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INHIBITION KINETICS OF HUMAN KIDNEY ALDOSE AND ALDEHYDE REDUCTASES BY ALDOSE REDUCTASE INHIBITORS

ARUNI BHATNAGAR, SIQI LIU, BALLABH DAS, NASEEM H. ANSARI and SATISH K. SRIVASTAVA*

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

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Abstract—Kinetic patterns of inhibition of homogenous human kidney aldose reductase (AR, EC 1.1.1.21) and aldehyde reductase II (AR II, EC 1.1.1.19) by statil, ICI 105552 [1-(3,4-dichlorobenzyl)-3-methyl-1,2-dihydro-2-oxoquinol-4-yl acetic acid], tolrestat, alrestatin, chromone carboxylic acid (CCA), quercetin, phenobarbital and sorbinil were studied. On the basis of the kinetic nature of inhibition, the inhibitors were classified into four distinct categories. For aldose reductase, sorbinil and phenobarbital were noncompetitive (NC; category I) and CCA and alrestatin were uncompetitive (UC; category II) to both the aldehyde substrate and NADPH. Quercetin and ICI 105552 were NC to the aldehyde and UC to NADPH (category III) and tolrestat and statil were UC to the aldehyde and NC to NADPH (category IV). For AR II, sorbinil and alrestatin were category I inhibitors, ICI 105552 and statil belong to category II, phenobarbital, tolrestat and CCA to category III, and quercetin to category IV. To determine the specificity of inhibition, the ratios of the inhibition constants (K_{ii}) for AR and AR II were calculated. A lower ratio indicates greater specificity. With aldehyde as the varied substrate the specificity ratios were: statil < ICI 105552 < alrestatin < tolrestat < quercetin < CCA < sorbinil < phenobarbital, and with NADPH as the varied substrate, ICI 105552 < statil < alrestatin < tolrestat < quercetin < CCA < sorbinil < phenobarbital. For AR, double-inhibition plots generated for one inhibitor from each kinetic category versus sorbinil showed that AR inhibitors of categories I–III bind to the same site on the protein molecule as sorbinil. However, tolrestat seemed to bind to a site different from the sorbinil binding site. For AR II, inhibitors from all the four categories appeared to bind to the same inhibitor binding site.

The therapeutic use of aldose reductase inhibitors (ARI) for the management of certain diabetic complications has been advocated for a number of years. The initial observations that tetramethylene glutarate [1], some flavonoids [2] and hydantoin [3, 4] derivatives could prevent sugar cataractogenesis in rats led to a considerable interest among investigators to synthesize compounds, which would be non-toxic, specific and irreversible inhibitors of aldose reductase. ARI have been shown to prevent or delay significantly sugar-induced cataractogenesis in rats [5, 6], decrease urinary protein excretion in diabetic rats [7], and improve motor nerve conduction velocity defect in diabetic rats [8, 9] and humans [10]. Several ARI are currently available and many have been tested for clinical use, albeit with limited success [10, 11]. The mechanism of action of these drugs has been proposed to be via inhibition of aldose reductase (AR, EC 1.1.1.21), an enzyme which catalyzes the reduction of glucose to sorbitol with the mediation of NADPH. Diabetic complications have been proposed to be due to an increase in the activity of AR in hyperglycemia, which in turn causes an increased diversion of NADPH for reducing glucose to sorbitol, leading to a generalized oxidative stress [12–15]. We had reported earlier that ARI such as sorbinil, alrestatin and quercetin are not specific to AR [16], but also inhibit aldehyde reductase II. Aldehyde reductase II (AR II, EC 1.1.1.19) is also a monomeric, NADPH-dependent oxidoreductase, and shares a number of properties with AR, including substrate specificity. For ARI to have therapeutic significance in the treatment of diabetic complications, it is necessary that the specificity, mechanism and the site of binding of ARI be studied for both AR and AR II.

In the present paper we report the kinetics of inhibition of AR and AR II, isolated from human kidney, by eight different ARI. The relative specificity of inhibition of AR and AR II was studied, and the inhibitors have been classified based upon the kinetic nature of inhibition.

MATERIALS AND METHODS

NADPH, sodium-D-glucuronate and DL-glyceraldehyde were obtained from the Sigma Chemical Co. Sorbinil (CP-45,634; d-6-fluoro-spiro[chroman-4,4′-imidazolidine]-2′,5′-dione) and CCA (7-hydroxy-4-oxo-4*H*-chromone-2-carboxylic acid) were gifts from the Pfizer Chemical Co. and The National Eye Institute respectively. Statil (ICI 128436; 3-[(4-bromo-2-fluorobenzyl)-4-oxo-3*H*-phthalazin-1-yl acetic acid]) and ICI 105552 [1-(3,4-dichlorobenzyl) -3-methyl- 1,2 -dihydro-2-oxoquinol-4-yl acetic acid] were obtained from Imperial Chemical Industries plc. Tolrestat (AY-27,773; *N*-

* Author to whom correspondence should be addressed.

methyl-*N*-[(5-trifluromethyl-6-methoxy 1-naphthalenyl)-thioxomethyl]glycine) and Alrestatin (AY-22,284; 1,3-dioxo-1*H*-benz-[ae]-isoquinoline-2(3*H*) acetic acid) were from Ayerst Laboratories; phenobarbital was purchased from the Sigma Chemical Co., and quercetin (3,3′,4′5,7-pentahydroxy flavone) from the Aldrich Chemical Company, Inc. All inhibitors except tolrestat were dissolved in ethanol. Tolrestat was dissolved in a sodium bicarbonate solution (28 mM). The stock solutions (10 mM) were diluted, so that the final concentration of ethanol in the cuvette for determining the enzyme activity was 0.01%. Small aliquots (10–20 μL) of tolrestat stock solution, when added to the enzyme reaction mixture, did not cause any change in the pH of the enzyme reaction mixture.

Human kidneys were obtained post mortem from the autopsy department of the University of Texas Medical Branch. The tissues were stored at −70°, until used. AR and AR II were purified to homogeneity by a method which was essentially similar to that used for purification of these enzymes from human lens [17]. The homogeneity of these enzymes was established by their migration as a single protein band, on β-mercaptoethanol/sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis at pH 8.6, performed according to Laemmli [18]. The purified enzymes were dialyzed thoroughly against 100 mM phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT). Protein concentration was determined by the method of Bradford [19], using bovine serum albumin as a standard.

AR activity was determined at 25° in a 1-mL system containing 100 mM phosphate (pH 6.0), 400 mM Li_2SO_4, 1 mM DTT, 0.1 mM NADPH and 10 mM glyceraldehyde. AR II activity was also determined at 25° in a 1-mL system containing 200 mM phosphate (pH 7.0), 0.1 mM NADPH and 15 mM glucuronate. For both the enzyme determinations the reference cuvette contained all the other additives except the enzyme.

The catalytic activities of AR and AR II were determined by monitoring the rate of NADPH oxidation at 340 nm on a Gilford-Response™ spectrophotometer, for a minimum of 4 min. All time–activity relationships were analyzed statistically and only those which were linear up to 1% were used for analysis. One unit of the enzyme catalyzes the oxidation of 1 μmol NADPH/min at 25°. When the effect of an inhibitor was studied, the enzyme was incubated with the inhibitor and the buffer for 1 min at 25° before the addition of the substrate. Equivalent concentrations of the inhibitor were added to the reference cuvette to account for any non-enzymatic changes in A_{340}.

To calculate the inhibition constants, initial velocities at different inhibitor concentrations were obtained at a variable concentration of the aldehyde substrate, at a fixed and saturating concentration of NADPH (0.1 mM), and then in the presence of a variable NADPH concentration, at a fixed concentration of the aldehyde (10 mM glyceraldehyde or 15 mM glucuronate). Inhibition patterns for variable NADPH and the aldehyde concentrations for the two enzymes were fitted to linear competitive (C), noncompetitive (NC) and uncompetitive (UC)

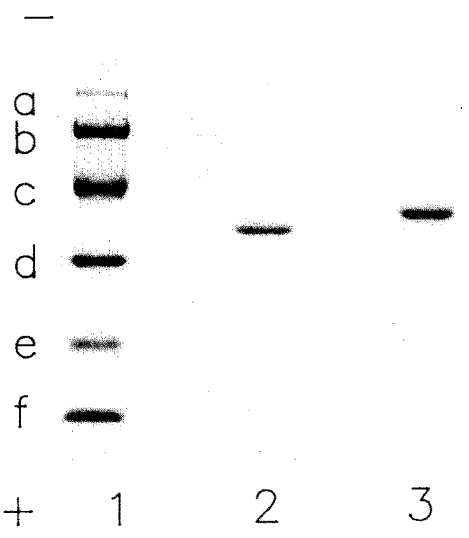

Fig. 1. β-Mercaptoethanol/sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis of human kidney aldose reductase and aldehyde reductase II. Lane 1, standard mixture of molecular weight marker proteins: A, phosphorylase *b* (M_r 94,000); b, bovine serum albumin (67,000); c, ovalbumin (43,000); d, carbonic anhydrase (30,000); e, soybean trypsin inhibitor (20,100); and f, α-lactalbumin (14,400). Lane 2, aldose reductase. Lane 3, aldehyde reductase II.

equations [(1), (2) and (3) respectively] using the statistical programs of Cleland [20]:

$$v = \frac{[S]}{K_s\,(1 + [I]/K_{is}) + [S]} \tag{1}$$

$$v = \frac{v\,[S]}{K_s(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \tag{2}$$

$$v = \frac{V\,[S]}{K_s + [S](1 + [I]/K_{ii})} \tag{3}$$

where v is the initial velocity, $[S]$ is the substrate concentration, V and K_s are the Michaelis constants, V_{max} and K_m, $[I]$ is the inhibitor concentration, K_{is} is the K_i slope, and K_{ii} is the K_i intercept. The best fit data were chosen on the basis of the lowest value of the standard error of the fitted parameters and the lowest value of σ, which is the sum of squares of the residuals divided by the degree of freedom, where degree of freedom is equal to the number of observations minus the number of parameters calculated. All graphs presented have the experimental data plotted as discrete points and the lines are drawn from the theoretical values generated by the curve-fitting routine. The double-inhibition plots were fitted by a weighted linear regression program, and the best fit lines are shown in the figures.

RESULTS

Aldose reductase and aldehyde reductase II purified from human kidney were apparently homogeneous, as indicated by the presence of a single protein band in each case on β-mercaptoethanol/sodium dodecyl sulfate/polyacrylamide gel electrophoresis. (Fig. 1). The calculated molecular

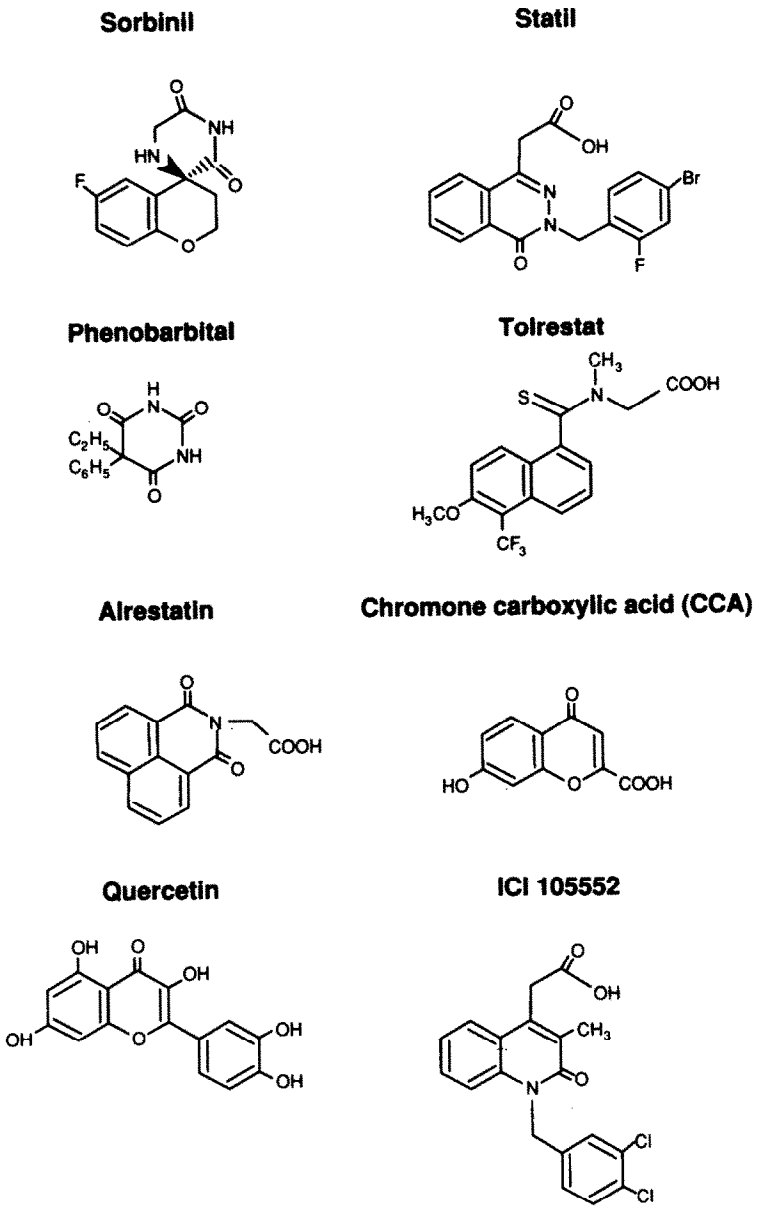

Fig. 2. Chemical structures of the inhibitors used in this study.

weight of kidney AR is 36,000 and of AR II 38,000. The specific activities were: AR 7 units/mg protein and AR II 24 units/mg protein. Inhibition characteristics of both of these enzymes were studied using a series of inhibitors shown in Fig. 2. For each inhibitor, inhibition patterns were generated first with NADPH as the varied substrate at a saturating concentration of the aldehyde substrate and then with the aldehyde as the varied substrate at a saturating concentration of NADPH. A similar experimental protocol was used for both AR and AR II. The various inhibition patterns obtained were analyzed as described in Materials and Methods. On the basis of the type of inhibition observed, the inhibitors were classified into four kinetically distinct categories as follows: (I) inhibitors which were UC to both the aldehyde and NADPH, (II) NC to both the substrates, (III) UC to the aldehyde substrate but NC to NADPH and (IV) NC to the aldehyde substrate and UC to NADPH. Tables 1 and 2 list the inhibitors of all the four categories for both AR and AR II. Figures 3 and 4 show double-reciprocal plots with NADPH and the aldehyde as the varied substrate at different inhibitor concentrations, for one inhibitor from each of the four categories. In the figures some data points have been omitted for clarity. The reciprocal plots for both AR and AR II were linear in the absence of the inhibitors but in the presence of some of the inhibitors a slight deviation from linearity was observed, especially at higher concentrations of the inhibitor.

In the case of AR, sorbinil and phenobarbital were

Table 1. Inhibition of human kidney aldose reductase by aldose reductase inhibitors

Category	Type of inhibition* A	B	Inhibition constants† (μM) Aldehyde variable K_{ii}	K_{is}	NADPH variable K_{ii}	K_{is}
I						
Sorbinil	NC	NC	1.24 ± 0.16	1.59 ± 0.75	0.51 ± 0.10	0.47 ± 0.21
Phenobarbital	NC	NC	4109 ± 716	181 ± 9.91	2209 ± 325	172 ± 23.3
II						
CCA	UC	UC	1.38 ± 0.13		0.51 ± 0.01	
Alrestatin	UC	UC	0.98 ± 0.24		0.30 ± 0.05	
III						
Quercetin	NC	UC	5.06 ± 0.51	1.95 ± 0.19	1.124 ± 0.23	
ICI 105552	NC	UC	0.03 ± 0.008	0.01 ± 0.005	0.011 ± 0.001	
IV						
Tolrestat	UC	NC	0.033 ± 0.003		0.024 ± 0.003	0.011 ± 0.006
Statil	UC	NC	0.001 ± 0.0003		0.007 ± 0.001	0.024 ± 0.002

* A: aldehyde was the varied substrate, and B: NADPH was the varied substrate.

† The concentration of NADPH was varied from 0.015 to 0.1 mM and that of DL-glyceraldehyde from 0.1 to 10.0 mM. The inhibition constant values are means ± SE. For each pattern the residual sum of squares (σ) was 0.001 to 0.008 for 18–23 degrees of freedom.

Table 2. Inhibition of human kidney aldehyde reductase II by aldose reductase inhibitors

Category	Type of inhibition* A	B	Inhibition constants† (μM) Glucuronate variable K_{ii}	K_{is}	NADPH variable K_{ii}	K_{is}
I						
Sorbinil	NC	NC	1.45 ± 0.16	9.82 ± 3.80	1.50 ± 0.21	7.08 ± 2.56
Alrestatin	NC	NC	48.4 ± 2.86	181.7 ± 15.6	61.65 ± 5.81	50.5 ± 6.43
II						
ICI 105552	UC	UC	7.33 ± 1.32		12.6 ± 1.28	
Statil	UC	UC	1.44 ± 0.18		2.54 ± 0.22	
III						
Phenobarbital	NC	UC	87.0 ± 4.29	144.31 ± 24.7	80.7 ± 2.91	
CCA	NC	UC	3.23 ± 0.44	6.51 ± 1.98	3.15 ± 0.25	
Tolrestat	NC	UC	0.67 ± 0.08	6.47 ± 1.39	2.13 ± 0.20	
IV						
Quercetin	UC	NC	41.3 ± 5.04		11.8 ± 2.23	32.42 ± 2.98

* A: Glucuronate was the varied substrate, and B: NADPH was the varied substrate.

† The concentration of NADPH was varied from 0.015 to 0.1 mM and that of sodium-D-glucuronate from 1.0 to 15.0 mM. The inhibition constant values are means ± SE. For each pattern the residual sum of squares (σ) was 0.001 to 0.008 for 18–23 degrees of freedom.

NC, whereas alrestatin and CCA were UC to both the substrates. Quercetin and ICI 105552 were NC to glyceraldehyde and UC to NADPH, whereas tolrestat and statil were UC to glyceraldehyde and NC to NADPH. For this series of inhibitors, the K_{ii} value, with glyceraldehyde or NADPH as the varied substrate, was minimal for statil and maximal for phenobarbital. The K_{ii} values in ascending order, with either NADPH or glyceraldehyde as the varied substrate, were: statil < ICI 105552 < tolrestat < alrestatin < sorbinil < CCA < quercetin < phenobarbital. The K_{is} values determined for the NC inhibition patterns were, in general, not well defined and since these values could not be determined for all the inhibitors, no comparison was made between inhibitors on the basis of the K_{is} values.

For AR II, sorbinil and alrestatin were NC, whereas ICI 105552 and statil were UC versus both the substrates. Phenobarbital, CCA and tolrestat were NC to glucuronate and UC to NADPH, and quercetin was UC to glucuronate and NC to NADPH. The K_{ii} values for each inhibitor in ascending order, with glucuronate as the varied substrate, were: tolrestat < statil < sorbinil < CCA < ICI 105552 < quercetin < alrestatin < phenobarbital. With NADPH as the varied substrate, the K_{ii} values in the ascending order were: sorbinil < tolrestat < statil < CCA < quercetin < ICI 105552 < alrestatin < phenobarbital. The K_{is} values of the inhibitors for AR II also were poorly defined and, therefore, not used for any comparative purposes.

To compare the specificity of the inhibitors for AR and AR II, the K_{ii} value of each inhibitor for AR was compared to the K_{ii} value of the same inhibitor for AR II. The ratios of K_{ii} AR/K_{ii} AR II are given in Table 3. The K_{ii} ratio was minimal for statil with

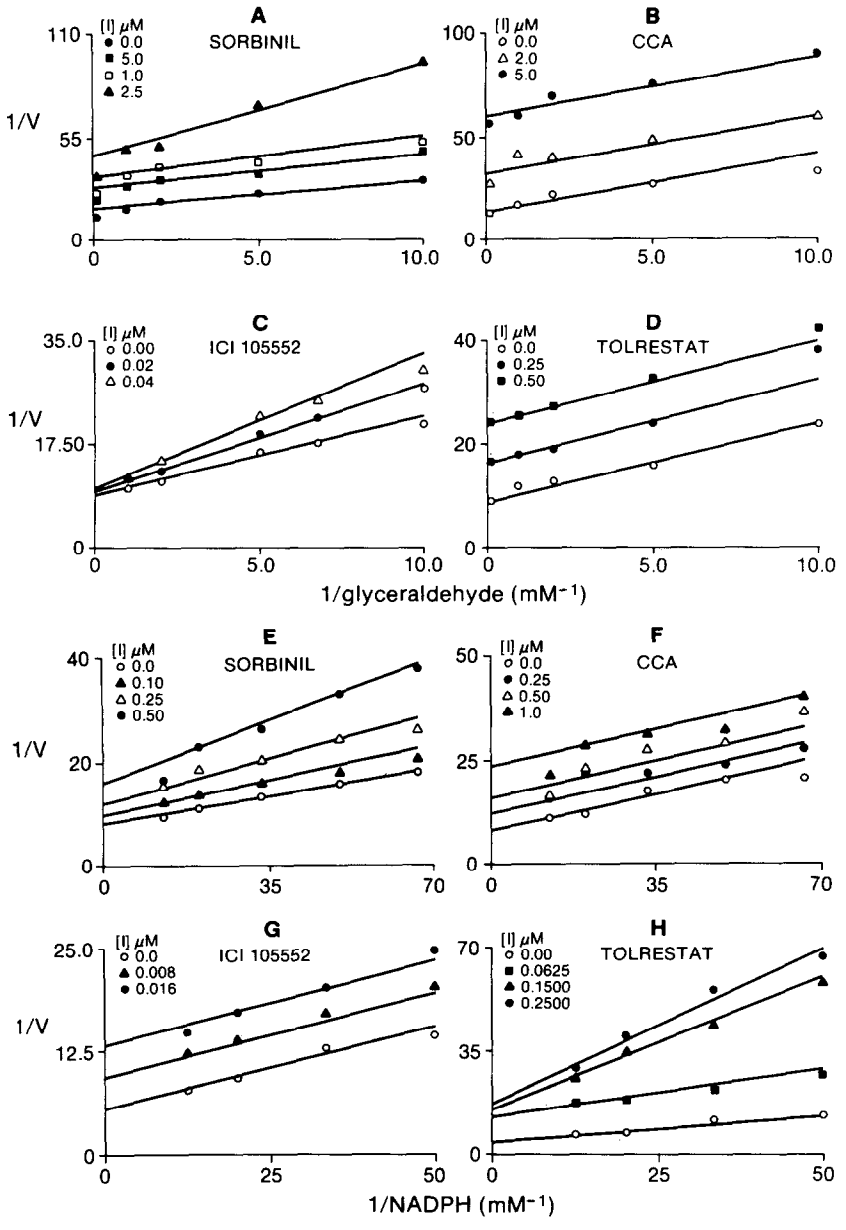

Fig. 3. Double-reciprocal plots of the inhibition of human kidney aldose reductase by sorbinil, CCA, ICI 105552 and tolrestat, with glyceraldehyde as the varied substrate (A–D) at a fixed concentration of NADPH (0.1 mM), and with NADPH as the varied substrate (E–H) at a fixed concentration of glyceraldehyde (10 mM). The concentration of the inhibitor [*I*] is given with each plot.

aldehyde as the varied substrate and for ICI 105552 with NADPH as the varied substrate. This indicates a greater specificity of these inhibitors for AR. Comparable K_{ii} values of sorbinil for AR and AR II indicate that this inhibitor does not distinguish between AR and AR II, whereas statil, ICI 105552 and alrestatin appear to be relatively more specific to AR, as their K_{ii} values for AR were at least two orders of magnitude lower than for AR II. Of the inhibitors tested in this study, phenobarbital was the most specific inhibitor of AR II as compared to AR.

To further study the nature of inhibition by ARI, double-inhibition experiments, using a combination of two inhibitors, were performed. The results of these experiments were analyzed according to the method of Yonetani and Theorell [21]. In this method v_0/v_i values are plotted against the concentration of one inhibitor [*I*] at different fixed concentrations of another [*X*]. If both inhibitors can simultaneously bind to the same form of the enzyme, an intersecting pattern will be obtained. If the lines plotted so are parallel to each other and the plot for [*X*] = 0 intercepts the v_0/v_i axis at 1, it indicates that the inhibitors are mutually exclusive, i.e. *X* and *I* do not bind to the same enzyme form. In this study, sorbinil was used as a standard inhibitor [*I*] and

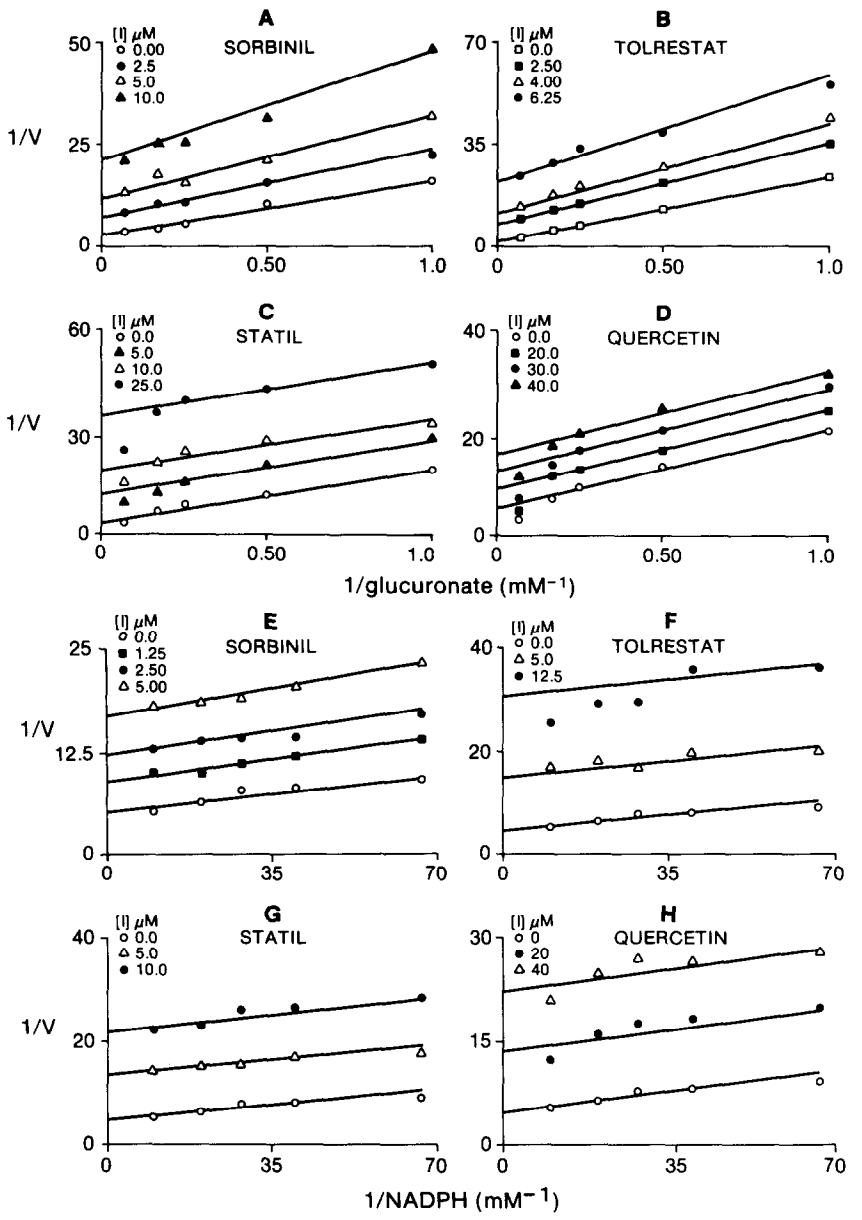

Fig. 4. Double-reciprocal plots of the inhibition of human kidney aldehyde reductase II by sorbinil, tolrestat, statil and quercetin, with glucuronate as the varied substrate (A–D) at a fixed concentration of NADPH (0.1 mM), and with NADPH as the varied substrate (E–H) at a fixed concentration of glucuronate (15 mM). The concentration of the inhibitor [*I*] is given with each plot.

Table 3. Comparison of K_{ii} values of human kidney aldose reductase and aldehyde reductase II for different aldose reductase inhibitors

Inhibitor	K_{ii} AR/K_{ii} AR II (aldehyde variable)	K_{ii} AR/K_{ii} AR II (NADPH variable)
Statil	0.001	0.003
ICI 105552	0.004	0.001
Alrestatin	0.014	0.005
Tolrestat	0.050	0.011
Quercetin	0.122	0.095
CCA	0.427	0.163
Sorbinil	0.855	0.343
Phenobarbital	46.960	27.272

K_{ii} values were obtained for aldose reductase and aldehyde reductase II from Tables 1 and 2 respectively.

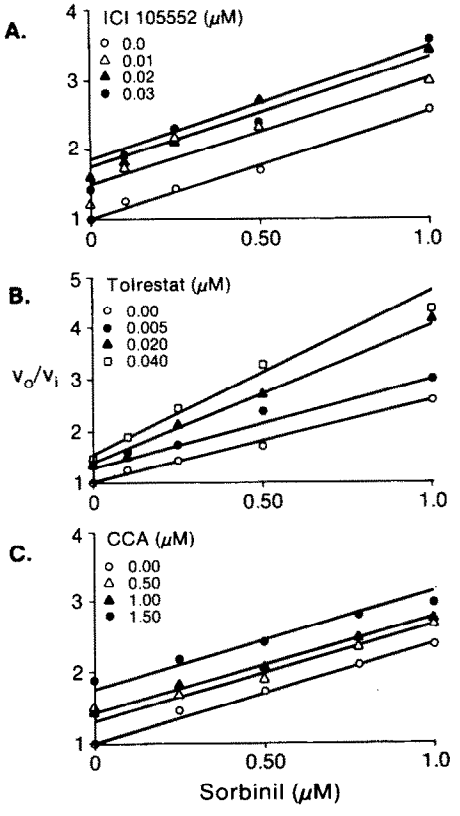

Fig. 5. Yonetani–Theorell plots of v_0/v_i (initial velocity in the presence of sorbinil/initial velocity in the presence of sorbinil + another inhibitor) versus [sorbinil] for human kidney aldose reductase. The other inhibitors were: ICI 105552 (A), tolrestat (B) and CCA (C). The concentration of glyceraldehyde was 10 mM and of NADPH 0.1 mM. The concentration of the inhibitor [*I*] is given with each plot.

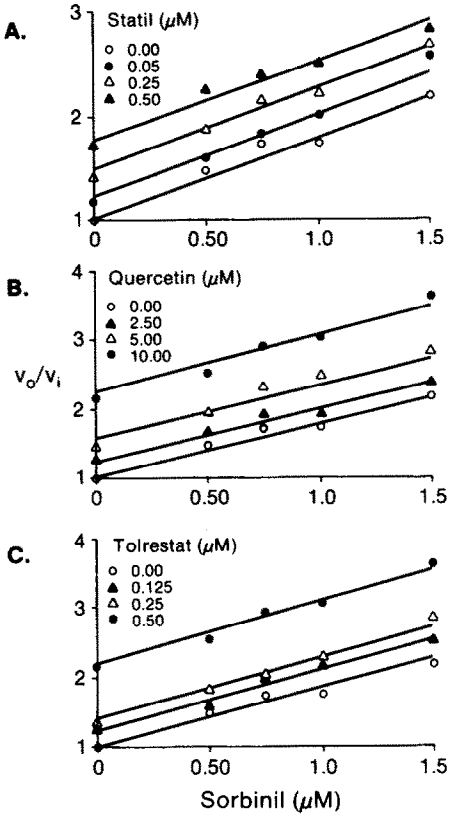

Fig. 6. Yonetani–Theorell plots of v_0/v_i versus sorbinil concentration for human kidney aldehyde reductase II. The other inhibitors used were: statil (A), quercetin (B) and tolrestat (C). The concentration of glucuronate was 15 mM and of NADPH 0.1 mM. The concentration of the inhibitor [*I*] is given with each plot.

double-inhibition patterns were studied in the presence of sorbinil and another inhibitor [*X*], one from each of the other three kinetic categories. Figure 5 shows Yonetani–Theorell [21] double-inhibition plots for AR. With sorbinil vs ICI 105552 and sorbinil vs CCA, the inhibition plots were parallel, whereas the plots for tolrestat vs sorbinil were not parallel. For AR II, sorbinil vs statil, sorbinil vs tolrestat, and sorbinil vs quercetin, double-inhibition plots were essentially parallel (Fig. 6).

DISCUSSION

A number of studies have shown that inhibition of aldose reductase leads to prevention or significant delay of hyperglycemia-induced tissue damage in insulin-independent tissues [10, 11, 20–25]. However, little *in vitro* data are available. It is not known, for example, how specific these inhibitors are for aldose reductase, and whether or not all the inhibitors bind to the same site on the enzyme protein and where, in the course of the reaction, these inhibitors bind. To answer some of these questions, we studied the kinetic nature of inhibition of AR by eight different ARI and, to assess the specificity of inhibition, kinetic inhibition constants were determined for both AR and AR II. Both these enzymes are members of a distinct class of NADPH-dependent aldo-keto reductases. The amino acid sequence of human AR has a 65% identity to that of AR II [26]. Although the two enzymes are immunologically and kinetically distinct [27–29], they have an overlapping substrate specificity [18, 27, 28]. It is, therefore, likely that aldose reductase inhibitors may also inhibit, with equal efficacy, AR II. Therefore, an important criterion of specificity for an ARI will be its specific inhibition of AR as opposed to AR II.

To minimize tissue and species differences human kidney AR and AR II were used. Both the enzymes were isolated under identical conditions, so that factors such as differences in purification protocol would not prevent a direct comparison of the data obtained for AR and AR II. This would make any clinical interpretation of the results easier.

Kador and Sharpless [30] have postulated a model for the inhibitor binding site of aldose reductase, and recently Butera *et al.* [31] have synthesized a computer-generated hybrid designed by the superimposition of tolrestat and ICI 105552, using sorbinil as a template, to discern a common pharmacophore.

In these studies it was tactically assumed that all the inhibitors tested bind to the same site on the enzyme molecule, an assumption that had never been tested directly. A comparison of the inhibitor binding site(s) of different inhibitors, using double-inhibition plots (Fig. 5), indicates that inhibitors of categories I, II and III are mutually exclusive and bind to the same site on the molecule. Although only one inhibitor from each category was tested, in view of their kinetic similarity, it is quite likely that all the inhibitors of one category would bind to the same site. The only inhibitor with non-parallel Yonetani–Theorell plots was tolrestat, indicating that either it does not bind to the common ARI-inhibitor-binding site or it has a binding site(s) different from, and in addition to, the sorbinil binding site. For AR II, all the inhibitors tested seemed to bind to the same site, as the Yonetani–Theorell plots versus sorbinil were parallel. The inhibitor binding sites of the two enzymes, however, may be different. Further experiments will be necessary to understand the differences in the binding sites of the two enzymes; nevertheless, our results offer an important clue. It has been reported that there is a proportional relationship between the ability of ARI to "undergo a charge transfer interaction and their ability to inhibit aldose reductase" [30]. The ability to undergo charge transfer reactions was estimated by the lowest empty molecular orbital (LEMO) calculations. LEMO is a quantum chemical index; a low LEMO score indicates a greater capability of the molecule to undergo charge transfer interactions. Our results (Table 3) show that molecules that are more specific to AR have lower LEMO values, e.g. alrestatin (LEMO value = 0.0095 [30]), whereas inhibitors less specific to AR have higher LEMO values, e.g. quercetin and sorbinil (LEMO values = 0.0510 and 0.0983 [30] respectively).

To interpret the kinetic nature of inhibition of each of the inhibitors tested, the kinetic reaction schemes for AR and AR II (Schemes I and II), proposed earlier [29], were used (Fig. 7).

Sorbinil and phenobarbital acted as NC inhibitors to AR when either aldehyde or NADPH was the varied substrate (category I, Table 1), indicating that these inhibitors could bind to the free enzyme form and all enzyme–substrate complexes along the reaction scheme. Thus, inhibitor binding does not prevent substrate binding, but may slow down the interconversion of the ternary complexes. An NC pattern of inhibition by sorbinil and other hydantoin derivatives has also been reported previously [3, 32, 33]. The K_{ii} values of sorbinil reported here (1–2 μM) are similar to those reported previously for partially purified AR isolated from human placenta [34] or bovine retina [35]. However, these values are much lower than those reported for the human brain (170–320 μM) [32]. Sorbinil is the most commonly used ARI, but in our study it displayed a poor specificity for AR (Table 3). Its K_{ii} value and the nature of inhibition of AR were identical to its inhibition of AR II. Therefore, this compound would obviously not differentiate between AR and AR II and would be a poor choice if a specific inhibition, *in vivo*, of AR is desired.

Inhibitors of category II, alrestatin and CCA, were

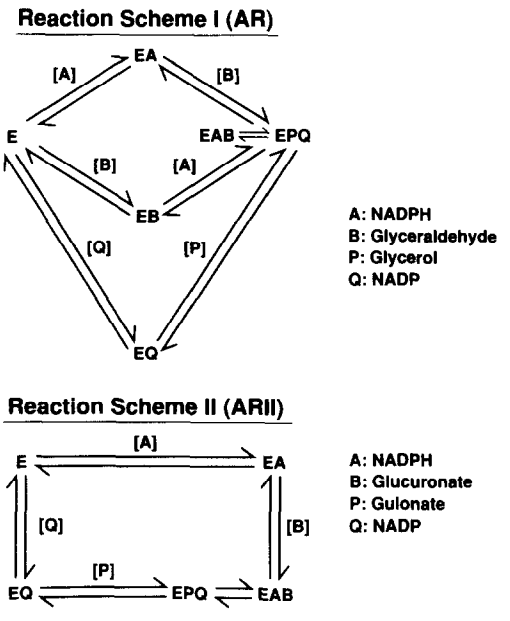

Fig. 7. Reaction kinetic schemes for aldose reductase and aldehyde reductase II.

UC to both the substrates and would probably not bind to the free enzyme, but only to the enzyme–substrate complexes. As shown in Fig. 7, the reaction scheme for AR has a random addition of substrates and an ordered release of products. If these inhibitors bind to the E:NADPH complex, they would be NC vs glyceraldehyde and vice versa; therefore, its seems likely that these inhibitors bind mostly to the E:NADPH:Glyceraldehyde complex. The kinetic similarity of inhibition by alrestatin and CCA may be due, in part, to the presence of adjacent carboxylic and carbonyl groups in their structures (Fig. 2). The K_{ii} values and the nature of inhibition by alrestatin reported here are in contrast to the studies reported earlier. O'Brien *et al.* [32] have reported an NC nature of inhibition of human brain AR by alrestatin with K_{ii} and K_{is} values ranging from 170 to 320 μM, whereas in this study the K_{ii} values were 0.3 to 1.0 μM. If the inhibition constants are any indication, the human brain and kidney enzymes appear to be fundamentally different in the nature of their inhibitor binding site(s).

The inhibitors of category III, quercetin and ICI 105552, were UC vs NADPH and NC vs glyceraldehyde. The K_{ii} and the K_{is} values when glyceraldehyde was the variable substrate were of the same magnitude. These inhibitors probably bind preferably to either E:Glyceraldehyde or E:NADPH complexes. An NC nature of inhibition of AR (glyceraldehyde variable) by quercetin also has been reported previously [36]. Of this category, ICI 105552 had a higher selectivity for AR.

Category IV inhibitors, tolrestat and statil, were NC vs NADPH and UC vs glyceraldehyde and probably bind to E:Glyceraldehyde, or to the ternary complex. The non-parallel nature of the Yonetani–Theorell double-inhibition plots (Fig. 5) indicates that tolrestat is different from other inhibitors in that

it does not bind to the site that binds other inhibitors. In this series of inhibitors tested, statil was found to have the lowest K_{ii} values (0.001 to 0.007 μM) for AR. As statil also displayed a very high selectivity for AR, from a purely kinetic point of view, it would be a relatively more specific inhibitor of AR.

For AR II, the kinetic categories were the same as for AR, but as AR II has a different reaction scheme, these categories would indicate different binding patterns. Sorbinil and alrestatin belong to the same category and may bind to any of the enzyme forms in the reaction scheme (Scheme I), as they were NC to both the substrates. For sorbinil, the K_{is} values were higher than the K_{ii} values, indicating that this compound has a higher affinity for the enzyme–substrate complexes than for the free enzyme form. Also as mentioned earlier, the K_{is} values were poorly defined which would indicate that there may be more than one enzyme form which could bind to the inhibitor. A noncompetitive nature of inhibition by sorbinil and alrestatin of AR II also has been observed for the human brain enzyme [32] and the sheep liver aldehyde reductase [37], with K_{ii} values similar to those of human placental AR II reported here. Category II includes statil and ICI 105552, both of which showed UC inhibition versus both the substrates. On the basis of the reaction scheme shown for AR II in Fig. 7, these inhibitors are expected to bind mainly to the E:NADP(H):Glucuronate complex. Phenobarbital, CCA and tolrestat seem to bind preferably only to E:NADPH as they were NC to glucuronate and UC to NADPH (Category III). A noncompetitive inhibition of AR II by phenobarbital, when aldehyde was the variable substrate, also has been observed for pig kidney [38] and ox brain [39] enzyme. In these studies the inhibition was suggested to be due to the presence of an E:NADP(H):Phenobarbital inactive complex, a conclusion also supported by the studies reported here. Quercetin was the sole member of category IV inhibitor and was UC to glucuronate and NC to NADPH. Quercetin acts as a UC inhibitor also to the ox brain aldehyde reductase when aldehyde is the varied substrate [39]. It probably binds to the E:NADPH:Glucuronate complex. The parallel nature of Yonetani–Theorell plots (Fig. 6) indicates that, for AR II, inhibitors of all the four categories bind to the same site.

From the studies on the kinetics of inhibition of both AR and AR II by various ARI, it is evident that all the ARI tested inhibit both the enzymes. However, the inhibition of these enzymes by ARI would depend on the affinity with which they bind to the different forms of the enzymes shown in Schemes I and II (Fig. 7). The results of this study indicate that inhibitors such as statil, ICI 105552 and tolrestat, which are more specific to AR may be so, in part, due to their greater affinity for the E:Aldehyde complex, which is present only in the AR reaction scheme. Therefore, it may be useful to synthesize inhibitors which bind exclusively to the E:Aldehyde complex in order to improve the specificity of inhibition of AR over AR II or other dehydrogenases, as most dehydrogenases have an ordered reaction scheme without a significant E:Aldehyde complex [40]. None of the inhibitors tested were competitive and, therefore, do not bind to the substrate binding sites. Thus, in order to design specific ARI, a more rational approach would be to study, in depth, the chemical mechanism of the reaction and to synthesize competitive, mechanism-based, inhibitors which would be analogues of the transition state of the substrates and cause irreversible inactivation of the enzyme.

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