Biochemical Pharmacology, Vol. 39, No. 2, pp. 337–346, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

PONALRESTAT: A POTENT AND SPECIFIC INHIBITOR OF ALDOSE REDUCTASE

WALTER H. J. WARD,* CHRISTOPHER M. SENNITT, HELEN ROSS, ANTHONY DINGLE, DAVID TIMMS, DONALD J. MIRRLEES and DAVID P. TUFFIN

ICI Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.

(*Received* 11 *April* 1989; *accepted* 29 *August* 1989)

Abstract—Many of the complications of diabetes appear to be closely linked to increased conversion of tissue glucose to sorbitol which is catalysed by aldose reductase (aldehyde reductase 2, ALR2). Inhibition of ALR2 could, therefore, lead to a reduction in the development of diabetic complications. Ponalrestat ["Statil" (a trademark, the property of Imperical Chemical Industries PLC), "Prodiax" (a trademark, the property of Merck, Sharp and Dohme), ICI 128436, MK538] inhibits ALR2 from a number of sources. Until now, the mechanism of this inhibition has not been fully elucidated. In this paper, we present a detailed mechanism for inhibition of bovine lens ALR2 by ponalrestat. Treatment of humans with some ALR2 inhibitors leads to side-effects, some of whcih may result from interactions with other enzymes. Aldehyde reductase (ALR1) is probably the most closely related enzyme to ALR2. Inhibition of ALR1 from bovine kidney was, therefore, investigated in order to assess the specificity of ponalrestat. The values of K_i and K_{ies} (apparent dissociation constants for inhibitor from enzyme–inhibitor and enzyme–inhibitor–substrate complexes, respectively) for the interactions of ponalrestat with ALR1 and ALR2 hae been calculated by non-linear fitting of kinetic data. These values indicate that ponalrestat does not compete with binding of glucose of NADPH to ALR2, nor with binding of glucuronate or NADPH to ALR1. Lack of competition and the structural dissimilarity of substrates and inhibitor make it unlikely that ponalrestat will utilize substrate binding sites on other enzymes, and so produce undesirable side-effects via such a mechanism. Ponalrestat is a potent inhibitor ($K_i = K_{ies} = 7.7$ nM) of ALR2 and follows a pure noncompetitive mechanism with respect to glucose. Efficacy, therefore, will not be decreased by development of hyperglycaemia. The compound is a mixed noncompetitive inhibitor of ALR1 when glucuronate is varied. The values of K_i and K_{ies} are 60 μM and 3 μM, respectively, so that inhibition tends towards uncompetitive. The selectivity of ponalrestat in favour of ALR2, therefore, lies in the range 390 to 7,800-fold, being higher at lower concentrations of glucuronate. The high selectivity of ponalrestat in favour of ALR2 rather than ALR1 suggests that the compound is unlikely to inhibit other enzymes which have less homology with ALR2.

Aldehyde reductase 2 (ALR2 or aldose reductase)† (EC 1.1.1.21) is strongly associated with pathogenesis of some of the symptoms of diabetes [1, 2]. The enzyme catalyses reduction of glucose to sorbitol and there is strong evidence suggesting that excessive flux through this pathway leads to damage in the nerve, retina, kidney and lens. Resultant mechanisms of tissue damage (see Ref. 2) appear to include: depletion of NADPH, decreased myoinositol, altered Na^+K^+ ATPase activity and cytoplasmic accumulation of sorbitol and its metabolite, fructose, which in turn leads to hypertonicity and osmotic uptake of water. Accordingly, suitable inhibitors of ALR2 have been sought as a therapeutic approach to reducing the development of diabetic complications [2, 3]. These compounds have not yet been fully evaluated in humans, but a possible problem which could arise is specificity for the target enzyme. High specificity for ALR2 may prove essential for a safe drug, especially since the enzyme is closely related to other enzymes in the aldo–keto reductase family [1]. The highest homology in structure and activity is seen with aldehyde reductase 1 (aldehyde reductase of ALR1, EC 1.1.1.2) [1, 4], and so specificity of inhibition has been characterized by investigating the effects on this enzyme.

The compound under investigation, ponalrestat (3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazin-1-yl) acetic acid), is a potent inhibitor of ALR2 preparations from a number of sources including rat, bovine or human cadaver lens, bovine retina and human placenta [5]. Until now, the mechanism of this inhibition has not been fully characterized. Our aims included measurement of the affinity of ponalrestat for bovine lens ALR2, and investigation of whether increased glucose concentrations in hyperglycaemia affect potency. We also present characterisation of the inhibition of bovine kidney ALR1 by ponalrestat. These data are used to assess specificity of inhibition.

* Author to whom correspondence should be addressed.

† Abbreviations: ALR1, aldehyde reductase 1 (aldehyde reductase); ALR2, aldehyde reductase 2 (aldose reductase); IC_{50}, concentration giving 50% inhibition of enzyme-catalysed reaction; EDTA, ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Materials

Reagents were purchased from the Sigma Chemical Co. (Poole, U.K.) and BDH (Poole, U.K.).

Methods

Preparation of ALR1. Partially purified ALR1 from bullock kidney was obtained using a procedure adapted from that of Daly and Mantle [6]. Kidneys from freshly slaughtered animals were kept on ice, cut into small pieces, and homogenized in 3 vol. 0.25 M sucrose, 2.0 mM EDTA (dipotassium salt), 2.5 mM 2-mercaptoethanol, 10 mM phosphate/NaOH, pH 7.2. The homogenate was centrifuged (16,000 *g* for 20 min at 4°) and the supernatant was retained. Ammonium sulphate precipitation was performed to 45% and then 75% saturation. The supernatant was retained after the first precipitation and the pellet was retained after the second. The pellet was redissolved in 10 mM phosphate/NaOH, pH 7.2, containing 2.0 mM EDTA (dipotassium salt) and 2.0 mM 2-mercaptoethanol, and then dialysed against the same buffer (2 × 10 L). DEAE-52 resin was added to the dialysate and then removed by centrifugation. The supernatant contained ALR1 and was diluted to around 20 mg/cm^3 before storage at −20°. All experiments were performed using a single batch of enzyme.

Assay of ALR1. The standard assay conditions were as follows: 0.1 M phosphate/NaOH, pH 7.2, 120 μM NADPH, 20 mM D-glucuronate, 5 μL dimethylsulphoxide (containing ponalrestat when added) per 3 cm^3 assay. The reaction, at 37° was initiated by addition of enzyme to about 0.16 mg protein/cm^3 and was followed spectrophotometrically by E_{340}, taking the extinction coefficient of NADPH as 6,200/M/cm.

Preparation of ALR2. Bovine lens ALR2 was partially purified using a procedure adapted from Hayman and Kinoshita [7]. Lenses from freshly slaughtered cattle were homogenized in distilled water containing 5 mM 2-mercaptoethanol and 0.5 mM EDTA (disodium salt), and then centrifuged (16,000 *g* for 20 min at 4°). The supernatant was precipitated with ammonium sulphate at 40%, 50% and then 75% saturation. The supernatant was retained after the first two precipitations, and ALR2 activity was pelleted in the final step. The precipitate was dispersed in 75% saturated ammonium sulphate at around 80 mg protein/cm^3, and then stored at −20°. All experiments were performed using a single batch of enzyme.

Assay of ALR2. The standard conditions were as follows: 67 mM phosphate/NaOH, pH 6.2; 75 μM NADPH; 0.40 M lithium sulphate, 167 mM D-glucose and 5 μL dimethylsulphoxide per 3 cm^3 assay (containing ponalrestat where appropriate). Reagents were incubated at 37°, and the assay was started by addition of enzyme (to approximately 1.3 mg protein/cm^3). Rate was followed spectrophotometrically as described for the ALR1 assay.

Fitting of kinetic data. Non-linear fitting was performed using the program Enzfitter [8]. This program runs on an IBM-PC and uses the Marquart algorithm (see Ref. 8). Standard errors are calculated by matrix inversion. All data were analysed using simple weighting. Non-linear fitting requires initial estimates of the variables. Like many programs for non-linear fitting, Enzfitter can calculate initial estimates using linear transformations of the data [8, 9]. The linear transformations of Dixon [10] and Henderson [11] were used in order to calculate initial estimates. Application of Dixon plots in this way has been described by Cleland [9].

Measurement of protein concentration. The Folin method was used.

THEORY

Determination of mechanism of inhibition using non-linear fitting

Different mechanisms of reversible inhibition are characterised by specific values for K_i* and K_{ies} (see Ref. 12). Competitive inhibition occurs when only K_i is finite. Conversely, inhibition is uncompetitive when only K_{ies} is finite. Pure noncompetitive inhibition occurs when $K_i = K_{ies}$, and the mechanism is mixed noncompetitive if K_i does not equal K_{ies}. Thus, calculation of the values of K_i and K_{ies} establishes the mechanism of inhibition. Experimental data can be analysed by non-linear fitting to rate equations describing each mechanism of inhibition. The most simple equation which accurately explains the data gives the most likely mechanism of inhibition [9, 13].

In general, a significant decrease in v is observed only when $[I] \gg [E]$. This allows the approximation in classical kinetics that $[I]_f = [I]$ which greatly simplifies mathematical analysis. However, the success of drug hunting programs which are searching for inhibitors of increased potency has increasingly caused the approximation $[I]_f = [I]$ to become inaccurate. Such compounds are said to follow tight binding kinetics [11, 14, 15] because the $[I]$ required to decrease activity is similar to $[E]$.

The equations for tight binding kinetics model the general case which holds whether or not tight binding kinetics occurs. Classical kinetics where $[I]_f$ approximates to $[I]$ is described accurately using the equations for tight binding kinetics. In this case, the value calculated for $[E]'$ is $\ll K_i$ and K_{ies}. Conversely, classical kinetics cannot describe tight binding because the assumption that $[I]_f$ approximates to $[I]$ is not valid. Thus, tight binding kinetics fit *only* to the complex equations, whereas classical inhibition fits *both* to the simplified equations and to the equations for tight binding kinetics.

When a compound follows classical kinetics, it is best not to fit to the equations for tight binding because they contain an extra variable. The value of this variable, $[E]$, is difficult to calculate in this case. Thus, inclusion of $[E]$ when a compound does not follow tight binding kinetics may lead to increased uncertainty in the calculated values of K_i and K_{ies}.

The methods described below calculate the values of K_i and K_{ies} when $[S]$ is constant and $[I]$ is varied.

* Symbols: E, enzyme; S, substrate; I, inhibitor; $[E]$, total concentration of enzyme; $[I]$, total concentration of inhibitor; $[I]_f$, concentration of inhibitor free in solution; K_m, Michaelis constant; V_{max}, velocity in the presence of saturating S; K_i, apparent dissociation constant of E–I complex; K_{ies}, apparent dissociation constant of I from E–I–S complex; v, velocity of enzyme catalysed reaction; v_0, velocity in absence of I; v_i, velocity in presence of I. Adding the primed symbol (′) indicates that the parameter is an apparent value which is specific to the experimental conditions at that time.

Table 1. Equations for non-linear fitting of v in the presence of I

Type of inhibition	Effect of increasing [S] on K_i'	General formula for K_i'	Equation number
Mixed noncompetitive	Increases or Decreases	$= \frac{K_i K_{ies}([S] + K_m)}{K_i[S] + K_{ies}K_m}$	(3)
Pure noncompetitive	None	$= K_i = K_{ies}$	(4)
Competitive	Increases	$= K_i([S] + K_m)/K_m$	(5)
Uncompetitive	Decreases	$= K_{ies}([S] + K_m)/[S]$	(6)

Two general equations describe $[I]$ dependence of v. The first of these applies only when $[I]_f$ approximates to $[I]$:

$$v_i = v_0/(1 + [I]/K_i') \quad (1)$$

whereas the second describes tight binding kinetics

$$v_i = v_0\{-0.5(K_i'/[E]' + [I]/[E]' - 1) + 0.5((K_i'/[E]' + [I]/[E]' - 1)^2 + 4K_i'/[E]')^{0.5}\} \quad (2)$$

The derivation of Eqn (2) is given by Williams and Morrison [15]. The $[S]$ dependence of K_i' is the same whether or not tight binding occurs and is given below. Eight equations describing different mechanisms of inhibition are produced as follows. Values for K_i' from Eqns (3–6) are substituted into Eqn (1) to give the relationships for classical inhibition. Rate equations for tight binding kinetics are given by substitution of values for K_i' from Eqns (3–6) into Eqn (2). For example, the rate equation for classical competitive inhibition is given by substitution of Eqn (5) into Eqn (1) giving

$$v_i = v_0/\{1 + [I]K_m/K_i([S] + K_m)\}.$$

Eight equations model four different mechanisms of inhibition, each with and without tight binding (Table 1). Non-linear fitting is employed treating v_i as the dependent variable, and $[I]$ as the independent variable. K_i and K_{ies} do not show a systematic variation which is dependent upon $[S]$ when data are analysed using equation(s) which correctly model mechanism of inhibition. Fitting to an incorrect equation can be detected because calculated values fail to overlap even when the standard error is taken into account.

In addition to calculation of values of K_i and K_{ies}, information on mechanism of inhibition is deduced from inspection of differences between experimental data and calculated best fit lines. There are two explanations for these differences: first, experimental error, which should exhibit a random distribution; second, systematic error which arises because data are being analysed using an incorrect equation. Inspection of the residuals between observed and calculated rates, therefore, gives an indication of whether the equation adequately describes the experimental data (see Refs 8 and 16).

RESULTS

Enzyme preparations are free from detectable cross contamination

Kidney is rich in ALR1 but low in ALR2. In contrast, lens contains a high concentration of ALR2 but very small levels of ALR1 [1]. Each enzyme preparation, although only partially purified, was thus expected to contain significant levels of only one aldehyde reductase. Measurement of substrate specificity and inhibitor sensitivity confirms no detectable contamination of the ALR2 preparation with ALR1 and vice-versa (Table 2). Properties of the bovine enzyme preparations are similar to those of homogeneous ALR1 and ALR2 purified from pig brain [17]. ALR1 turns glucuronate over rapidly, whereas xylose is a poor substrate, and glucose is not used to any detectable extent. ALR2 reduces

Table 2. Substrate specificity and inhibitor sensitivity of preparations of ALR1 and ALR2

Characteristic	Bovine kidney ALR1	Porcine brain ALR1	Bovine lens ALR2	Porcine brain ALR2
Activity using:				
20 mM D-glucuronate	100%	100%	100%	100%
50 mM D-xylose	7%	2%	89%	160%
163 mM D-glucose	0.7%	No activity	110%	140%
Inhibition by:				
0.5 mM valprote	87%	84%	31%	27%
1.0 mM Na barbitone	75%	74%	11%	30%

Values quoted for the bovine enzyme preparations are the means of duplicates and were measured under the standard ALR1 assay conditions described in Materials and Methods. Rate using glucuronate is taken as 100% in substrate specificity data. Inhibition data relate to the use of 20 mM D-glucuronate as substrate. The following values correspond to 100% rates: for ALR1, 16 nmol/min/mg; for ALR2, 1.7 nmol/min/mg. Values quoted for porcine enzymes are calculated from the data of Cromlish and Flynn [17] using enzymes purified to homogeneity. For inhibition data, Cromlish and Flynn [17] utilized 60 mM D-glucuronate for ALR1, and 85 mM D-xylose for ALR2.

Table 3. Substrate dependence of ALR2 and ALR1

Enzyme	Substrate	K_m (mM)	V_{max} (nmol/min/mg)
ALR1	D-Glucose	No significant activity	
ALR1	D-Glucuronate	6.6	21
ALR2	D-Glucose	48	3.8
ALR2	D-Glucuronate	13	2.8

Aldehyde concentration dependence of rate was measured at 125 μM NADPH for ALR1 and 75 μM NADPH for ALR2. Glucuronate was varied from 5 to 50 mM for both enzymes, and glucose was varied from 30 to 460 mM for ALR2. The rate of oxidation of NADPH by ALR1 in the presence of 163 mM D-glucose is <0.16 nmol/min/mg. NADPH-dependence of ALR1 was measured at 8–55 μM NADPH and 20 mM glucuronate. For ALR2, NADPH-dependence was studied at 8–1750 μM NADPH and 30 or 167 mM glucose. Under all conditions, each enzyme displays a K_m for NADPH <5 μM, and so the value could not be measured precisely (see Results). Experimental details are given in Materials and Methods.

each of the sugars at a similar rate. ALR1 is markedly inhibited by either valproate or sodium barbitone, whereas ALR2 shows a substantially smaller decrease in rate in the presence of either of these compounds.

Kinetic characteristics of ALR1 and ALR2

Both enzymes catalyse NADPH-dependent reduction of aldehydes. The two enzymes each display a similar value of K_m for glucuronate, but V_{max} is considerably greater for ALR1 (Table 3). Only ALR2 can utilize glucose to any significant extent (Table 3).

Both ALR1 and ALR2 display values of K_m for NADPH of <5 μM under all conditions tested (Table 3). Each enzyme is, therefore, saturated whenever there is sufficient substrate to measure rate by following E_{340}. This prevented precise determination of the value of K_m. All of these kinetic characteristics of ALR1 and ALR2 are similar to those previously reported for these enzymes from other sources [6, 17, 18].

Ponalrestat is a mixed noncompetitive inhibitor of ALR1

The [I] dependence of v was fitted to each of the eight different equations given above (Table 1) in order to calculate values for K_i and K_{ies}. These equations model the following types of inhibition both with and without tight binding: mixed noncompetitive, pure noncompetitive and uncompetitive. The values calculated for K_i and K_{ies} are given in Table 4. First, considering the models where tight binding does not occur. Data fit well to the model for mixed noncompetitive inhibition, because the values of K_i and K_{ies} do not vary systematically according to glucuronate concentration. Variation in K_{ies} with [S] is only just greater than the calculated standard errors when fitting to uncompetitive inhibition. The standard errors are larger for mixed noncompetitive inhibition, but this may be because this model contains a larger number of variables which cannot be determined independently, rather than being due to the model being inappropriate. When inhibition is assumed to be pure noncompetitive or competitive, there is a change in K_i when [S] increases. This indicates that these models do not accurately describe the mechanism of inhibition. Similar conclusions are obtained when the data are fitted to equations which allow for tight binding kinetics. Thus, the mechanism of inhibition may be mixed noncompetitive or uncompetitive, and tight binding may or may not be occurring.

The magnitude of K_m observed decreases by a factor of approximately two in the range of inhibitor concentrations used (Fig. 1), whereas the apparent value of V_{max} falls by around six-fold (Fig. 1). These observations rule out uncompetitive inhibition since such a mechanism would cause the observed values of K_m and V_{max} to fall by the same factor (see Ref. 12). These data demonstrate that inhibition is mixed noncompetitive, but there is a tendency towards uncompetitive since V_{max} is more affected than K_m.

Table 4. Values of K_i and K_{ies} determined for inhibition of ALR1 by ponalrestat

	D-Glucuronate concentration (mM)		
	5	25	50
When $[I]_t$ approximates to [I]			
(a) K_i (μM)	61 ± 10,000	30 ± 10,000	200 ± 7000
K_{ies} (μM)	3.0 ± 0.4	3.1 ± 0.2	3 ± 10
(b) K_i (μM)	6.7 ± 0.4	3.8 ± 0.1	3.7 ± 0.2
(c) K_i (μM)	3.7 ± 0.2	0.80 ± 0.03	0.43 ± 0.02
(d) K_{ies} (μM)	2.9 ± 0.2	3.0 ± 0.1	3.3 ± 0.1
When tight binding occurs			
(a) K_i (μM)	50 ± 200	30 ± 500	*
K_{ies} (μM)	3 ± 1	3 ± 30	*
(b) K_i (μM)	6.7 ± 0.5	3.8 ± 0.4	*
(c) K_i (μM)	3.8 ± 0.1	0.81 ± 0.03	*
(d) K_{ies} (μM)	2.9 ± 0.2	3.0 ± 0.3	*

Enzyme activity was determined as described in Materials and Methods. Ponalrestat was varied from 0 to 23 μM. Data were analysed by non-linear fitting to the equations given in Table 1. Type of inhibition: (a) mixed noncompetitive; (b) pure noncompetitive; (c) competitive; (d) uncompetitive. Values are quoted as best fit ± SE.

* Data would not fit to equations describing these mechanisms of inhibition.

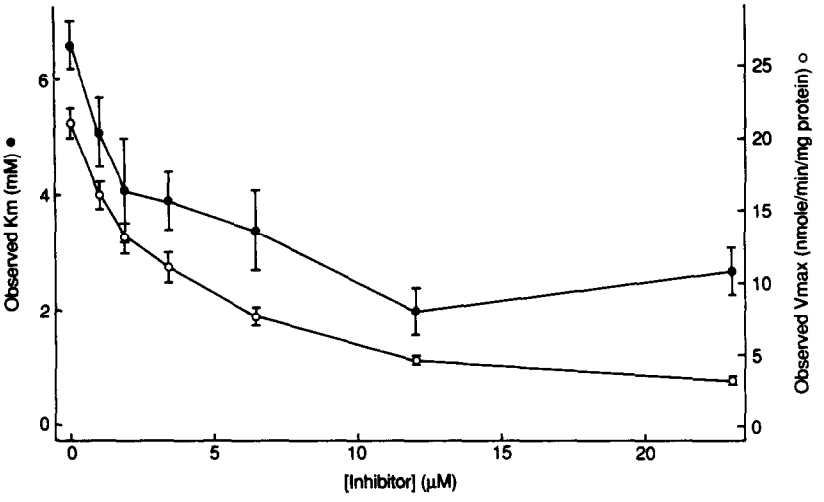

Fig. 1. Glucuronate dependence of ALR1 when ponalrestat is varied. (●) Observed K_m; (○) observed V_{max}. Values are quoted as best fit ± SE. Rates were determined at 5–50 mM glucuronate and 125 μM NADPH, other experimental details as described in Materials and Methods.

This shows that the affinity of the enzyme for ponalrestat is highest when ALR1 is complexed to glucuronate. The tendency towards uncompetitive inhibition is confirmed by direct calculation of K_i and K_{ies} (Table 4), but the data presented so far do not resolve whether tight binding kinetics are being followed.

Ponalrestat is not a tight binding inhibitor of ALR1

Two approaches: fitting of raw data (Fig. 2) and inspection of residuals are consistent with ponalrestat not being a tight binding inhibitor of ALR1. No improvement in fit is seen when tight binding is included in the kinetic analysis. Non-linear fitting to the rate equation for mixed noncompetitive tight binding kinetic leads to calculation of apparent $[E]$ = 0.05 ± 0.70 μM (best fit ± SE) in the presence of 5–25 μM inhibitor. (The large degree of uncertainty in the apparent $[E]$ arises because it is $\ll [I]$.) Thus, tight binding kinetics are not required to accurately model the system. This conclusion is confirmed by statistical analysis. Rate equations for tight binding kinetics require an additional variable, $[E]$, when compared to classical kinetics. The validity of using this additional variable can be measured using a variance ratio, or F-test [16]. Application of this procedure indicates that use of the additional variable is not justified at any concentration of glucuronate. The value obtained for F at each concentration of glucuronate indicates that the improvement in fit obtained is not statistically significant ($P > 0.1$).

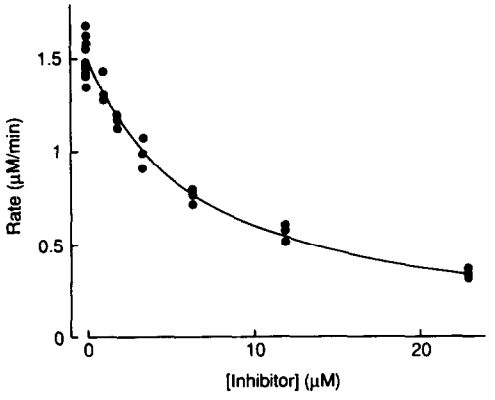

Fig. 2. Inhibition of ALR1 by ponalrestat at 5 mM D-glucuronate. Activity was measured as described in Materials and Methods. (●) Observed values. The best fit lines are shown, and these were calculated assuming mixed noncompetitive inhibition (see Theory). The lines with and without tight binding are superimposable. Similar results were obtained at other concentrations of glucuronate.

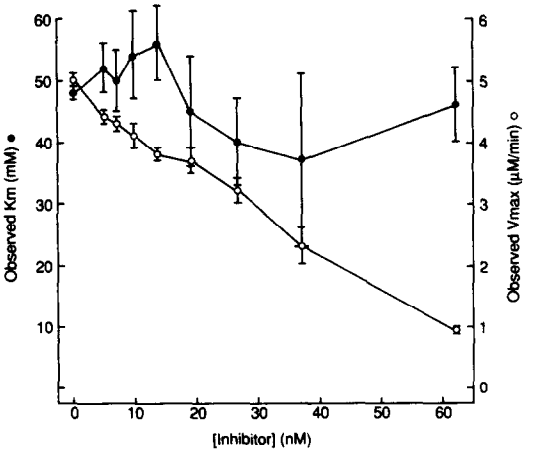

Fig. 3. Glucose dependence of ALR2 when ponalrestat concentration is varied. Data were analysed by non-linear fitting to the Michaelis–Menten equation. (●) Observed K_m; (○) observed V_{max}. Values are shown as best fit ± SE. Glucose concentration was 30–460 mM and NADPH concentration was 75 μM, other experimental details are given in Materials and Methods.

Table 5. Values of K_i and K_{ies} determined for inhibition of ALR2 by ponalrestat when glucose is varied

	Glucose concentration (mM)					
	30	60	100	167	278	460
When $[I]_f$ approximates to $[I]$						
(a) K_i (nM)	30 ± 50	26 ± 1	30 ± 300	30 ± 500	30 ± 700	30 ± 100
K_{ies} (nM)	30 ± 70	27 ± 1	30 ± 300	30 ± 200	30 ± 100	30 ± 100
(b) K_i (nM)	29 ± 2	27 ± 2	28 ± 2	29 ± 2	30 ± 2	30 ± 2
(c) K_i (nM)	18 ± 1	12 ± 1	9.0 ± 0.6	6.3 ± 0.4	4.4 ± 0.3	2.8 ± 0.2
(d) K_{ies} (nM)	11 ± 1	15 ± 1	19 ± 1	22 ± 2	26 ± 2	27 ± 2
When tight binding occurs						
(a) K_i (nM)	10 ± 100	7.5 ± 0.1	7 ± 800	5.0 ± 1.4	10 ± 1000	*
K_{ies} (nM)	10 ± 400	7.2 ± 0.1	6 ± 400	5.5 ± 0.5	7 ± 100	8.6 ± 100
(b) K_i (nM)	10 ± 1	7.3 ± 0.8	6.0 ± 0.6	5.5 ± 0.3	7.1 ± 0.8	10 ± 1
(c) K_i (nM)	6.3 ± 0.6	3.2 ± 0.4	1.9 ± 0.2	1.2 ± 0.2	1.0 ± 0.1	0.96 ± 0.02
(d) K_{ies} (nM)	3.9 ± 0.4	4.0 ± 0.4	4.0 ± 0.4	4.3 ± 0.6	6.0 ± 0.7	9.1 ± 1.0

Experimental details are given in Materials and Methods. Data were analysed by non-linear fitting to the equations given in Table 1. Type of inhibition: (a) mixed noncompetitive; (b) pure noncompetitive; (c) competitive; (d) uncompetitive. Calculated values ± SE are quoted. Ponalrestat was varied from 0 to 200 nM.

* $7 \times 10^3 \pm 12 \times 10^6$ nM.

Ponalrestat is a pure noncompetitive inhibitor of ALR2

The dependence of rate upon $[I]$ was fitted to each of the eight different models given in Table 1. The values obtained for K_i and K_{ies} are shown in Table 5. First, considering the models in the absence of tight binding kinetics: the data fit well to the equation for mixed noncompetitive inhibition, because the measured values of K_i and K_{ies} do not vary with $[S]$. The standard errors calculated for K_i and K_{ies} are the largest for this equation because this mechanism contains the largest number of variables whose values cannot be determined independently from each other. However, the magnitude of K_i is similar to that of K_{ies}, suggesting that inhibition could be pure noncompetitive. This is supported by the observation that the fit to pure noncompetitive inhibition is good since the value of K_i is independent of $[S]$. When inhibition is assumed to be competitive, the magnitude of K_i decreases with increasing $[S]$. This indicates that inhibition is not competitive. When fitting to uncompetitive inhibition, the value of K_{ies} measured shows an increase with increasing $[S]$, showing that the data are not described accurately by this rate equation. Similar conclusions are obtained when the data are fitted to equations which allow for tight binding kinetics (Table 5). Note that the increased standard error when fitting to tight binding kinetics is not necessarily due to the model being incorrect, but may arise because there is an extra variable in the model whose value cannot be determined independently using these data.

The value of K'_m does exhibit some variation when $[I]$ is varied (Fig. 3). However, variation is random rather than systematic and is only of a similar magnitude to the standard error. Conversely, the magnitude of V'_{max} clearly decreases with increasing $[I]$ (Fig. 3). Thus, the observed values of V'_{max} and K'_m are consistent with pure noncompetitive inhibition [12], but it is unclear whether or not tight binding is occurring.

Inhibition of ALR2 by ponalrestat follows tight binding kinetics

In order to establish whether ponalrestat follows tight binding kinetics, data were fitted to each of the two equations for pure noncompetitive inhibition (see Table 1). When data are analysed using the equation for tight binding kinetics there is a random error distribution (Fig. 4, continuous line), suggesting that the rate equation gives an accurate description of the system. Conversely, a systematic error distribution is seen when the same data are fitted to the simplified equation for classical kinetics (Fig. 4, broken line).

So far, only indirect evidence for tight binding kinetics has been presented. Direct evidence was

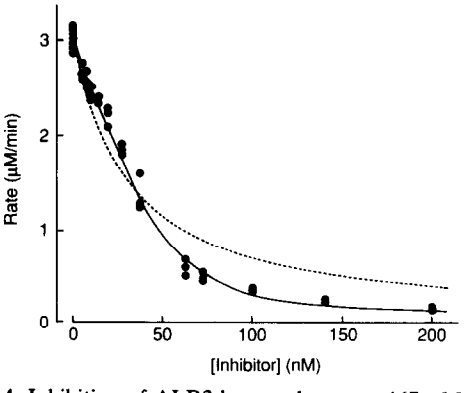

Fig. 4. Inhibition of ALR2 by ponalrestat at 167 mM glucose. Experimental procedures were as given in Materials and Methods. Best fit lines are shown, and these were calculated by non-linear fitting assuming pure noncompetitive inhibition as described in Theory. (●) Observed values; (- - - -) assuming that $[I]_f = [I]$; (——) assuming tight binding. Similar data were obtained at other concentrations of glucose.

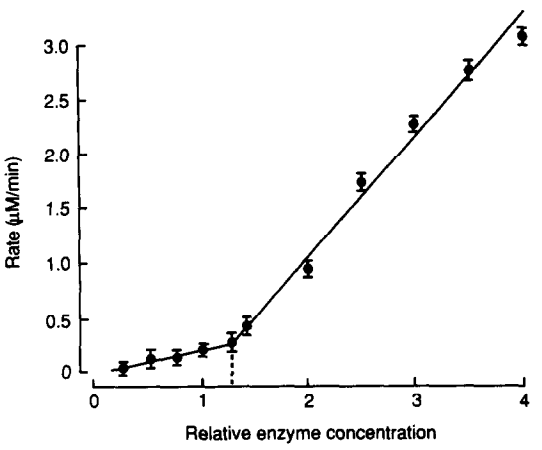

Fig. 5. Titration of bovine lens ALR2 against a fixed concentration of ponalrestat (50 nM). Activity was assayed at 167 mM glucose and 75 μM NADPH as described in Materials and Methods. Each point represents mean ± SE for six determinations.

obtained by measuring the concentration of ponalrestat binding sites. Using a fixed ponalrestat concentration (50 nM), a titration curve was constructed for rate against relative $[E]'$ (Fig. 5). The initial enzyme concentration was decreased by a factor of 1.5 below that used for kinetic characterization (data in Table 5) in order to allow titration with this ponalrestat concentration. An inflection is seen indicating that the concentration of binding sites is 50 nM in this experiment when $[E]'$ is raised by 1.3-fold above the initial assay concentration. These data, therefore, show that the concentration of binding sites routinely used is $50 \times 1.5/1.3 = 57$ nM so that kinetic behaviour is influenced by tight binding. This value for $[E]'$ is in close agreement with that determined by non-linear fitting of rate when ponalrestat concentration is varied (50 ± 5 nM).

The validity of fitting to tight binding kinetics is confirmed by statistical analysis. Application of an *F*-test procedure [16] indicates that treatment of $[E]$ as a variable is justified at all substrate concentrations because the improvement in fit obtained is highly significant ($P < 0.01$).

Ponalrestat does not compete with binding of NADPH to ALR1 or ALR2

Substrate concentration should be varied in a range both below and above the value of K_m in order to determine the mechanism of inhibition (see Table 1 and Ref. 9). This was not possible when investigating dependence of rate upon NADPH concentration (see above). However, data from the following experiments demonstrate that ponalrestat does not compete with binding of NADPH to either ALR1 or ALR2.

The magnitude of IC_{50} (K_i') is given by Eqn (1) for ALR1 and Eqn (2) for ALR2. All experiments were performed under conditions when NADPH concentration $\gg K_m$ so that $K_i' = K_{ies}$ for mixed noncompetitive inhibition. $K_i' = K_i = K_{ies}$ when inhibition is pure noncompetitive. For a competitive inhibitor, $K_i' = [S]K_i/K_m$, and when inhibition is uncompetitive, $K_i' = K_{ies}$.

Calculated IC_{50} values (Table 6) have a relatively large standard error when compared with parameters calculated when aldehyde concentration is varied (Tables 4 and 5). This is becuase nonenzymic loss of NADPH increases with concentration and becomes significant when compared with the rate of the enzyme-catalysed reaction which does not increase because there is sufficient coenzyme to give a rate of V_{max} at all concentrations. For both ALR1 and ALR2, the value of K_i' is constant, within the range of the standard errors, when NADPH concentration is increased (Table 6). This demonstrates that ponalrestat is not competitive with respect to NADPH, but the actual mechanism of inhibition is not elucidated as the data are consistent with mixed noncompetitive, pure noncompetitive and uncompetitive inhibition. The magnitude of IC_{50} obtained for ALR1 is between K_i and K_{ies} for glucuronate dependence (Table 4), but the value is closer to that of K_{ies} as would be expected since glucuronate concentration is $>K_m$. The magnitude of K_i' obtained for ALR2 agrees with the value calculated from study of glucose concentration dependence (Table 5).

Table 6. Values of IC_{50} for ponalrestat when concentration of NADPH is varied

Enzyme	Aldehyde	NADPH concentration (μM)	K_i'
ALR1	20 mM D-Glucuronate	8	3.4 ± 0.2 μM
		13	3.3 ± 0.2 μM
		21	3.1 ± 0.2 μM
		55	3.8 ± 0.2 μM
ALR2	30 mM D-Glucose	50	9.6 ± 3.7 nM
		250	9.2 ± 1.7 nM
		1750	6.4 ± 1.4 nM
	167 mM D-Glucose	50	5.4 ± 1.4 nM
		250	7.7 ± 1.9 nM
		1750	3.7 ± 1.4 nM

Experimental details are given in Materials and Methods. For ALR1, ponalrestat concentration was varied from 0 to 12.5 μM, and for ALR2, ponalrestat concentration was varied from 0 to 200 nM. Data were analysed as described in Results, assuming that only ALR2 followed tight binding kinetics. K_i' is quoted as calculated value ± SE.

DISCUSSION

Characterization of enzyme inhibitors by non-linear fitting of data from steady kinetics

In enzyme kinetics experiments, there is generally a non-linear relationship between rate and concentration of substrate or inhibitor. However, data are not traditionally fitted to the non-linear relationship, but to a linear transformation. The work presented above illustrates the values of using non-linear fitting rather than linear transformations in order to analyse enzyme kinetic data (see Refs 8, 9 and 13). Non-linear fitting is more accurate, and allows estimation of the standard errors for the calculated parameters. The magnitude of the standard error can be used as a measure of confidence in a calculated value.

Enzyme inhibition data are frequently analysed using the Dixon plot [10, 12] which is highly dependent upon data obtained at high inhibitor concentration where the relative error in measured rate is likely to be highest. Application of any linear transformation, such as the Dixon plot or Henderson plot, assumes a random distribution of error in the transformed data. This assumption is not usually justified. In non-linear regression, data are used with minimal manipulation, so tht complex error distributions do not usually occur. Application of non-linear fitting, therefore, greatly increases the accuracy of the calculated parameters. Linear transformations are, however, valuable in enzyme kinetics. They allow clear presentation of data in a form where systematic errors and multiple kinetic phases can often be visualized. Furthermore, linear transformations have been used in the present work in order to provide the initial estimates required to begin non-linear fitting.

IC_{50} values may vary according to the experimental conditions

IC_{50} values are commonly used as estimates of potency for enzyme inhibitors. The observed IC_{50} value is equal to K_i' when $[I]_f$ approximates to $[I]$. This allows Eqn (1) to be used to calculate IC_{50}. When tight binding kinetics apply, the observed value of IC_{50} equals $K_i' + [E]'/2$. This observed value is not a reliable estimate of potency first because K_i' is difficult to measure when its value is less than that of $[E]'$, and second because $[E]'$ may vary between assays. Tight binding leads to a detectable distortion of observed IC_{50} values when $K_i' < 5[E]'$. Thus, tight binding kinetics will be seen in the present assay for any inhibitor of ALR2 with a value of $K_i' < 250$ nM. For such compounds, increase in potency does not give rise to the expected decrease in IC_{50}. In the extreme, when $K_i' \ll [E]'$, further increase in potency has no apparent effect upon the observed IC_{50} value. K_i' can be regarded as the true IC_{50} in the absence of perturbation resulting from tight binding. The magnitude of K_i' should be calculated by fitting to Eqn (2) when tight binding occurs. In most mechanisms of inhibition, the value of IC_{50} also varies according to $[S]$ (Table 1). In fact, the $[S]$ dependence of K_i' values determined by fitting to equations for pure noncompetitive inhibition suggests a mechanism of inhibition. This is because variation of K_i' according to $[S]$ is characteristic of certain mechanisms (see Table 1, and Ref. 13). IC_{50} values are, therefore, only qualitative estimates of potency which are specific to the assay conditions. This can lead to incorrect ranking of compounds and incorrect assessment of selectivity against different enzymes (see below).

The value of $[E]'$ calculated in tight binding kinetics indicates the maximum possible concentration of active enzyme in the assay. However, the value must be treated with caution because any species that binds I will appear to be enzyme. Thus, the value of $[E]'$ represents the sum of active enzyme and contaminants that bind I.

Inhibition of ALR1 by ponalrestat

Ponalrestat causes mixed noncompetitive inhibition of ALR1 when glucuronate concentration is varied. The values of K_i and K_{ies} are 60 and 3 μM, respectively, which explains why inhibition tends towards uncompetitive. Prior binding of glucuronate causes an increase in the affinity of the enzyme for ponalrestat. The inhibitor prevents catalysis by ALR1. Inhibition could follow an alternative mechanism if a different substrate is used. The value of K_{ies}, in particular, is likely to be dependent on the substrate employed, because the aldehyde is a component of the enzyme–inhibitor complex. The substrate may contribute functional groups directly to the inhibitor binding site, and may also change the predominant conformation of the enzyme so that the protein contributes different groups to the inhibitor binding site. Glucuronate is a naturally occurring substrate of ALR1; thus mixed noncompetitive inhibition is likely to operate *in vivo*. However, other substrates may also be utilized *in vivo* and then the mechanism of inhibition may change.

Inhibition of ALR2 by ponalrestat

Many of the complications of diabetes are thought to result from reduction of glucose to sorbitol by ALR2 (see Ref. 1). We have shown that ponalrestat causes pure noncompetitive inhibition of ALR2 ($K_i = 7.7$ nM) when glucose is varied. Ponalrestat inhibits the enzyme by preventing catalysis and does not appear to affect binding of glucose. The ALR2 concentration in the assays is arround 50 nM so that tight binding kinetics are followed [11, 14, 15]. The observation that inhibition follows tight binding kinetics may explain why previously reported Lineweaver–Burk plots [5] were not linear in the presence of ponalrestat. Some inhibitors are tight binding because dissociation of the enzyme–inhibitor complex is very slow relative to the time scale of the assay. Such "slow binding" inhibitors do not follow steady state kinetics under these conditions [19]. However, there is no evidence for ponalrestat following slow binding kinetics because (i) initial rate of the inhibited reaction is the same whether or not enzyme is preincubated with inhibitor, and (ii) tight binding kinetics alone are sufficient to accurately model observed rates.

Ponalrestat is a potent inhibitor of ALR2 since the value of K_i is only 7.7 nM. The potency will remain unchanged when glucose levels rise because inhibition is not competitive. Lack of competition with

glucose shows that ponalrestat does not bind to the glucose binding site of ALR2, presumably due to insufficient structural similarity between the substrate and the inhibitor. Ponalrestat is, therefore, unlikely to bind to the substrate binding sites on other enzymes that utilize glucose and so is unlikely to produce side-effects via such a mechanism.

Structure of the binding site for ponalrestat

Kinetic studies can give only indirect information on enzyme structure. Caution is, therefore, necessary when using kinetic data to make deductions about enzyme structure. Ponalrestat does not compete with the binding of glucose or NADPH to ALR2, nor with the binding of glucoronate or NADPH to ALR1. This suggest that the inhibitor binding site on each enzyme does not overlap with the binding site for any of the substrates. Ponalrestat inhibits both ALR1 and ALR2 by decreasing catalytic rate and not by interfering with binding of substrates. These data are consistent with conservation of the inhibitor binding site between ALR1 and ALR2. Conservation is not complete because the binding affinity for ALR2 is much greater than that for ALR1. Nonetheless similarity between the inhibitor binding sites on the two enzymes might be reflecting homology in amino-acid sequence as reported for human ALR1 and ALR2 [20], and for rat ALR2 and human ALR1 [4].

The mechanism of inhibition has been charactrised for several ALR2 inhibitors: sorbinil, tolrestat and AL1576 (Alcon) (for review see Ref. 2). Like ponalrestat, these compounds also inhibit ALR1, but show less selectivity [2, 21]. Inhibition of both enzymes is noncompetitive or uncompetitive in all cases which is consistent with none of these compoundes interacting with substrate binding sites on the enzymes. Data from kinetic experiments, molecular modelling, structure–activity relationships and protein modification experiments suggest that there is a degree of overlap between the sites where different compounds bind to ALR2, and a model describing pharmacophore requirements of the inhibitor binding site has been postulated [22]. Ponalrestat binds very tightly to ALR2, so that the inhibitor could be used to protect side-chains in its binding site from chemical modification. This approach could help to identify groups in the inhibitor binding site.

Ponalrestat shows high specificity in favour of inhibition of ALR2 rather than ALR1

The design of these experiments allows measurement of the selectivity of ponalrestat for three reasons. First, the enzyme preparations are not cross contaminated to any detectable extent (Table 1). Second, the ALR2 assay does not detect ALR1 activity since ALR1 does not appear to utilize glucose (Table 2). Third, the highest ponalrestat concentration used in the ALR2 experiments (0.2 μM) does not lead to detectable inhibition of ALR1. Conversely, the ALR1 assay is not influenced by ALR2 activity because the lowest ponalrestat concentration tested (1.0 μM) is sufficient to inhibit any ALR1 activity present to negligible levels.

Table 7. Selectivity of ponalrestat against ALR1 and ALR2

Measured parameter	Enzyme		Selectivity: ratio of ALR1/ALR2
	ALR1	ALR2	
IC_{50} (nM)	3500	32*	110
K_i (nM)	60,000	7.7	7800
K_{ies} (nM)	3000	7.7	390

D-Glucose was the varied substrate for ALR2, and D-glucuronate for ALR1. IC_{50} values were determined under standard assay conditions (see Materials and Methods). Values of K_i and K_{ies} were calculated and described in the Theory.

* Value determined by fitting to Eqn (1) which does not allow for tight binding.

IC_{50} values are often used as estimates of potency of enzyme inhibitors, but this approach is not reliable because the observed IC_{50} values can be dependent upon $[E]$ and $[S]$ in the assay (Table 1). Inhibition of ALR2 by ponalrestat gives an IC_{50} value which is independent of glucose concentration because $K_i = K_{ies}$, but IC_{50} increases with increasing $[E]$ due to tight binding. The measured IC_{50} value was 32 nM, but calculations show that this would fall to 7.7 nM when $[E] \ll K_i$ (see above). The observed IC_{50} value for ponalrestat against ALR1 decreases with increasing glucuronate concentration, but is independent of $[E]$. IC_{50} equals K_i (60 μM) when glucuronate concentration $\ll K_m$, but, when the substrate is saturating, $IC_{50} = K_{ies}$ (3 μM).

The value of IC_{50} can also vary according to the K_m value of the substrate being used (Eqn 3, Table 1). Different substrates can have very different value of K_m. Ox kidney ALR1 has a $K_m = 1.3$ mM for glucuronate and a $K_m = 0.018$ mM for 4-carboxybenzaldehyde [6], a difference of 72-fold. Accordingly it is important, wherever possible, to measure potency of inhibitors when using the substrate of importance in the disease condition. Ponalrestat has been reported to display higher IC_{50} against bovine lens ALR2 when 4-nitrobenzaldehyde is used as a substrate, relative to when glucose is employed [23].

These considerations mean that the IC_{50} ratio can only be a qualitative estimate of selectivity since values obtained are not absolute for either enzyme. The dependence upon aldehyde concentration and $[E]$ can be eliminated by comparison of K_i and K_{ies} (Table 7). These values were all measured at saturating NADPH concentrations and could be different when the coenzyme is not saturating. Even K_i and K_{ies} are apparent dissociation constants and do not provide an absolute measure of binding affinity. Microscopic equilibrium constants are required in order to evaluate these absolute parameters. These constants could be measured by equilibrium dialysis or using pre-steady state kinetics. However, each of these techniques requires large amounts of homogeneous enzyme. ALR2 has been reported to change properties during purification [23, 24] such that results obtained with homogeneous enzyme preparations may not be indicative of behaviour *in vivo*. We conclude that the values of K_i and K_{ies}, although not absolute measures of potency, represent the most reliable parameters currently available.

At the NADPH concentration used, comparison of IC_{50} values underestimates the selectivity of ponalrestat in favour of ALR2 by a factor or 3.5 to 71-fold (Table 5). The true selectivity falls in the range 390 to 7,800-fold being higher at lower glucuronate concentration. The significance of this selectivity is not clear because the physiological roles of ALR1 and ALR2 have not been fully established (see Ref. 1). However, ALR1 is probably the most closely related enzyme to ALR2 [1, 4, 17, 20]. Ponalrestat shows high selectivity for ALR2 rather than ALR1, suggesting that the compound has even less affinity for other enzymes which are less closely related to ALR2 than is ALR1.

The kinetic characteristics of ponalrestat are compatible with maintenance of potent and selective inhibition of ALR2 during hyperglycaemia

When glucose is varied, ponalrestat is a pure non-competitive inhibitor of ALR2. This mechanism is particularly appropriate where the goal is to inhibit the enzyme in diabetic patients, because elevated levels of glucose will not decrease the potency of ponalrestat. The compound shows a high specificity for inhibition of ALR2 rather than the closely related enzyme, ALR1. Further, ponalrestat is not a competitive inhibitor of ALR1 or ALR2, confirming that it does not resemble the structure of any of the substrates: glucose, glucuronate and NADPH. This is a valuable property in a therapeutic agent since structural similarity could cause binding to any other enzyme that uses the given substrate. Such binding is one of the many mechanisms which could cause undesirable side-effects upon treatment with the drug.

REFERENCES

1. Flynn TG, Aldehyde reductases: monomeric NADPH-dependent oxidoreductases wtih multifunctional potential. *Biochem Pharmacol* **31**: 2705–2712, 1982.
2. Kador P, The role of aldose reductase in the development of diabetic complications. *Medicinal Res Rev* **8**: 325–352, 1988.
3. Sima AAF, Bril V, Nathaniel V, McEwan TAJ, Brown MB, Lattimer SA and Greene DA, Regeneration and repair of myelinated fibres in sural nerve biopsy specimens from patients with diabetic neuropathy treated with sorbinil. *New Eng J Med* **319**: 548–555, 1988.
4. Carper D, Nishimura C, Shinohara T, Dietzchold B, Wistow G, Craft C, Kador P and Kinoshita JH, Aldose reductase and rho-crystallin belong to the same protein superfamily as aldehyde reductase. *FEBS Lett* **220**: 209–213, 1987.
5. Stribling D, Mirrlees DJ, Harrison HE and Earl DCN, Properties of ICI 128436, a novel aldose reductase inhibitor and its effects on diabetic complications in the rat. *Metabolism* **34**: 336–344, 1985.
6. Daly AK and Mantle TJ, Purification and characterisation of multiple forms of aldehyde reductase in ox kidney. *Biochem J* **205**: 373–380, 1982.
7. Hayman S and Kinoshita JH, Isolation and properties of lens aldose reductase. *J Biol Chem* **240**: 877–882, 1965.
8. Leatherbarrow RJ, Enzfitter. Elsevier Science Publishers, Amsterdam, 1987.
9. Cleland WW, The statistical analysis of enzyme kinetic data. *Adv Enzymol* **29**: 1–32, 1967.
10. Dixon M, The determination of enzyme inhibitor constants. *Biochem J* **55**: 170–171, 1953.
11. Henderson PJF, A linear equation that describes the steady state kinetics of enzymes and subcellar particles interacting with tightly bound inhibitors. *Biochem J* **127**: 321–333, 1972.
12. Segel IH, *Enzyme Kinetics*. John Wiley and Sons, New York, 1975.
13. Duggleby RG, Determination of inhibition constants, IC_{50} values and the type of inhibition for enzyme-catalyzed reactions. *Biochem Med Metabol Bio* **40**: 204–212, 1988.
14. Cha S, Tight binding inhibitors. I. Kinetic behaviour. *Biochem Pharmacol* **24**: 2177–2185, 1975.
15. Williams JW and Morrison JF, The kinetics of reversible tight binding inhibition. *Methods Enzymol* **69**: 437–467, 1979.
16. Ellis KJ and Duggleby RG, What happens when data are fitted to the wrong equation? *Biochem J* **171**: 513–517, 1978.
17. Cromlish JA and Flynn TG, Identification of pig brain aldehyde reductases with the high-K_m aldehyde reductase, the low-K_m aldehyde reductase and aldose reductase, carbonyl reductase, and succinic semialdehyde reductase. *J Neurochem* **44**: 1485–1493, 1985.
18. Halder AK and Crabbe MJC, Bovine lens aldose reductase; purification, kinetics and mechanism. *Biochem J* **219**: 33–39, 1984.
19. Morrision JF and Walsh CT, The behaviour and significance of slow binding enzyme inhibitors. *Adv Enzymol* **61**: 201–301, 1988.
20. Bohren KM, Bullock B, Wermuth B and Gabbay KH, The aldo-keto reductase superfamily. cDNAs and deduced amino acid sequences of human aldehyde and aldose reductases. *J Biol Chem* **264**: 9547–9551, 1989.
21. Sato S, Kador PF and Kinoshita JH, Rat kidney aldehyde reductase: purification and comparison with rat lens aldose reductase. In: *Polyol Pathway and its Role in Diabetic Complications* (Eds. Sakamoto N, Kinoshita JH, Kador PF and Hotta N), pp. 72–81. Elsevier Science Publishers, Amsterdam, 1988.
22. Kador PF, Kinoshita JH and Sharpless NE, The aldose reductase inhibitor site. *Metabolism* **35**: 109–113, 1986.
23. Poulsom R, Comparison of aldose reductase inhibitors *in vitro*. Effects of enzyme purification and substrate type. *Biochem Pharmacol* **36**: 1577–1581, 1987.
24. Vander Jagt DL, Stangebye LA, Hunsaker LA, Eaton RP and Sibbitt WL Jr, Purification of aldose reductase from human placenta and stabilization of the inhibitor binding site. *Biochem Pharmaol* **37**: 1051–1056, 1988.