
70 - 78	553.86	1105.70	1105.61	0.08	1	REDLFIVSK	45
71 - 78	475.81	949.60	949.51	0.08	0	EDLFIVSK	54
79 - 90	781.00	1559.99	1559.86	0.13	0	LWPTFFERPLVR	28
102 - 117	660.42	1978.25	1978.04	0.22	0	LSYLDVYLIHWPQGFK	70
118 - 128	618.82	1235.63	1235.57	0.06	1	SGDDLFPKDDK	67
118 - 125	439.76	877.50	877.42	0.08	0	SGDDLFPK	68

136 - 155	1141.67	2281.33	2281.08	0.25	0	ATFLDAWEAMEELVDEGLVK Oxidation (M)	71
136 - 155	1133.69	2265.36	2265.09	0.27	0	ATFLDAWEAMEELVDEGLVK	63
156 - 169	788.98	1575.95	1575.80	0.15	0	ALGVSNFSHFQIEK	92
170 - 177	441.82	881.63	881.57	0.06	0	LLNKPGLK	48
196 - 203	496.30	990.58	990.50	0.09	0	LIQYCHSK	4
204 - 233	810.02	3236.05	3235.64	0.41	0	GITVTAYSPLGSPDRPWAKPE DPSLLEDPK	5
243 - 251	500.36	998.70	998.62	0.08	1	KTAAQVLIR	72
244 - 251	436.30	870.59	870.53	0.06	0	TAAQVLIR	57
257 - 263	391.80	781.58	781.51	0.07	0	NVIVIPK	36
270 - 280	676.41	1350.80	1350.72	0.08	0	IVENIQVFDFK	84
270 - 294	986.96	2957.84	2957.48	0.36	1	IVENIQVFDFKLSDEEMATILS FNR	47
281 - 294	821.47	1640.93	1640.77	0.16	0	LSDEEMATILSFNR Oxidation (M)	69
281 - 294	813.49	1624.96	1624.78	0.19	0	LSDEEMATILSFNR	73

C

1 MATFVELSTK AK**MPIVGLGT WK**SPLGKVKE AVK**VAIDAGY R**HIDCAYVYQ

51 NEHEVGEAIQ EKIQEKAVK**R EDLFIVSKLW PTFFERPLVR** KAFEKTLKDL

101 K**LSYLDVYLI HWPQGFKSGD DLFPKDDK**GN AIGGK**ATFLD AWEAMEELVD**

151 EGLVKALGVS NFSHFQIEKL LNKPGLKYKP VTNQVECHPY LTQEK**LIQYC**

201 HSKGITVTAY SPLGSPDRPW AKPEDPSLLE DPKIKEIAAK HK**KTAAQVLI**

251 RFHIQR**NVIV IPK**SVTPAR**I VENIQVFDFK LSDEEMATIL SFNR**NWRACN

301 VLQSSHLEDY PFDAEY

[a] MW = Molecular mass in Dalton (Da); pI = isoelectric point.

[b] Mr = Monoisotopic mass of neutral peptide; Δ = fragment mass difference between expected and calculated peptide mass in Da.

[c] Sequence coverage of the AKR1B10 primary structure is 59 %. Sequenced peptides are depicted in bold face.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 2

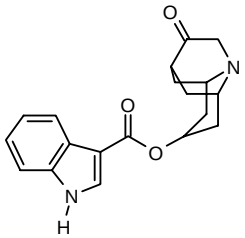
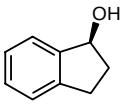
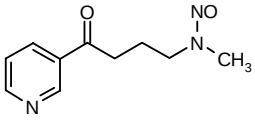
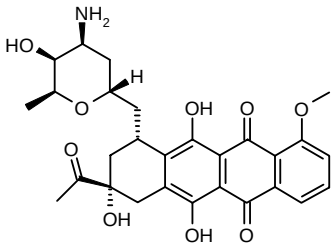
Oxidative and reductive activities: native AKR1B10 was incubated with the given substrates and the reaction rate was determined as detailed in "Materials and Methods"

Substrate	Concentration [mM]	Reaction rate [nmol / (min mg)]
DL-glyceraldehyde	1.0	450; 490
(*S*)-1-indanol	0.5	1100; 1000
(*R*)-1-indanol	0.5	110
naltrexone	0.05	4
dolasetron	0.05	94

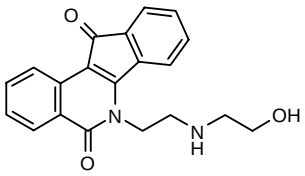
DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 3

Kinetic constants for native and recombinant AKR1B10 with ketones and (S)-indanol. Data obtained with the recombinant enzyme are in brackets.

substrate		conc. [mM]	K_m [a] [mM]	k_{cat} [b] [min^{-1}]	k_{cat}/K_m [a] [$mM^{-1} \cdot min^{-1}$]	df / r^2 [c]
	dolasetron	0.010 – 0.27	0.080	11	130	6 / 0.9994
		0.020 – 0.40	0.090	16	180	3 / 0.9995
		0.020 – 0.42	0.060	6.9	115	7 / 0.962
	(*S*)-1-indanol	0.25 - 4.0	0.76 ± 0.05 (2.2 ± 0.04)	85 ± 2 (39 ± 3)	112 (18)	7 / 0.986
	NNK	0.05 – 1.5	0.41 ± 0.06 (0.63 ± 0.08)	0.19 ± 0.009 (0.18 ± 0.011)	0.47 (0.29)	7 / 0.966
	dauno-rubicin [d]	0.15 – 2.0	1.1 ± 0.18	1.4 ± 0.16	1.3	9 / 0.980

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

	oracin [e]	0.02-0.75	0.14 ± 0.02 (0.29 ± 0.03)	0.06 ± 0.006 (0.0067 ± 0.0003)	0.43 (0.023)	7 / 0.980

[a] K_m- and k_{cat}/K_m-values ± standard deviation were determined from four independent experiments in the given concentration range as detailed in "Materials and Methods".

[b] k_{cat} calculated for Mr 36 kDa.

[c] df = degrees of freedom; r = regression coefficient.

[d] Data were fitted to the eqation $v = v_{max} \frac{S^n}{S^n + K_{0.5}^n}$, where v = reaction rate, S = substrate concentration, $K_{0.5}^n$ = substrate concentration at half maximal rate and n = Hill coefficient (n = 1.5).

[e] Data were fitted to the eqation $v = v_{max} \frac{S}{K_m + S(1 + \frac{S}{K_S})}$, where K_S = substrate inhibition constant (K_S = 1.2 mM).

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 4

Inhibition of dolasetron reduction [a]

Inhibitor	Concentration [µM]	Inhibition [%]
lithocholic acid	1	5
	20	84
phenolphthalein	10	22
medroxyprogesterone acetate	5	5
	20	39
	50	58
dexamethasone	100	54
flufenamic acid	100	40
ibuprofen	100	14
barbital	1000	24
sulfobromophthalein	20	0

[a] Native AKR1B10 (0.5 µg in 0.36 ml) was incubated with 0.05 mM dolasetron and the indicated concentrations of inhibitors dissolved in methanol resulting in a final methanol concentration of 1 or 2 % (see Materials and Methods). Positive controls contained equal amounts of methanol without inhibitor.

Fig. 1

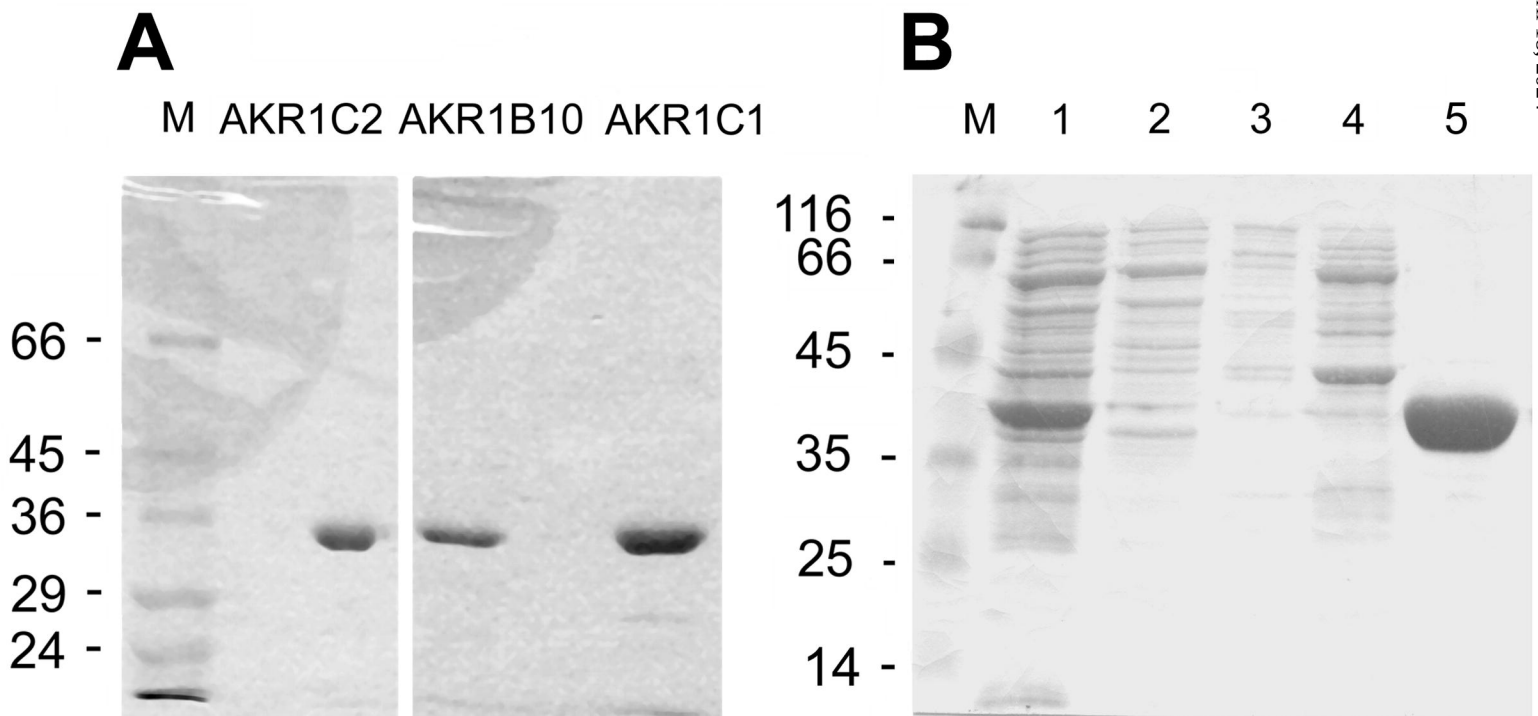


Fig. 2

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.

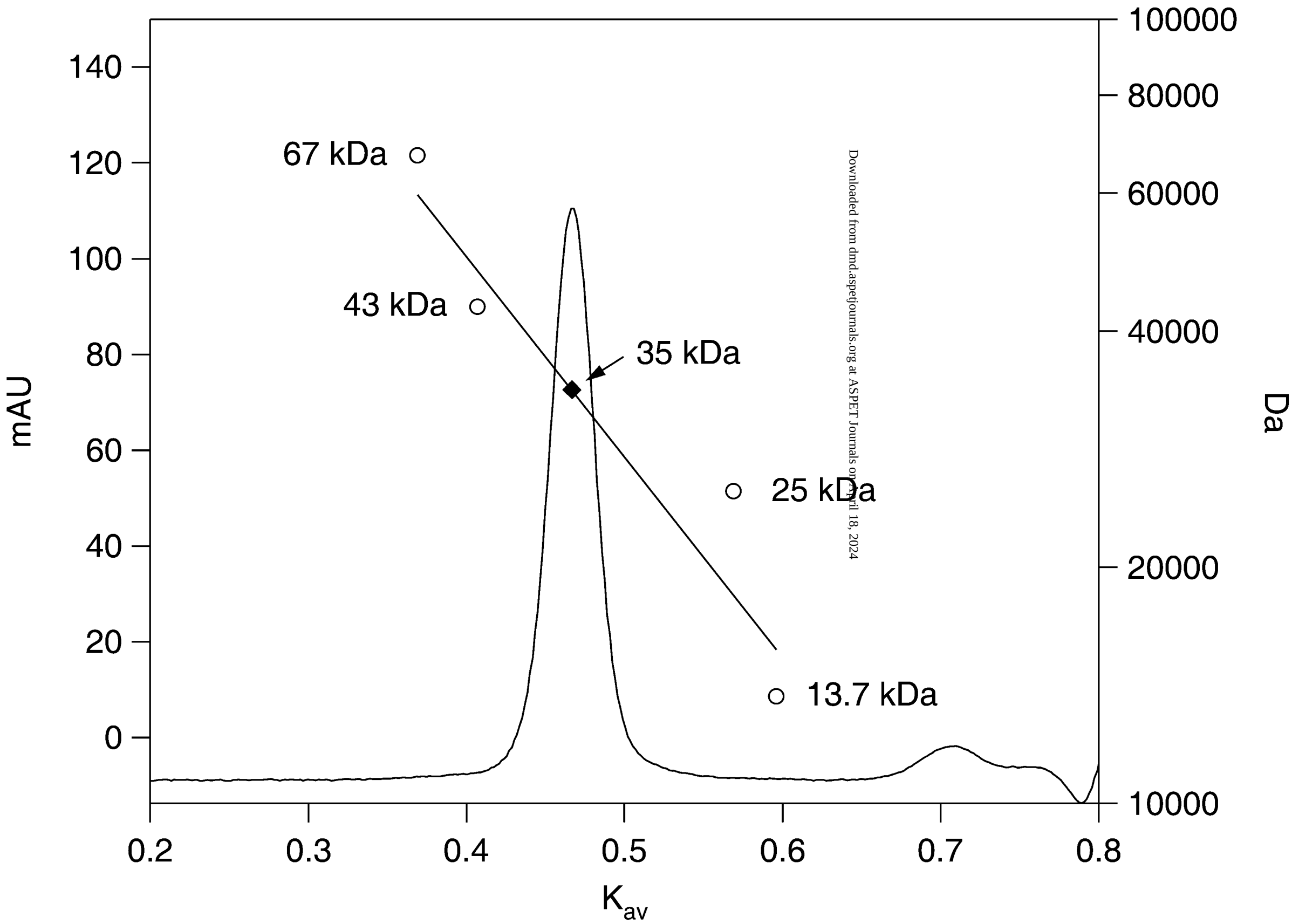


Fig. 3

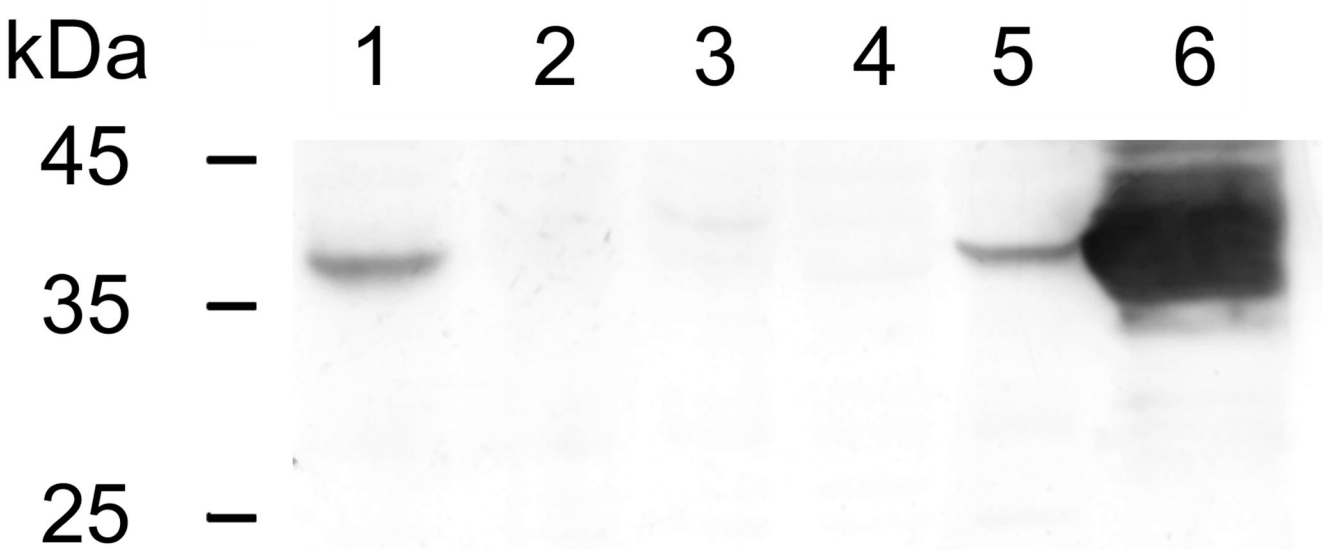


Fig. 4

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.007971
This article has not been copyedited and formatted. The final version may differ from this version.