Biochemical Pharmacology, Vol. 59, pp. 249–260, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00319-6

High-affinity Stereoselective Reduction of the Enantiomers of Ketotifen and of Ketonic Nortriptyline Metabolites by Aldo–Keto Reductases from Human Liver

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ABSTRACT. Aldo–keto reductases (AKR) form an enzyme superfamily catalyzing the reduction of carbonyl compounds and in some cases the reverse oxidation of alcohols as well. In particular, a role in drug metabolism has been considered for the AKR1C family, but published data failed to reveal low K_m drug substrates. Moreover, structure–activity relationships using chemically related substrates have not been established. In the present investigation, a modified procedure was developed for the isolation of AKR1C1, 1C2, and 1C4 (dihydrodiol dehydrogenases 1, 2, and 4) from human liver cytosol along with carbonyl reductase (EC 1.1.1.184), a member of the short-chain alcohol dehydrogenase superfamily. The kinetics of NADPH-dependent reduction by the closely related enzymes AKR1C1 and 1C2 were studied with the structurally similar substrates (*R*)- and (*S*)-ketotifen and E- and Z-10-oxonortriptyline by HPLC measurement of the products. K_m values varied between 2.6 and 53 μM and V_{max} values between 5 and 313 mU/mg protein; substrate inhibition with K_i around 30 μM occurred in the reduction of E- and Z-10-oxonortriptyline by AKR1C1. The reactions were strictly stereospecific with production of one enantiomeric alcohol from each ketotifen enantiomer and of the (+)-enantiomers of E- and Z-10-hydroxynortriptyline. Enzymatic $NADP^+$-dependent oxidation of the alcohols mirrored the reduction with regard to stereochemical specificity. All four ketones were no or poor substrates of carbonyl reductase, whereas haloperidol was reduced by this enzyme with low affinity, but high efficiency. BIOCHEM PHARMACOL **59**;3:249–260, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. aldo–keto reductase, human; dihydrodiol dehydrogenase; carbonyl reductase; ketone reduction, stereoselectivity; structure–activity relationship; nortriptyline metabolites; ketotifen; haloperidol

Redox reactions between endobiotic and xenobiotic carbonyl compounds and the corresponding alcohols can be catalyzed by enzymes from various superfamilies. In man, a number of enzymes belonging to the aldo–keto reductase [1] and short-chain alcohol dehydrogenase superfamilies [2] have been characterized and found to share some enzymatic properties in the absence of significant sequence homologies [3]. Recently, a nomenclature system has been proposed for the aldo–keto reductases that is based on structural similarities and comprises seven families with the mammalian aldo–keto reductases in family 1 (AKR1†) [1]. While human aldehyde reductase (AKR1A1) and aldose reductase (AKR1B1) catalyze the reduction of biogenic aldehydes [4], hexoses and xenobiotic aldehydes [5, 6], members of the AKR1C subfamily prepared from human liver cytosol, reduce a variety of ketonic substrates [7, 8]. Their ability to mediate the reversible oxidation of 3α- and 20α-hydroxysteroids led to the designation of AKR1C1 as 20α-hydroxysteroid dehydrogenase and of AKR1C4 as 3α-hydroxysteroid dehydrogenase type I [1]. Since they are further able to oxidize araliphatic alcohols and dihydrodiols derived from aromatic hydrocarbons [7], AKR1C1, 1C2, and 1C4 have been termed dihydrodiol dehydrogenase (DD) 1, 2, and 4, respectively. This $NADP^+$-dependent reaction may increase xenobiotic toxicity, since all three enzymes oxidize the two enantiomers of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene to the highly reactive *o*-quinone benzo[*a*]pyrene-7,8-dione [9]. The high affinity of AKR1C2 towards lithocholate led to its description as bile acid binder [10], and AKR1C4 was previously known as chlordecone reductase due to its enzymatic activity towards the insecticide chlordecone [11]. Though the aldo–keto reductases exhibit high affinity in reducing 3- or 20-oxosteroids [7, 8, 12], their physiological role is still unclear.

CR (EC 1.1.1.184) has been detected in and purified from various human tissues under different designations [3, 4, 13], the most detailed information being available on the

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† *Abbreviations:* AKR, aldo–keto reductase; CL_{int}, intrinsic clearance; CM, carboxymethyl; CR, carbonyl reductase; DD, dihydrodiol dehydrogenase; DEAE, diethylaminoethyl; HL, human liver; 10-OH-NT, 10-hydroxynortriptyline; 10-Oxo-NT, 10-oxonortriptyline; and PBE, polybuffer exchanger.

Received 22 February 1999; accepted 1 August 1999.

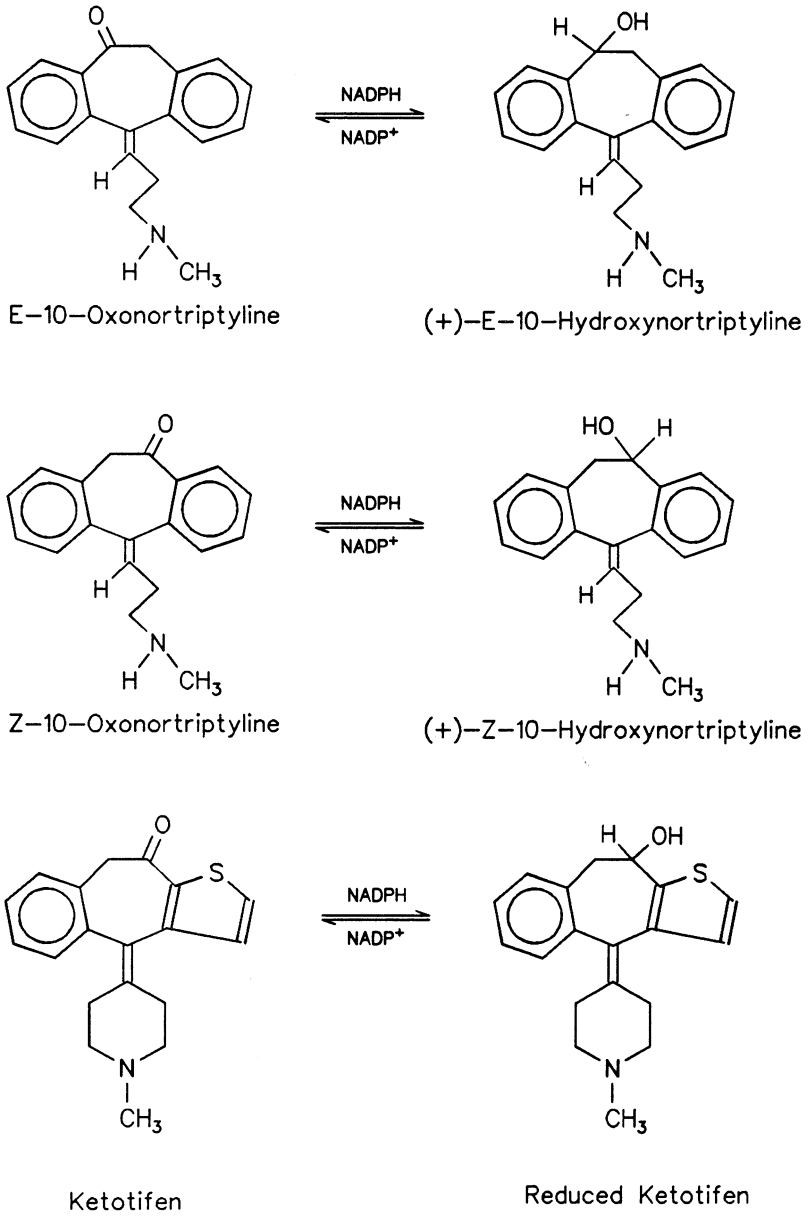

FIG. 1. Structural formulas of ketones and alcohols interconverted by aldo–keto reductases. Ketotifen consists of the *(R)*-(+)- and *(S)*-(−)-enantiomers, from which the (+)- and (−)-enantiomers, respectively, of reduced ketotifen are produced.

enzyme isolated from brain [14]. Though it is able to catalyze redox reactions at the 9- and 15-positions of prostaglandins [13, 14], a contribution to prostaglandin metabolism *in vivo* is unlikely in view of the high K_m values [15]. CR is, however, efficient in reducing quinones and may have a role in their detoxication [16]. It also reduces 3- and 17-oxosteroids and a variety of aldehydes and ketones [14, 17].

The reduction kinetics of a number of drugs from various pharmacological groups have been measured using purified AKR1C1, 1C2, 1C4, CR, and aldehyde reductase (AKR1A1) [18]. Catalytic efficiencies varied considerably, but none of the ten drugs was reduced by any of the five enzymes with a K_m value below 190 μM. Values in the low micromolar range had, however, been observed in studying the reduction of ketonic nortriptyline metabolites in human liver cytosol [19]. When the secondary alcohols E- or Z-10-hydroxynortriptyline, which are major metabolites of the antidepressants amitriptyline and nortriptyline [20, 21], were incubated with human liver microsomes in the presence of NAD^+ or other cofactors, the only products detected were the ketones E- and Z-10-Oxo-NT, respectively (Fig. 1, [19]). The (+)-enantiomers of the chiral alcohols were oxidized preferentially, and the reverse NADPH-dependent reduction by human liver cytosol converted E- and Z-10-Oxo-NT nearly exclusively to the (+)-enantiomers of E- and Z-10-hydroxynortriptyline. This reduction was also catalyzed by liver cytosol from rabbit, but not from rat or guinea pig. Preferential incorporation of the pro-4R hydrogen of tritium-labeled NADPH pointed to the

involvement of an aldo–keto reductase [19]. E-10-Oxo-NT occurs as a minor amitriptyline metabolite in human urine [22].

The present investigation aimed at identifying the human liver enzyme(s) catalyzing the high-affinity enantioselective reduction and therefore necessitated their preparation from cytosol. A procedure from the literature [7] was modified in a way so as to enable the simultaneous isolation of CR. The enantiomers of ketotifen, which bear a great chemical similarity to the 10-Oxo-NT isomers, were included in order to obtain more data on the structure–activity relationships of the enzymatic reactions. The chirality of the antiasthmatic drug ketotifen (Fig. 1) is due to a lack of symmetry in a molecule with a non-planar seven-membered ring. The stability of the conformers is ascribed to a high energy barrier caused by the oxo group that prevents chiral inversion at room temperature [23]. Racemic ketotifen has been demonstrated to undergo ketone reduction to the alcohol reduced ketotifen as a major metabolic pathway in man *in vivo* and in human hepatocytes [24, 25]. Cytosolic enzymes were assumed to be responsible, because no reduction took place in human liver microsomes [26]. Nothing has been published on the stereochemistry of the alcohol(s). The reduction kinetics of haloperidol were also studied, since this is an araliphatic ketone as are the other substrates and was reported to have been reduced by AKR1C1 with a relatively low K_m value [18]. Some of the results have been the subject of a preliminary report [27].

MATERIALS AND METHODS

Materials

Human liver samples were kindly supplied by Prof. W. Lauchart, Department of Surgery, University of Tübingen. These were either samples from livers excluded from transplantation for medical reasons or excess normal tissue obtained on partial hepatectomy for tumor metastases. They were cut into pieces of 5–10 g and stored at −80°. (*R*)-(+)- and (*S*)-(−)-ketotifen and racemic reduced ketotifen (free bases) were generously donated by Novartis Pharma AG, E- and Z-10-Oxo-NT and synthetic racemic E- and Z-10-OH-NT by A. Jørgensen, and haloperidol and reduced haloperidol by Janssen Pharmaceutica. 10-OH-NT enantiomers were obtained as described previously [19]. Pyridine nucleotide cofactors and glucose-6-phosphate dehydrogenase were purchased from Boehringer, Sephadex G-100, Q Sepharose, CM Sepharose, DEAE Sepharose, Polybuffer Exchanger PBE 94, and the Polybuffers 74 and 96 from Pharmacia, hydroxyapatite Bio-Gel HTP from Bio-Rad, Reactive Red 120 agarose, (*S*)-(+)- and (*R*)-(−)-1-indanol, and the molecular weight standard Dalton Mark VII-L for protein electrophoresis from Sigma–Aldrich, a 50-mL ultrafiltration chamber and filters YM10 from Amicon, the carrier ampholytes Servalyt 6–9 and 9–11 from Serva, and Resolyte 3.5–10 and 6–9.5 from Merck.

Enzyme Isolation

Liver homogenates were prepared with four volumes of buffer (250 mM sucrose, 20 mM Tris–HCl, 5 mM EDTA, adjusted to pH 7.4 at 37°) and cytosol was obtained by fractionated centrifugation, in the last step at 85,000 *g* for 1 hr. Samples were concentrated to half their volumes by ultrafiltration and stored at −80°. Quantities corresponding to 5–7 g of liver were applied to a 2.5 × 100-cm column with Sephadex G-100 in 25 mM Tris–HCl buffer pH 7.4 and eluted with the same buffer within 36 hr in fractions of 5 mL. These were tested for Z-10-Oxo-NT reduction in the presence of NADPH, and active fractions were concentrated to about 0.5 mL by ultrafiltration. As in all further fractionations, the medium for storage of the concentrates was changed to buffer A (see below) by twice adding 3 mL and concentrating to 0.5 mL. Samples were stored at −80° in 1–1.5 mL buffer A.

Separation of gel filtrates into three fractions was achieved on a 2.5 × 10-cm column with Q Sepharose [7]. The column was eluted at 1 mL/min with 100 mL buffer A (400 mL 6.25 mM Tris + 0.625 mM EDTA adjusted to pH 8 with HCl and 100 mL glycerol, with 2-mercaptoethanol added to a concentration of 5 mM before use) followed by a linear gradient to 0.1 M NaCl in buffer A within 4 hr (240 mL) and 100 mL 0.5 M NaCl in buffer A. Fractions (5 mL) were measured for absorption at 280 nm, tested for enzymatic activities, and combined to the following concentrates: Q1 (fractions 10–24) containing Z-10-Oxo-NT reductase and (*S*)-1-indanol oxidase activities; Q2 (fractions 42–62) with Z-10-Oxo-NT and 4-benzoylpyridine reductase activities; Q3 (fractions 63–75) with (*S*)-1-indanol oxidase and 4-benzoylpyridine reductase activities.

Ion-exchange chromatography on 1 × 13 cm CM Sepharose served for purifying Q1 and Q2 by elution at 1 mL/min with 10 mM sodium phosphate + 0.5 mM EDTA adjusted to pH 6.5 and mixed with 5 mM 2-mercaptoethanol. A linear NaCl gradient was started from the beginning (Q1) or after 20 min (Q2) and attained 0.1 M within 60 min. The column was purified by 0.5 M NaCl in phosphate buffer. Enzymatic activities were present in single peaks: Q1–CM eluted with 0.1 M NaCl and Q2–CM eluted with 0.05–0.09 M NaCl. Pure AKR1C1 (DD1 of Hara *et al.* [7]) was obtained from Q1–CM by chromatofocusing on a 0.9 × 56-cm column with PBE 94. The column was equilibrated with 25 mM imidazole adjusted with HCl to pH 7.8 at room temperature and containing 5 mM 2-mercaptoethanol. The sample was eluted in 3-mL fraction at 10 mL/hr with Polybuffer 96 diluted with 12 volumes of water, adjusted to pH 6 with HCl, and mixed with 5 mM 2-mercaptoethanol. Following a protein peak without enzymatic activity, AKR1C1 was eluted in fractions 21–30 at a pH of 8.1 measured at 0°. AKR1C2 (DD2 of Hara *et al.* [7]) and CR contained in Q2–CM were separated on a 1.1 × 9.5-cm column of Bio-Gel HTP equilibrated with 10 mM Tris–HCl pH 7.5 containing 5 mM 2-mercaptoethanol. At a rate of 1 mL/min, a linear gradient was run by which the Tris

buffer was replaced by 0.2 M sodium phosphate pH 7.5 within 2 hr. 4-Benzoylpyridine reductase activity (corresponding to CR) was eluted with 0.06–0.1 M phosphate and Z-10-Oxo-NT reductase activity (AKR1C2) with 0.11–0.16 M phosphate. The two fractions were purified separately by chromatofocusing on PBE 94 equilibrated with 25 mM imidazole pH 7.4 containing 5 mM 2-mercaptoethanol. Polybuffer 74 diluted with 7 volumes of water, adjusted to pH 5, and mixed with 5 mM 2-mercaptoethanol served for elution in 3-mL fractions. CR I and II were eluted in two peaks in fractions 24–32 at pH 7.6 and in fractions 39–45 at pH 7.4–7.25 that were collected separately. AKR1C2 was contained in fractions 32–39 at pH 7.45–7.3.

Q3 was applied to a 3-mL column of Reactive Red 120 agarose [11] equilibrated with 10 mM Tris–HCl pH 7.5 containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol. After 17 hr at 4°, the majority of protein and a small quantity of 4-benzoylpyridine reductase activity were eluted with 20 mL of the above buffer containing 20% glycerol. Addition of 2.5 M NaCl to 25 mL of the buffer with glycerol led to the elution of 4-benzoylpyridine reductase and (*S*)-1-indanol oxidase activities (Q3–R). For their separation by chromatofocusing, Polybuffer 74 diluted eightfold was adjusted to pH 4 and contained 20% glycerol, while 2-mercaptoethanol was omitted. Around pH 7.1 and 6.9, peaks containing CR II and III appeared, were collected separately, and purified on CM Sepharose as described above for Q1. Further elution of the PBE column resulted in three peaks of (*S*)-1-indanol oxidase activity between pH 6.3 and 5.6 that contained AKR1C4 (DD4 of Hara *et al.* [7]). From the first peak, AKR1C4 I was purified on 1.1 × 9.5 cm of Bio-Gel HTP (see above), the enzyme being eluted at 0.05–0.07 M phosphate. For final purification, it was loaded on a 1 × 10-cm column with DEAE Sepharose equilibrated with 10 mM Tris–HCl pH 7.9 containing 5 mM 2-mercaptoethanol. A linear gradient of NaCl in this buffer achieved 0.1 M within 60 min and AKR1C4 I was eluted at 0.08 M NaCl. The second (*S*)-1-indanol oxidase peak was loaded on a column of 10 mL Reactive Red 120 agarose. After 30-min equilibration, elution was started with 10 mM Tris–HCl pH 7.5 containing 5 mM 2-mercaptoethanol and 20% glycerol to which a linear gradient of NaCl was added that attained 3 M after 150 min. During the subsequent 90 min of isocratic elution, AKR1C4 II was obtained as a peak with a sharp rise and slow decline. The same purification was attempted with the third (*S*)-1-indanol oxidase peak, but contaminating proteins could not be removed completely. Protein was measured according to Bradford [28] with bovine serum albumin as standard. The purity of protein fractions was checked by SDS–PAGE [29]. For isoelectric focusing, the polyacrylamide gel contained Servalyt and Resolyte buffers in the pH range 3.5–11. Partial amino acid sequencing was carried out on tryptic digests by 4base lab.

Measurement of Enzyme Activities

All tests were carried out at 37° in a medium containing 100 mM Tris–HCl adjusted to pH 7.4 at 37°, 8 mM $MgCl_2$, and 25 mM KCl. When reductase activities were determined by HPLC measurement of the alcoholic products, the total volume was 0.36 mL and NADPH was generated from 0.2 mM $NADP^+$, 2 mM glucose 6-phosphate, and 1 U/mL glucose-6-phosphate dehydrogenase. The corresponding oxidative reactions were measured in the presence of 0.8 mM $NADP^+$. After a preincubation of 5 min, the reaction was started by the addition of substrate and stopped after 4–20 min by mixing with 0.04 mL 2 N perchloric acid and cooling on ice. When haloperidol was the substrate, reactions were stopped by sulfuric acid in order to prevent precipitation of haloperidol perchlorate. Following centrifugation, the supernatant was injected for HPLC. Substrate concentrations were chosen to comprise the range from 4- to 5-fold below to 4- to 5-fold above the K_m value. For testing of fractions obtained in protein purification, 10 μM Z-10-Oxo-NT was incubated for 10 min. For reductase determination by the optical test at 339 nm in a volume of 0.6 mL, NADPH was added at 0.1 mM, while oxidase activity was measured with 0.25 mM $NADP^+$. Substrate concentrations were 1 mM for 4-benzoylpyridine, 0.25 mM for menadione, and 0.5 mM for (*S*)-1-indanol.

Chromatographic Analyses

Of the supernatants containing alcoholic or ketonic reaction products, 0.2 mL was applied to an HPLC clean-up column of 5 × 6 mm filled with coarse C_{18} silica gel particles by pumping 30 mM perchloric acid adjusted to pH 2.5 with NaOH (1.5 mL/min) for 2 min. Adsorbed substances were transferred to the separation column by running eluent in the reverse direction for 1 min, and the clean-up column was conditioned with buffered perchloric acid for 2.5 min. Separation took place on a 4.6 × 250-mm column with 5 μm C_{18} silica gel (Nucleosil 5 C_{18}, Macherey–Nagel,) eluted at 1.2 mL/min with 10 mM perchloric acid adjusted to pH 2.5/acetonitrile. The volume ratios and detection wavelengths were varied according to product resulting in the following retention times: 70:30 reduced ketotifen (240 nm) 8.3–11.1 min, ketotifen (300 nm) 14 min; 64:36 E-10-OH-NT (240 nm) 7.3 min, E-10-Oxo-NT (310 nm) 9.8 min; 64:36 Z-10-OH-NT (240 nm) 8.8 min, Z-10-Oxo-NT (310 nm) 10.5 min; and 62:38 reduced haloperidol (220 nm) 10.3 min, haloperidol 13 min. Evaluation was based on peak heights of products and external standards, the latter giving strictly linear calibration curves when 0.2–5 μM solutions were injected. Reduced ketotifen was eluted as two peaks connected by a low plateau. The sum of the heights of the two peaks was used for quantitation. The isomers contained in the two peaks are interconvertible, as shown by sampling from the HPLC eluate and re-injection. The same elution pattern as the original

resulted whether the first peak, the plateau, or the second peak had been recovered. A similar separation followed by rapid isomerization occurred when HPLC was carried out with a buffer of pH 6 instead of pH 2.5. Enantiomer analysis of E- and Z-10-OH-NT produced by ketone reduction was performed as described previously [30].

Separation of Ketotifen and Reduced Ketotifen Enantiomers

The ketotifen enantiomers could be separated by HPLC without a precolumn on β-cyclodextrin-bonded silica gel (ChiraDex LiChroCART 4 × 250 mm, Merck). Using 10 mM phosphoric acid adjusted to pH 4 with NaOH/acetonitrile (95:5) as eluent at 1 mL/min, (*S*)-ketotifen had a retention time of 7 min and (*R*)-ketotifen of 16.8 min. The alcohols resulting from enzymatic reduction of the two enantiomers were eluted at 7.7 and 16 min, respectively, and each exhibited a reversible isomerization to a product eluted shortly after the void volume. The chiral column served for semipreparative separation of synthetic reduced ketotifen into the enantiomers with retention times of 7.7 and 16 min. Their opposite optical configurations were confirmed by measuring circular dichroism in the Jasco J-720 spectropolarimeter. By extrapolation from the spectra and by direct measurement of optical rotation at 589 nm, it could be shown that reduced (*S*)-ketotifen was levorotatory. Racemization of the alcohols occurred with a half-time of 50 min in 0.1 M HCl at room temperature. Slow racemization was observed in 0.01 M HCl at 4°, while a decomposition product was also visible, probably resulting from elimination of water. The enantiomers were optically stable in near-neutral solution at 4°.

For investigations of the stereoselectivity of the enzymatic redox reactions, alkalinized incubates of the ketotifen enantiomers were extracted with *tert*-butyl methyl ether. By TLC of the extract residue on silica gel in *tert*-butyl methyl ether/1-butanol/triethylamine (5:1.6:0.4, v/v), reduced ketotifen (R_F (retardation factor) 0.65) was separated from excess ketotifen (R_F 0.50) and isolated by suspending the UV-absorbing band in 2 N ammonia and extracting with *tert*-butyl methyl ether. It was subjected to enantiomer analysis by HPLC and to enzymatic reoxidation with subsequent enantiomer analysis of the formed ketotifen following its extraction from the incubate.

Calculations

Kinetic parameters were calculated according to the Michaelis–Menten equation by non-linear least-squares regression analysis (Fig. P, Biosoft). Intrinsic clearance (CL_{int}) was calculated as V_{max}/K_m or, if substrate concentrations were far below K_m, as reaction rate/substrate concentration. In cases of substrate inhibition, a formula for non-competitive inhibition was used. Substrate concentrations used for kinetic calculations were those attained at the mid-time of incubation in order to correct for substrate

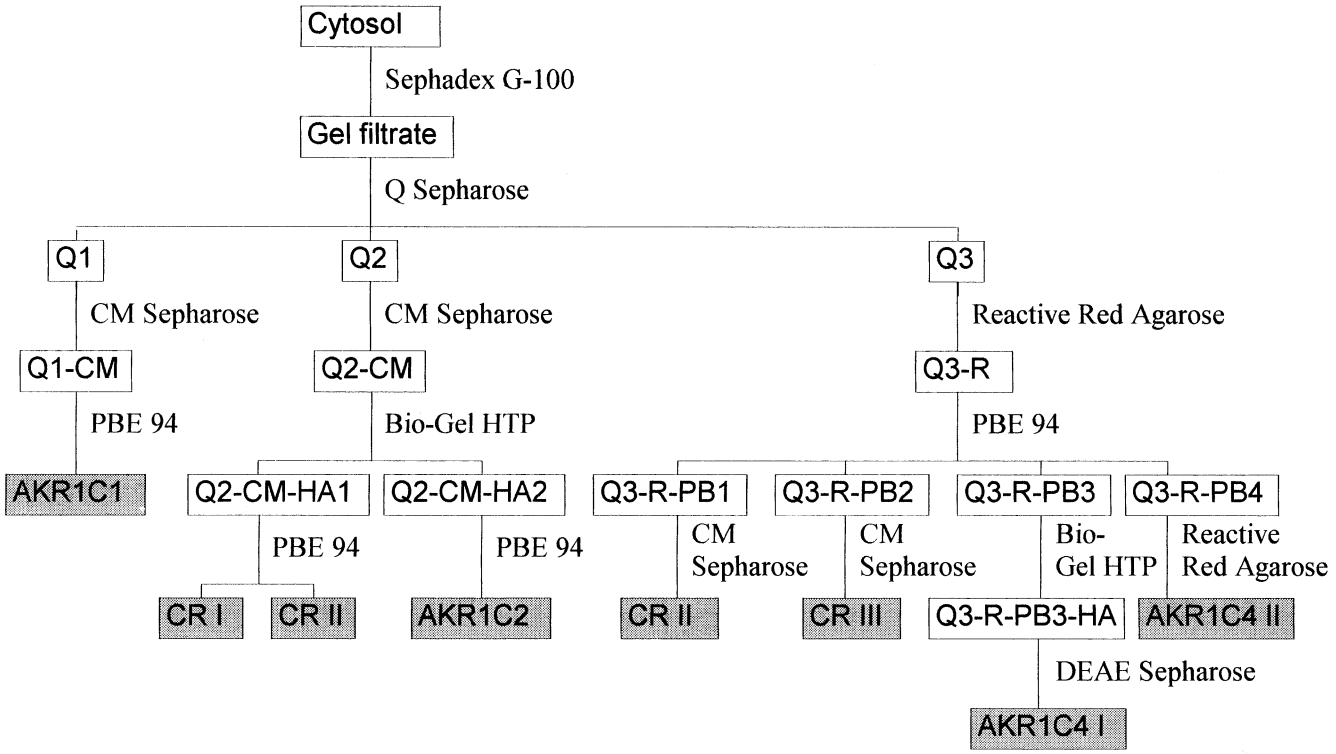

FIG. 2. Fractionation scheme for the preparation from human liver cytosol of pure aldo–keto reductases and carbonyl reductase isoforms (shown on grey background).

TABLE 1. Fractionation of cytosol from HL 17 (25 g) and specific activities of fractions measured with Z-10-Oxo-NT (10 μM), (*S*)-1-indanol (0.5 mM), or 4-benzoylpyridine (1 mM)

Purification by	Resulting fraction or enzyme	Protein (mg)	Specific activity (mU/mg) with		
			Z-10-Oxo-NT	(*S*)-Indanol	4-Benzoylpyridine
—	cytosol	1700	0.8		
Sephadex G-100	gel filtrate	82	30	230	200
Q Sepharose	Q1	9.1	53	1130	
CM Sepharose	Q1–CM	5.1	69	1215	
PBE 94	AKR1C1	2.4	190	2100	
Q Sepharose	Q2	28	31	195	350
CM Sepharose	Q2–CM	11	55	330	
Bio-Gel HTP	Q2–CM–HA1	1.3			3200
PBE 94	CR I	0.11			7000
	CR II	0.15			5000
Bio-Gel HTP	Q2–CM–HA2	4.8	88	450	
PBE 94	AKR1C2	1.6	145	780	
Q Sepharose	Q3	30	3.3	97	167
React. Red Agar.	Q3–R	8		460	590
PBE 94	Q3–R–PB1	0.18			5200
CM Sepharose	CR II	0.1			7000
PBE 94	Q3–R–PB2	0.6			1700
CM Sepharose	CR III	0.12			6300*
PBE 94	Q3–R–PB3	1.5		710	
Bio-Gel HTP	Q3–R–PB–HA	0.6		1140	
DEAE Sepharose	AKR1C4 I	0.3		1300	
PBE 94	Q3–R–PB4	0.9		700	
React. Red Agar.	AKR1C4 II	0.55		1150	

*Specific activity of an enzyme exhibiting a contamination on electrophoresis.
HA, Bio-Gel HTP; R, Reactive Red 120 agarose; and PB, PBE 94.

consumption. Variations given are standard deviations (SD).

RESULTS

Enzyme Purification

The fractionation scheme is illustrated in Fig. 2 and the enzyme quantities recovered are presented in Table 1. Z-10-Oxo-NT reductase activities measured in gel filtrates exceeded those found in cytosol by a factor of 2–3, apparently due to the removal of inhibitors. Recoveries of enzyme activities in the subsequent steps usually were between 40 and 90%, and in the cases of AKR1C1 and 1C2 were the same when measured with Z-10-Oxo-NT and (*S*)-1-indanol as substrates.

The aldo–keto reductases AKR1C1 and 1C2 (DD1 and DD2) were obtained in eletrophoretically pure form with estimated molecular masses of 36 kDa (Fig. 3) in good agreement with values predicted from cDNAs of about 36.8 kDa [12]. The preparation could be simplified in comparison to one described previously [7] by omission of the initial ammonium sulfate precipitation and of one purification step for AKR1C1. In contrast to an earlier finding [7], this protein was eluted as a single peak from CM Sepharose. Partial amino acid sequencing revealed that a tryptic fragment of AKR1C2 matched amino acids 305–318 of DD2 [12]. While this sequence is common to AKR1C1 and AKR1C2, the two proteins could be unambiguously differentiated by their order of elution from Q Sepharose (Fig. 2 and Ref. 7) and the pI 8.0 value of AKR1C1 (8.4 in Ref. 12) in comparison to pI 7.25 of AKR1C2 (7.6 in Ref. 7). Moreover, in accordance with the literature, AKR1C1 oxidized (*S*)-1-indanol with a much lower K_m and higher specific activity than AKR1C2 and required far higher concentrations of lithocholic acid and ibuprofen for inhibition of (*S*)-1-indanol oxidation (Table 2). For AKR1C4 (DD4), the separation into three forms by chromatofocusing [31] with different pI [7] was confirmed. Those purified to homogeneity exhibited molecular masses of 36 kDa (Fig. 3) and pI values of 6.3 (AKR1C4 I) and 5.9 (AKR1C4 II), respectively. Their enzymatic properties with regard to the oxidation of (*S*)- and (*R*)-1-indanol, acenaphthenol and cyclohexanol, as well as to the inhibitory potencies of medroxyprogesterone acetate and dexamethasone, were in good agreement with data published on the mixture of isoforms (Table 2).

Enzymes identified as CR with high specific activities in reducing 4-benzoylpyridine or menadione were recovered from the Q2 and Q3 fractions. In accordance with Inazu *et al.* [17], chromatofocusing led to the separation of three isoforms that differed by their isoelectric points. The most basic form (pI 7.8, CR I) was present in the Q2 fraction only and the most acidic form (pI 6.7, CR III) in the Q3 fraction, while both fractions contained the form with intermediate pI (7.3, CR II). CR III exhibited a small

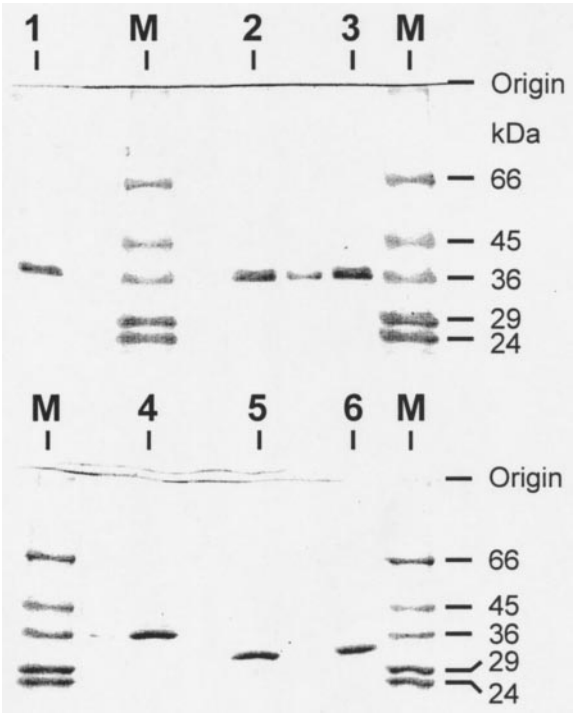

FIG. 3. Purity of isolated enzymes as demonstrated by SDS–PAGE. Lane 1: AKR1C1; lane 2: AKR1C2; lane 3: AKR1C4 I; lane 4: AKR1C4 II; lane 5: CR I; lane 6: CR II; lanes M: molecular weight markers. In some cases, empty lanes contained enzymes spilled over from adjacent lanes.

admixture of a protein of 36 kDa on gel electrophoresis, while the main bands had apparent molecular masses of 32–33 kDa (Fig. 3) in accordance with predicted masses of about 30.4 kDa [32]. Partial amino acid sequence analysis of CR I from a Q2 fraction and of CR II from a Q3 fraction resulted in sequences that matched amino acids 113–119 of CR [32]. The two preparations were also compared with regard to their substrate spectra. The relative rates at which they reduced 1 mM 4-benzoylpyridine, 0.25 mM menadione, and 1 mM 4-nitrobenzaldehyde were 1:1.8:0.56 and 1:1.6:0.54, respectively. All forms were inhibited by 80–90% by 10 μM rutin, a potent inhibitor of CR [14], whereas 1 mM barbital, an aldehyde reductase inhibitor, reduced the activity by only 20–30%. The enzymes were nearly devoid of Z-10-Oxo-NT reductase or (*S*)-1-indanol oxidase activity, but they slowly reduced E-10-Oxo-NT at concentrations around 100 μM.

Kinetics of Reductive and Oxidative Reactions

The kinetics of alcohol production from the ketotifen enantiomers and the isomers of 10-Oxo-NT differed between the closely related AKR1C1 and 1C2 and between substrates. Each reaction was investigated with purified enzymes from three different livers and the data were in good agreement (Table 3). The highest affinities towards both enzymes (K_m around 3 μM) were observed with Z-10-Oxo-NT closely followed by that of (*S*)-ketotifen towards AKR1C2, whereas the K_m value for its reduction by AKR1C1 was 15-fold higher. V_{max} values varied 20-fold among the four ketotifen–enzyme combinations and 12-fold among those of 10-Oxo-NT isomers and enzymes. AKR1C1 was inhibited by high concentrations of Z- and E-10-Oxo-NT, and a small inhibitory effect was exerted by E-10-Oxo-NT on AKR1C2 (Table 3). The differences among the four substrates in the reduction kinetics by AKR1C1 are illustrated by Fig. 4. AKR1C4 I produced traces of alcoholic metabolites from Z-10-Oxo-NT and (*S*)-ketotifen, but none from (*R*)-ketotifen.

AKR1C1 and 1C2 reduced all four substrates with strict product stereoselectivity. The Z-10-OH-NT resulting from incubation of Z-10-Oxo-NT with AKR1C1 contained 98% of the (+)-enantiomer and that formed by AKR1C2 at least 99%. (+)-E-10-OH-NT represented 97% or more of the alcohol produced from E-10-Oxo-NT by AKR1C1, AKR1C2, or CR. One alcohol represented 98% or more of the reduction products of each of the ketotifen enantiomers, reduced ketotifen with the shorter retention time in HPLC (7.7 min) being the product of the faster eluting (*S*)-ketotifen. The fact that the two isomers of reduced ketotifen were enantiomers could be demonstrated by their circular dichroism spectra. Reduced (*S*)-ketotifen exhibited a negative band around 230 nm and a positive one around 260 nm, while the reverse applied to reduced (*R*)-ketotifen.

Complete kinetic data on haloperidol reduction at physiological pH could not be obtained, because the solubility of haloperidol is too low [33] to achieve saturation of AKR1C1, AKR1C2, or CR. Since the reaction rate depended linearly on the substrate concentration in the range 5–100 μM, CL_{int} values could be derived from the ratio rate/concentration, resulting in 0.015 and 0.03 mL/min per mg protein for AKR1C1 and 1C2, respectively ($r^2 > 0.99$, $P < 0.001$). K_m values with these enzymes were around 1 mM. The isoforms CR I, II, and III of carbonyl reductase exhibited CL_{int} of 4.2, 3.9, and 2.7 mL/min per mg protein, respectively, and an attempt to saturate CR I with haloperidol resulted in a K_m value above 1 mM.

With regard to stereoselectivity, the enzymatic oxidation of alcohols to ketones mirrored the corresponding reductions. With $NADP^+$ as cofactor, 20 μM (−)-Z-10-OH-NT was oxidized by AKR1C1 and 1C2 at about 1% the rate of the (+)-enantiomer, while with (−)-E-10-OH-NT the corresponding values were below 0.5% of those of (+)-E-10-OH-NT. The oxidation of the enantiomers of reduced ketotifen with AKR1C1 led to ketotifen that consisted of 95% of the enantiomer from which the alcohol had been produced; using AKR1C2, the enantiomeric purity of the ketotifen isomers was at least 99%. Kinetic studies (Table 4) revealed that, except in the case of reduced (*S*)-ketotifen oxidation by AKR1C1, the K_m values of the alcohols exceeded those of the corresponding ketones 4- to 14-fold, whereas the V_{max} values were higher such that CL_{int} mostly was of the same order of magnitude for the two opposite reactions.

TABLE 2. Comparison of kinetic parameters for the oxidation of 1-indanol and other substrates by aldo–keto reductases measured in the present investigation and reported in the literature

Parameter	Enzyme	Present work	Data from literature
(*S*)-1-Indanol K_m (μM)	AKR1C1	37*	38 [12]
	AKR1C2	400*	500 [7]
	AKR1C4 I	305*	260 [7]
Oxidation rate of 0.5 mM (*S*)-1-indanol (mU/mg)	AKR1C1	310; 330	a: 626 [7] b: 235 [7]
	AKR1C2	74; 88	58 [7]
	AKR1C4 I	196; 240	217 [7]
Oxidation rate of other substrates (% of (*S*)-1-indanol oxidation rate)			
0.5 mM (*R*)-1-indanol	AKR1C1	15*	a: 14 [7] b: 13 [7]
	AKR1C2	20*	27[7]
	AKR1C4 I	65*	88 [7]
0.5 mM 1-acenaphthenol	AKR1C1	260*	a: 264 [7] b: 404 [7]
	AKR1C2	543*	601 [7]
	AKR1C4 I	112*	103 [7]
	AKR1C4 II	86*	
0.5 mM cyclohexanol	AKR1C1	5*	a: 0 [7] b: 0 [7]
	AKR1C2	0*	1.5 [7]
	AKR1C4 I	40*	23 [7]
	AKR1C4 II	39*	
Inhibition of oxidation of 0.5 mM (*S*)-1-indanol (%) by 1 μM medroxyprogesterone acetate	AKR1C1	66*	a: 62 [7] b: 62 [7]
	AKR1C2	70*	57 [7]
	AKR1C4 I	91*	94 [7]
	AKR1C4 II	97*	
Inhibition of oxidation of 1 mM (*S*)-1-indanol (%) by			
100 μM dexamethasone	AKR1C2	33*	14 [8]
	AKR1C4 I	92*	91 [8]
	AKR1C4 II	90*	
25 μM lithocholic acid	AKR1C1	43*	50 [12]
0.07 μM lithocholic acid	AKR1C2	36*	50 [12]
950 μM ibuprofen	AKR1C1	61*	50 [12]
6.9 μM ibuprofen	AKR1C2	36*	50 [12]
100 μM ibuprofen	AKR1C2	91*	86 [8]

*Values measured at 37°; other data were obtained at 25°.

DISCUSSION

To our knowledge, this is the first report describing a procedure for the parallel isolation of aldo–keto reductases and CR from human liver. Wermuth [14] described separation by DEAE Sepharose chromatography of purified CR from human brain into three fractions with isoelectric points of about 8.5, 8, and 7; these are in fair accordance with the pI values of 7.8, 7.3, and 6.7 observed herein. Less purified preparations contained an additional protein of pI 5.2 with very similar enzymatic properties. CR from human testis was separated by chromatofocusing into fractions designated CR I, II, and III eluting at pH 7.74, 7.63 and 7.28 [17], values only slightly higher than those observed here. Modification of the native most basic enzyme form by binding of 2-oxo-mono- or -dicarboxylic acids to a lysine residue and autocatalytic reduction of the condensation product is probably responsible for the occurrence of multiple forms [34]. The relative velocities in the reduction of 4-benzoylpyridine, menadione, and 4-nitrobenzaldehyde were in good agreement with those found with CR from human brain [17].

The aldo–keto reductases AKR1C1 and 1C2 were obtained by a simplified procedure. Only one form of AKR1C1 was detected in disagreement with the findings of Hara *et al.* [7], who described two forms (DD1a and DD1b), but in accordance with a later paper by the same group [12] in which only one native DD1 from human liver is mentioned. Its pI value was found to be 8.4 and thus

TABLE 3. Kinetic parameters for the reduction of *(R)*- and *(S)*-ketotifen and of Z- and E-10-Oxo-NT by aldo–keto reductases

Substrate	Enzyme	K_m (μM)	V_{max} (mU/mg)	K_i (μM)	CL_{int} (mL/min per mg)
(*R*)-Ketotifen	AKR1C1	10.9 (2.0)	5 (1)		0.44
	AKR1C2	7.5 (0.1)	58 (8)		7.7
(*S*)-Ketotifen	AKR1C1	53 (2.6)	92 (10)		1.7
	AKR1C2	3.6 (0.2)	100 (5)		28
Z-10-Oxo-NT	AKR1C1	2.6 (0.5)	313 (46)	32 (2.5)	120
	AKR1C2	3.1 (0.3)	173 (3)		56
E-10-Oxo-NT	AKR1C1	6.4 (1.3)	140 (10)	30 (5)	22
	AKR1C2	10.2 (1.4)	26 (0.7)	250 (126)	2.6

Values are means (SD) of three experiments with AKR1C1 isolated from cytosol of HL 15, 17, and 23, and with AKR1C2 from HL 15, 17, and 24. Enzyme quantities were 0.6–12 μg per 0.36 mL incubate and incubation times 4–20 min. With 6–10 substrate concentrations, non-linear regression analysis gave r^2 values of more than 0.99 ($P < 0.001$).

slightly higher than that of 8.0 determined here, whereas Hara *et al.* [7] gave pI 9.1 and 9.7 for DD1a and DD1b, respectively. A lower pI value (7.25) for AKR1C2 was also observed now than by the previous authors (pI 7.6) [7]. Though AKR1C1 and 1C2 differ only by seven amino acids [12, 35], they differ with regard to specificities for steroidal substrates and inhibitor sensitivities. These differences could largely be abolished by replacing Leu-54 in AKR1C1 by Val, the corresponding amino acid in AKR1C2 [35].

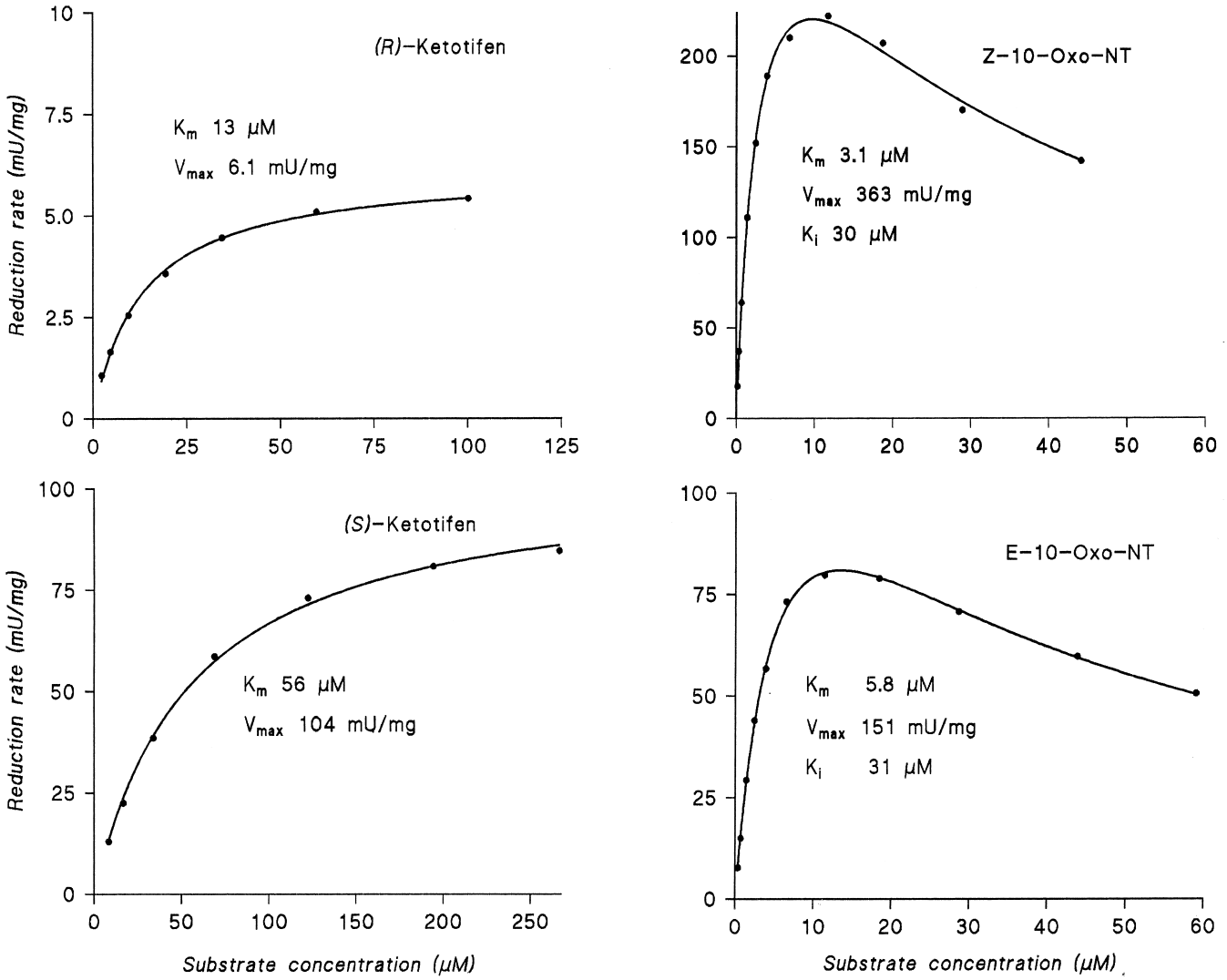

FIG. 4. Kinetics of the reductions of *(R)*- and *(S)*-ketotifen, Z- and E-10-Oxo-NT by AKR1C1 from HL 23. For incubation conditions see Table 3.

TABLE 4. Kinetic parameters for the oxidation of reduced *(R)*- and *(S)*-ketotifen and of (+)-Z- and (+)-E-10-OH-NT by aldo–keto reductases

Substrate	Enzyme	K_m (μM)	V_{max} (mU/mg)	CL_{int} (mL/min per mg)
Reduced (*R*)-ketotifen	AKR1C1	114	140	1.2
	AKR1C2	64	690	11
Reduced (*S*)-ketotifen	AKR1C1	37	140	3.8
	AKR1C2	13	680	52
(+)-Z-10-OH-NT	AKR1C1	20	620	31
	AKR1C2	43	350	8.1
(+)-E-10-OH-NT	AKR1C1	26	625	24
	AKR1C2	46	132	2.9

Data were derived from one or two experiments with enzyme quantities of 1–5.5 μg per 0.36 mL incubate and 6–8 substrate concentrations, incubation time 3–10 min. Non-linear regression analysis gave r^2 values of more than 0.99 ($P < 0.001$).

Whereas AKR1C2 reduces some ketonic steroids with K_m values in the low or submicromolar range [8, 12], an investigation with drug substrates from various classes resulted in K_m values between 190 and 3200 μM for AKR1C1 and 1C2 [18]. Thus, the structurally similar substrates Z- and E-10-Oxo-NT and (*S*)- and (*R*)-ketotifen (Fig. 1) are distinguished by their low K_m values of 2.6–53 μM (Table 3). These values, in conjunction with the high NADPH/$NADP^+$ ratio in cytosol, may be the reason why the corresponding alcohols are predominantly detected in human urine. This applies to ketotifen [24, 25], which is administered as the racemic ketone, as well as to the isomers of 10-Oxo-NT, which are produced from E- and Z-10-OH-NT in human liver microsomes and cytosol under physiological conditions [19]. The preferential substrates of the oxidative reactions are the (+)-enantiomers of E- and Z-10-OH-NT which, although not considered major metabolites, still accounted for 11.5 and 2.4%, respectively, of total amitriptyline metabolites recovered from patient urine [36], thus exceeding by far the minor quantities of ketones detected [22]. The assumption of ketotifen reduction by cytosolic enzymes [26] could be confirmed by the present data. AKR1C1 apparently possesses an additional binding site with relatively high affinity for the 10-Oxo-NT isomers, the occupation of which results in an inhibition of the catalytic activity.

The reason for the reversible isomerization of the reduced ketotifen enantiomers at room temperature and near-neutral pH is not clear. An analogous phenomenon has been described with two hydroxylated metabolites of the antihistamine loratadine, which bear great chemical similarity to reduced ketotifen. They also possess a piperidine ring linked by a double bond to the central seven-membered ring of a tricyclic system, and on the basis of ^1H NMR data the authors interpret the isomerization as a piperidylidene ring flip from one face of the tricyclic system to the other [37]. The racemization of the enantiomers of reduced ketotifen in acidic solutions may be due to intermediate removal of the protonated hydroxy group with formation of a benzylic cation. Either this adds OH^- at either of two possible positions, or H^+ is removed from the adjacent carbon atom with production of an unsaturated compound, the latter reaction being the slower one. In contrast, racemization has never been observed with the 10-OH-NT enantiomers, which eliminate water to form the dehydro compounds on heating under acidic conditions [38].

When haloperidol reduction by purified enzymes was measured under physiological conditions, CL_{int} values between 4.2 and 2.7 mL/min per mg protein were calculated for the CR isoforms I–III at the low substrate concentrations achievable without substrate precipitation [33]. While these values are compatible with V_{max}/K_m 736 U/mg/M (=0.736 mL/min per mg protein) at 25° found by Ohara *et al.* [18] for CR, the efficiencies of the aldo–keto reductases were much lower in the present investigation. For AKR1C1, a CL_{int} value about 250-fold lower than that for CR I was found, and for AKR1C2 it was 140-fold lower. In contrast, V_{max}/K_m values reported previously [18] were lower by factors of 3 and 12 only, with DD2 (AKR1C2) being the less active enzyme. Moreover, in the present study, a K_m value with AKR1C1 around 1 mM was estimated that distinctly exceeds that of 0.19 mM derived from measurements at 25° and pH 6 [18]. Haloperidol reduction has been shown to occur with high stereospecificity in human brain and liver cytosol, with reduced haloperidol containing more than 99% of the (*S*)-(−)-enantiomer [33]. Since CR was also isolated from brain [14], the stereospecific haloperidol reduction can be assumed to be mainly catalyzed by this enzyme in cytosol from liver and brain. In contrast to the high predominance of alcohols over ketones among urinary nortriptyline metabolites [22], comparable steady-state plasma concentrations of haloperidol and reduced haloperidol are attained during haloperidol treatment, though with large interindividual variations. Lower haloperidol/reduced haloperidol ratios following administration of the alcohol point to a higher reduction than back-oxidation efficiency [39]. The latter reaction is catalyzed by CYP3A4 in human liver microsomes [40, 41], and the fact that it also requires NADPH may be the reason for the relatively low alcohol/ketone ratios in patients.

In conclusion, carbonyl groups adjacent to aromatic rings in the tricyclic compounds (*R*)- and (*S*)-ketotifen as well as E- and Z-10-Oxo-NT are reduced by aldo–keto reductases AKR1C1 and 1C2 purified from human liver with high affinity, specific activity, and product stereospecificity to chiral alcohols. These can be re-oxidized to the corresponding ketones with the same degree of stereospecificity. In haloperidol, the carbonyl group is also adjacent to an aromatic ring, but its reduction is carried out by aldo–keto reductases with low affinity and low efficiency. In contrast, the CR isoforms reduce haloperidol with low affinity but high efficiency, as evidenced by CL_{int} values.

The authors wish to thank the persons and institutions named above who provided materials. They are grateful to Dr. D. Kloor, Department of Pharmacology, for valuable suggestions concerning protein purification, to Prof. V. Schurig and Mr. H. Czesla, Department of Chemistry, for help with chiral chromatography, and to Prof. W. Voelter and Mrs. M. Geiger, Department of Physiological Chemistry, for the opportunity to measure CD spectra and their help in interpretation. The cooperation of Mrs. G. Wiatr and Mrs. S. Vetter in protein electrophoresis and the advice of Prof. J. Kömpf, Department of Human Genetics, in isoelectric focusing are gratefully acknowledged.

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