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INHIBITION OF HEXONATE DEHYDROGENASE AND ALDOSE REDUCTASE FROM BOVINE RETINA BY SORBINIL, STATIL, M79175 AND VALPROATE

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Abstract—Aldose reductase inhibitors (A.R.I.s), developed as potentially therapeutic agents for the treatment of complications of long-term diabetes, were found to be potent inhibitors of aldose reductase (ALR2) partially purified from bovine retina (IC_{50} values: Statil 0.89 μM, Sorbinil 2 μM, M79175 >1 μM). These compounds varied, however, in their ability to inhibit hexonate dehydrogenase (ALR1), a closely related enzyme isolated from the same source (IC_{50} values: Statil >1 μM, Sorbinil 3.9 μM, M79175 0.18 μM). Statil and Sorbinil were active against ALR2 at very low concentrations (approx. 5% inhibition at 100 pM), but did not inhibit ALR1 at ≤10 nM. In contrast, M79175 (structurally very similar to Sorbinil) and M7HEQ (a flavonoid) were preferential inhibitors of ALR1. Valproate, a compound of value in the treatment of epilepsies, was a poor inhibitor of ALR2 (18% at 1 mM). Furthermore, valproate was found to be a relatively poor inhibitor of ALR1, particularly in comparison with M79175.

Hexonate dehydrogenase (L-gulonate: $NADP^+$ oxidoreductase EC 1.1.1.19) and aldose reductase (alditol: $NADP^+$ oxidoreductase EC 1.1.1.21) are closely related but distinct monomeric aldehyde reductases (EC 1.1.1.2) capable of catalysing the reduction of 4-nitrobenzaldehyde (4-NB) with NADPH [1, 2]. According to the nomenclature proposed recently by Turner and Flynn [3] for the aldehyde reductases; hexonate dehydrogenase, which is specific for NADPH and is very reactive with D-glucuronate but not D-xylose, is to be known as ALR1. Aldose reductase, which prefers NADPH to NADH [4] and is much more active with D-xylose than D-glucuronate, is to be known as ALR2.

ALR1 and ALR2 exhibit very broad and partially overlapping substrate specificities, which have resulted in each of these cytosolic [5] enzymes being known by various names. ALR1 has been described as high-K_m aldehyde reductase [6], glucuronate reductase, L-hexonate dehydrogenase [7–9], mevaldate reductase (EC 1.1.1.33) and lactaldehyde reductase (EC 1.1.1.55) [10].

ALR1 is sensitive to inhibition by many barbiturates [2, 3, 11, 12]. The anticonvulsant drug sodium valproate inhibits ALR1 from rat [5, 6], pig [13, 14] and bovine-brain [15].

Potent orally active inhibitors of ALR2, aldose reductase inhibitors (A.R.I.s) have been developed [16–19] with a view to preventing or ameliorating the development and progression of complications secondary to long-term diabetes mellitus [20].

The retina, which can be affected severely by diabetes [20], contains at least two NADPH-dependent aldehyde reductases [8, 21]. It has been found more difficult to demonstrate effective inhibition of ALR2 in the retina than in other tissues of the rat [18, 22]. This might be due to the presence of the blood–retinal barrier, or to a difference in the susceptibility of retinal enzymes to those in other tissues. In general, human placental ALR2 is less sensitive to inhibitors than is human lens ALR2 [16–19].

The present study was carried out to determine the relative effects of the potentially therapeutic agents Sorbinil, Statil and M79175 upon ALR1 and ALR2 of bovine retina. Comparisons made with valproate and a flavonoid revealed that the A.R.I.s, particularly M79175, were also effective inhibitors of bovine retinal ALR1.

MATERIALS AND METHODS

Materials. Eyes from healthy young bullocks were obtained within 6 hr of enucleation. Retinas were removed swiftly and stored overnight at −20°. DEAE-Sephacel, NADPH (type X), D(+)-xylose, D-(+)-galactose (<0.01% glucose), sodium D-glucuronate, benzamidine hydrochloride, soybean trypsin inhibitor, bovine serum albumin (fraction V), turkey egg albumin (grade VI), menadione and PMSF (phenyl-methyl-sulphonylfluoride) were purchased from Sigma Chemical Company Ltd. (Poole, U.K.); 4-nitrobenzaldehyde (99%, recrystallised from water before use), valproic acid (98%, 2-propylpentanoic acid) and sodium azide from Aldrich Chemical Co. Ltd. (Gillingham, U.K.); PD-10 gel filtration columns from Pharmacia Ltd. (Milton Keynes, U.K.). All other chemicals purchased were of analytical grade, where possible, from Fisons plc (Loughborough, U.K.). Statil (ICI 128436; 3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazin-1-yl acetic acid) and ICI 105552 (sodio-(1-(3,4-dichlorobenzyl)-3-methyl-1,2,-dihydro-2-oxoquinol-4-yl acetate) were provided by ICI plc (Pharmaceuticals Division, Alderley Park, U.K.); Sorbinil (CP-45, 634; d-6-fluoro-spiro[chroman-4,4'-imidazoline]-2',5'-dione) by Pfizer Central Research (Sandwich, U.K.);

M79175 (2R.4S 6-fluoro-2-methyl-spiro[chroman-4,4′-imidazoline]-2′-5′-dione) by Eisai Co. Ltd, Tokyo, Japan; and M7HEQ ((7-*O*-β-hydroxyethyl)quercetin) originated from Zyma (Macclesfield, U.K.).

Buffer A for homogenisation of retinas contained triethanolamine–HCl buffer (10 mM), magnesium chloride (10 mM), benzamidine hydrochloride (760 μM) and soybean trypsin inhibitor (0.2 g/l); after degassing under reduced pressure 2-mercaptoethanol (2 mM) was added. Buffer B for ion exchange chromatography contained triethanolamine–HCl buffer (10 mM), magnesium chloride (10 mM), and 2-mercaptoethanol (1 mM) after degassing. Buffers A and B were pH 7.5 at 26°.

Enzyme assays. Routine assays of aldehyde reducing activity were performed at 24–26° in sodium–potassium phosphate buffer (0.1 M), Li_2SO_4 (0.4 M), bovine serum albumin (0.5 g/l), sodium azide (0.2 g/l) and NADPH (160 μM). 4-NB (0.5 mM), was added in methanol (final concentration 1.3% v/v) which did not affect the assay. Incubations were carried out in a final volume of 750 μl (semi-micro u.v. cuvettes; Hughes and Hughes Ltd, Romford, U.K.) at pH 6.2. Oxidation of NADPH was monitored at 340 nm in a Perkin–Elmer model 552 spectrophotometer. One Unit of enzyme activity catalyses the oxidation of 1 μmol NADPH/min at 25°. Xylose (0.1 M) or glucuronate (20 mM) were used as alternative substrates to discriminate between aldehyde reducing enzymes.

Protein assay. Protein concentration was determined with a coomassie blue-binding method [23], using turkey egg albumin as standard.

IC_{50} *determinations.* The concentration of inhibitor required to produce 50% inhibition of 0.5 mU of enzyme activity in the absence of Li_2SO_4, with 4-NB (0.5 mM) as substrate was determined in sodium-potassium phosphate buffer (0.1 M) containing NADPH (160 μM) and 2-mercaptoethanol (1 mM). 4-NB was chosen as both ALR1 and ALR2 display reasonable activity with 4-NB at 0.5 mM, enabling the IC_{50} values to be determined using a single substrate, which is commonly used for members of the aldehyde reductase family. DL-glyceraldehyde is often used as substrate in studies of ALR2 but it has disadvantages. D- and L-glyceraldehyde are both substrates for ALR1 but give different rates of reaction [24]. D-Glucose would be the substrate of choice for determining IC_{50} values against ALR2, but would require approx. 10-fold the amount of enzyme per estimation for ALR2 and an even greater amount for ALR1. Inhibitors were dissolved initially in either methanol (Statil, menadione), water (ICI 105552), dilute NaOH (10 mM for Sorbinil, M79175; 20 mM for valproic acid) or alkaline methanol (M7HEQ), then diluted with water. The dilutions used did not affect the final pH. Oxidation of NADPH was assessed at 340 nm during 10 min periods in the absence then in the presence of substrate in a final volume of 750 μl, pH 6.4. Preliminary experiments with partially purified bovine lens aldose reductase, Sorbinil and M79175 revealed that the effectiveness of these inhibitors was not altered by pre-incubation with enzyme, as found previously by Tipton and Ryle for ICI 105552 and valproate [25]. Five concentrations of each inhibitor were tested in triplicate. Mean replicate variation (difference between worst replicate and triplicate mean as percentage of triplicate mean) was 4.6 ± 0.99% (SEM, N = 6 compounds) for assays with ALR1, 4.5 ± 0.53% (SEM, N = 6 compounds) for assays with ALR2, when inhibition was <90%. IC_{50} values were determined by linear regression after inspection of plots of % activity vs. $\log_{10}$[inhibitor].

Enzyme purifications. Frozen retinas (40 g) were thawed at approx. 5° in Buffer A (100 ml). PMSF dissolved in acetone was added (1 mM final) and the tissue was homogenised gently (PTFE/glass). The mixture was centrifuged (30,000 *g*, 20 min, 4°) and an ammonium sulphate cut (40–75% saturation) was obtained from the resulting supernate by dissolving powdered ammonium sulphate at room temperature (242 g/l for 40% saturation, and an additional 241 g/l to obtain 75% saturation), cooling to approx. 4° for 30 min, followed by centrifugation for each precipitation step. The final precipitate was desalted (3× PD-10) into Buffer B, then applied to a column of DEAE-Sephacel (22 mm diameter, 100 ml) pre-equilibrated with Buffer B at 26°. Elution was at 20 cm/hr with Buffer B for 200 ml followed by a linear gradient of KCl (0–0.5 M) in Buffer B over 500 ml. Fractions (10 ml) were screened for activity with 4-NB, D-(+)-xylose and sodium-D-glucuronate. Two peaks of 4-NB reducing activity were found. Consecutive fractions containing >20 mU/ml were pooled, and proteins were precipitated overnight at 5° with ammonium sulphate (90% saturated). Precipitates were recovered by centrifugation and washed with ammonium sulphate solution (90% saturated) containing sodium azide (0.2 g/l). IC_{50} values were determined for both enzyme pools. Aliquots of the aldose reductase pool were desalted (PD-10) into fresh Buffer B before use; this step was omitted for the hexonate dehydrogenase pool as the final concentration of ammonium sulphate was negligible.

Kinetic studies with galactose. Galactose has been used to increase polyol levels rapidly in erythrocytes [18] and other tissues [17–19] in order to assess A.R.I.s. There is, however, controversy over the identity of the enzyme responsible for polyol production in erythrocytes [1, 9, 21, 26]. The affinities of partially purified bovine retinal ALR1 and ALR2 for galactose were determined in the presence of NADPH (160 μM), without Li_2SO_4. Initial rate data for a wide range of galactose concentration were examined graphically [27].

RESULTS

Enzyme purification

Ion exchange chromatography of the ammonium sulphate cut from bovine retina yielded two peaks of 4-NB reducing activity (Fig. 1). The first peak eluted before the KCl gradient and displayed considerable activity with glucuronate, but minimal activity with xylose. In Pool 1 the ratio of 4-NB reducing activity to glucuronate reducing activity was approximately 1:1, that for 4-NB to xylose was 17:1, and for glucuronate to xylose, 16:1. The ratios implied that Pool 1 contained ALR1. The second peak, eluted at

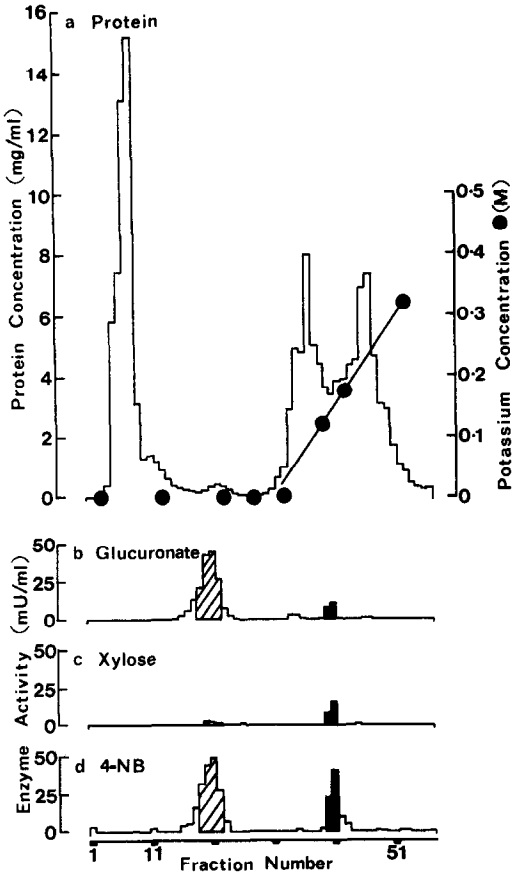

Fig. 1. Separation of enzyme activity on DEAE-Sephacel. (a) Protein (histogram, mg/ml) and K^+ (●, M) concentrations in fractions. Enzyme activity (mU/ml) with (b) sodium-D-glucuronate, (c) D-xylose, (d) 4-nitrobenzaldehyde. See Methods for conditions of chromatography and assays. Cross hatched bars ▨ show fractions pooled for ALR1, solid bars ■ show fractions pooled for ALR2.

approximately 125 mM KCl, showed substantial 4-NB and xylose reducing activities in proportion to glucuronate. In Pool 2 the activity ratios were: 4-NB vs. glucuronate 3.4:1, 4-NB vs. xylose 2.8:1, and glucuronate vs. xylose 0.8:1. Pool 2 therefore contained aldose reductase (ALR2). The specific activity of Pool 1 was 121 mU/mg protein with 4-NB as substrate; this represented approximately 140-fold purification of ALR1 compared with centrifuged retinal homogenate. ALR2 was purified approx. 25-fold, based on assays with xylose as substrate.

The activity of ALR1 with any of the 3 substrates, was increased moderately (5–20%) in the presence of Li_2SO_4 (0.4 M). The activity of ALR2 with xylose was not increased by Li_2SO_4, with 4-NB it was increased by approximately 40%.

The 40% saturated ammonium sulphate precipitate contained no excess 4-NB reducing activity over the small amounts of activity detected with glucuronate or xylose. Thus, this precipitate was substantially free of any other NADPH:4-NB reductases.

Chromatography at 5° compared with 26° gave poorer resolution of ALR1 and ALR2 from other proteins and conferred no advantages, although a third, minor but well resolved peak of 4-NB activity eluted between ALR1 and ALR2. This activity was unaffected by Li_2SO_4, was absent with either glucuronate or xylose as substrates, and might correspond with that observed in human brain [28].

Inhibition of hexonate dehydrogenase (ALR1)

Valproate gave 92% inhibition when present at 1 mM (97% at 3.16 mM), and an IC_{50} value of 98 μM was obtained. These figures reaffirmed the identity of the enzyme [2, 6, 13–15]. It was clear, however, that in comparison with the other compounds tested, valproate was a relatively poor inhibitor of ALR1. The percentage inhibition obtained for each compound at 1 μM were: M79175 83%, M7HEQ 39%, Sorbinil 21%, Statil 20%, ICI 105552 12%, valproate 1.3% at 3.16 μM. The percentage inhibitions obtained at 100 nM were: M79175 39%, M7HEQ 4.6%, Sorbinil 3.1%, Statil 1.3%. M79175 was the only compound with effects detectable at below 100 nM (4.5% inhibition at 10 nM and 1 nM), and gave the best IC_{50} value (Table 1).

Inhibition of aldose reductase (ALR2)

Statil and Sorbinil were the best inhibitors of ALR2. The percentage inhibitions obtained at 100 nM were: Statil 36%, Sorbinil 24%, ICI 105552 18%, M79175 17%. Statil and Sorbinil retained appreciable degrees of inhibition at 1 nM (15% and 7% respectively), and at 100 pM both compounds gave approximately 5% inhibition of bovine retinal ALR2. Menadione gave an IC_{50} value of 25 μM when tested in 19% methanol.

Selectivity of inhibitors (Table 2)

The ratio of IC_{50} values obtained against each enzyme gave an indication of the selectivity of each inhibitor for ALR1 or ALR2. Inspection of the degrees of inhibition obtained at several concentrations suggested that Statil, ICI 105552 and Sorbinil are preferential inhibitors of ALR2, particularly at low inhibitor concentrations. Conversely, valproate and the flavonoid M7HEQ selectively

Table 1. IC_{50} of various inhibitors tested against partially purified hexonate dehydrogenase and aldose reductase from bovine retina

Inhibitor*	IC_{50} (μM)† Hexonate dehydrogenase (ALR1)	Aldose reductase (ALR2)
Statil	>1.0‡	0.89
Sorbinil	3.9	2
M79175	0.18	>1.0§
ICI 105552	14	3.1
Valproate	98	>3160‖
M7HEQ	1.5	7.4

* See Materials for full names.
† Determined with 4-nitrobenzaldehyde as substrate.
‡ 20% inhibition at 1 μM.
§ 27% inhibition at 1 μM.
‖ 28% inhibition at 3.16 mM.

Table 2. Selectivity of various inhibitors tested against partially purified hexonate dehydrogenase and aldose reductase from bovine retina

Inhibitor*	IC_{50}‡ ratio	Selectivity index† Concentration of inhibitor 10 nM	100 nM	1 μM	10 μM
Statil	>1.12	∞	29.4	2.60	n.t.
Sorbinil	1.95	∞	7.9	2.07	n.t.
M79175	<0.18	1.88	0.44	0.32	n.t.
ICI 105552	4.52	∞	∞	2.63	1.54
Valproate§	<0.03	n.t.	n.t.	0	0.07
M7HEQ	0.20	n.t.	0	0.03	0.58

* See Materials for full names.

† Ratio (% inhibition of aldose reductase)/(% inhibition of hexonate dehydrogenase).

‡ Ratio IC_{50} hexonate dehydrogenase/IC_{50} aldose reductase.

§ Determined at 3.16 μM and 31.6 μM.

inhibited ALR1. M79175 gave greater inhibition of ALR1 than ALR2 at 1 μM and 100 nM, thus the selectivity of M79175 differed considerably from that of Sorbinil.

Kinetic studies with galactose

Hexonate dehydrogenase (ALR1) exhibited very little activity with galactose; the apparent K_m was approx. 1 M. The galactose used contained <0.01% glucose. Aldose reductase (ALR2) exhibited two distinct K_m (36 mM and 240 mM) in Lineweaver–Burk, Eadie–Hofstee and Eisenthal–Cornish–Bowden plots.

DISCUSSION

The order of elution of ALR1 and ALR2 from DEAE-Sephacel was as expected from the work of Gabbay [8, 21]. Gabbay and Cathcart isolated two "isoenzymes" of ALR2 from several bovine tissues, including the retina [21]. Recently, Flynn and coworkers produced evidence that ALR2 can exist as two distinct proteins, one of which appears only in tissues of non-castrated males [13, 29]. This raised the important possibility that the two forms of ALR2 need not be equally sensitive to A.R.I.s; although the "isoenzymes" isolated by Gabbay and Cathcart from bovine kidney were inhibited equally by TMG (3,3′-tetramethylene glutaric acid [21]). In the present work, retinas from castrated bovine animals were used and precautions were taken to prevent degradation of ALR2 during its partial purification. The enzyme eluted as one species from DEAE-Sephacel, but the subsequent demonstration of two apparent K_m for galactose might indicate the presence of two enzyme forms. However, two K_m have been observed for homogeneous bovine lens ALR2 [29] and for human brain ALR2 [12].

M7HEQ was more effective against ALR1 than ALR2 (Tables 1 and 2), as found generally for flavonoids, with the exception of quercitrin and rutin [6, 12, 31]. Certain flavonoids are also reasonable inhibitors of ALR3 and succinic semialdehyde reductase [1, 12, 14, 31]; TMG also inhibits this latter enzyme [12].

ICI 105552 was a reasonable inhibitor of ALR2, and of the four A.R.I.s tested was the least effective inhibitor of ALR1. The IC_{50} value of 3.1 μM against ALR2 is greater than that of 0.05 μM reported for bovine brain ALR2 [25]. This compound exhibits significant activity *in vivo* in the rat [22, 32], and in cultured MKE cells (88% inhibition of sorbitol accumulation with 10 μM ICI 105552 [33]).

Valproate (1 mM) gave 18% inhibition of ALR2 and 92% inhibition of ALR1 from bovine retina. Against these enzymes from bovine brain this concentration resulted in 37% inhibition of ALR2, and would give near total inhibition of ALR1 [15, 25]. Valproate is the drug of choice for certain forms of epilepsy, although its mode of action is uncertain [36]. It has been suggested that valproate's effects may be due in part to its ability to inhibitit ALR1 [15]. M79175 might therefore seem suitable for investigating this hypothesis, particularly if M79175, like Sorbinil, does not inhibit ALR3 or succinic semialdehyde reductase [14]. Neither M79175 nor Sorbinil are particularly effective against electrically-induced convulsions in the rat; ED_{50} values ≥ 30 mg/kg p.o. (Nomura and Nakai, personal communication, 1985).

Menadione (vitamin K_3), a substrate for ALR3 [3], was not a substrate for ALR2; in fact it inhibited bovine retinal ALR2 with an IC_{50}value of 25 μM. This value might not be directly comparable with those in Table 1, as menadione was tested in a higher concentration of methanol. Menadione was not tested against ALR1. It is of interest that menadione is a competitive inhibitor of ALR2 from chicken muscle [35] and competitive inhibitors of ALR2 are uncommon [36].

Sorbinil is already in clinical trial against diabetic retinopathy in man [37]. The IC_{50} value of 2 μM reported here for bovine retina ALR2 compares remarkably well with 2.1 μM obtained by Russell and coworkers for extracts of a human retinal cell line [38]. Retinal ALR2 therefore appears more sensitive to Sorbinil than does ALR2 from human erythrocytes (IC_{50} approx. 10 μM [26]), human brain (IC_{50} approx. 200 μM; derived from Fig. 3 of [39]) or pig muscle and brain (IC_{50} approx. 10 μM [13, 14]). Conversely, ALR2 from human lens (IC_{50} 0.22 μM), human placenta (IC_{50} 0.68 μM) and rat lens (IC_{50} 0.07 μM) are more sensitive [16]. Plasma Sorbinil levels averaged 27 μM (7.4 μg/ml [40]) in diabetic patients receiving 200 mg daily. In another study, a single topical application of 0.5 mg Sorbinil produced approximate peak drug levels of 50 μM in aqueous humour, and 6 μmol/kg in human lens [41]. No data are published of Sorbinil levels in animal retina after treatment with Sorbinil.

Statil gave an IC_{50} value of 0.89 μM for inhibition of bovine retinal ALR2, slightly lower than that of Sorbinil. Recently, Stribling and colleagues reported a value of 19 nM for bovine retina [18]. Their data were obtained using an ammonium sulphate cut which would have contained additional NADPH consuming enzymes; although their IC_{50} value of 25.9 nM obtained similarly for bovine lens (which does not contain ALR1) compares well with 40 nM, obtained by the author for bovine lens ALR2 after DEAE-Sephacel chromatography (unpublished).

The present results suggest that at low concentrations Statil and Sorbinil are preferential inhibitors of bovine retinal ALR2, although both compounds give significant inhibition of ALR1 over a wide range of concentrations. O'Brien and coworkers [39] found human brain ALR2 to be rather insensitive to both Sorbinil and Alrestatin (IC_{50} values estimated at 200 μM). This lack of sensitivity might be connected with the existence of a heat-stable dissociable factor in some preparations of ALR2, which impairs markedly the sensitivity of the enzyme to Alrestatin and Sorbinil, but not to flavonoids [42]. Other groups have determined that Sorbinil at 10 μM inhibits ALR1 preferentially to ALR2 for pig brain (selectivity 0.45 [14]) and human erythrocyte (selectivity 0.71 [26]).

The inhibitory properties of M79175 and Sorbinil were surprisingly different in view of their nearly identical structures. Sorbinil was marginally more effective than M79175 against ALR2 from bovine retina. Conversely, M79175 is more effective than Sorbinil against ALR2 from rabbit lens [36]. ALR2 is sensitive to the absolute configuration of inhibitor molecules [11, 17], but both compounds are believed to possess the more active *S*-configuration at the spiro junction [17, 36]. M79175 does however possess an additional chiral centre of unknown importance.

In conclusion, Statil and Sorbinil were particularly effective inhibitors of ALR2 from bovine retina. These compounds were more selective in their inhibition of ALR2 over ALR1 than valproate, M79175 or M7HEQ, yet they were still excellent inhibitors of bovine retinal ALR1 when compared with valproate. In view of the rapid evolution of the aldehyde reductase family [35] it is important to extend these studies to human retinal enzymes to avoid the need to extrapolate results between species.

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