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Fibrates inhibit aldose reductase activity in the forward and reverse reactions

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Abstract

Fibrates such as bezafibrate, gemfibrozil, clofibric acid, ciprofibrate and fenofibrate, are ligands for peroxisome proliferator-activated receptor α (PPARα), and are used as therapeutic agents in the treatment of hyperlipidemia. Synthesis and accumulation of sorbitol in cells due to aldose reductase (AR) activity is implicated in secondary diabetic complications. In pursuit of finding a lead compound identification to design an effective AR inhibitor employing fragment-based design-like approach, we found that this class of compounds and their nearest neighbors could inhibit AR. Bezafibrate and gemfibrozil displayed a mixed non-competitive inhibition pattern in the glyceraldehyde reduction activity and pure non-competitive inhibition pattern in the benzyl alcohol oxidation activity of AR. Clofibric acid, ciprofibrate and fenofibrate showed pure non-competitive inhibition patterns in the forward reaction. In the reverse reaction, clofibric acid displayed a non-competitive inhibition pattern while ciprofibrate and fenofibrate displayed competitive inhibition patterns. This finding reveals for the first time a novel attribute of the fibrates in the regulation of AR activity and may be useful as lead compounds to control the function of AR in the progression and treatment of secondary diabetic complications in addition to other clinical conditions. Alternatively, these findings demonstrate that AR plays a significant role in the fibrate metabolism under various scenarios.

Keywords: Aldose reductase inhibitors; Oxidoreductase; Fibrates; Polyol pathway; Hyperglycemia; Diabetes complication

1. Introduction

Sorbitol accumulation in the cells through aldose reductase (AR) catalyzed reduction of glucose to sorbitol is the first step of the polyol pathway. Under hyperglycemic conditions this event is implicated in the pathogenesis of most of the diabetic complications [1–7]. The second enzyme in the polyol pathway is sorbitol dehydrogenase (SDH), which has a lower rate constant than AR. This causes the sorbitol that is formed not to be readily metabolized and leads to accumulation within the cell [8,9]. This increase in sorbitol accumulation causes cell edema and an increase in cell permeability. This results in a loss of potassium, free amino acids, and myo-inositol, and in the build-up of sodium and chloride concentrations within the cell [10]. This, in turn, causes the cells to swell and form vacuoles, which ultimately results in a loss of integrity [10]. This process has been implicated in many diabetic complications including retinopathy, neuropathy, nephropathy and cataract formation [11,12]. Furthermore, AR has been implicated in diabetic autonomic neuropathy (DAN) that has an increased cardiovascular mortality rate compared with patients without DAN [13].

Fibrates (Fig. 1) were developed and effectively used clinically to treat dyslipidemia [14–16]. Studies in rodents and humans reveal major mechanisms that are implicated in the modulation of lipoprotein phenotypes by fibrates through the activation of PPARα [17–27]. In the present study, comprehensive kinetic characterization of fibrates in regulating AR activity is revealed. This report delves on the possible role of fibrates in impeding the polyol pathway.

2. Materials and methods

2.1. Overexpression of human AR

DNA encoding the open reading frame of His-tagged human AR cloned into the bacterial expression vector

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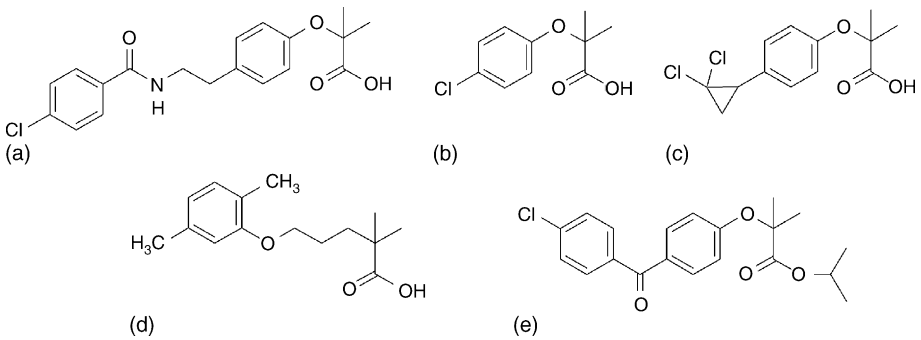

Fig. 1. Chemical structures of fibrates. (a) Bezafibrate for *p*-[4-(chlorobenzoyl-aminoethyl)-phenoxy]-β-methylpropionic acid, (b) gemfibrozil for 2,2-dimethyl-5-[2,5-dimethylphenoxy]-pentanoic acid, (c) clofibric acid for 2-(4-chlorophenoxy)-2-methyl-propionic acid, (d) ciprofibrate for 2-[*p*-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropanoic acid and (e) fenofibrate for 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid isopropyl ester.

pET15b (Novagen) was provided by Drs. Dino Moras and Alberto Podjarny. His-tagged recombinant human AR (hAR) was expressed in *E. coli* BL21 by growing the culture in Luria-Bertani broth supplemented with ampicillin (50 μg/ml) at 37 °C with shaking and induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) after the culture reached an attenuance (A_{600}) of 0.7–0.8. The cells were harvested by centrifugation, washed, resuspended in 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 1 mM 2-mercaptoethanol, and lysed by sonication at 50% power in the pulse mode for 10 min. To separate and isolate His-tag hAR, the centrifuged supernatant of the sonicated cell lysate was passed through 5 ml of Talon metal affinity matrix (Clontech), and eluted with 50 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl, 1 mM 2-mercaptoethanol and 150 mM imidazole. The eluted protein was dialyzed overnight using Spectra/Por at 4 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol, and the His-tag was removed using the thrombin cleavage kit (Novagen) as per the manufacturer's instruction. The His-tag removed hAR was further purified by passing it through the DEAE Sephadex A25 column. The eluates that contained the AR (as determined by the SDS-PAGE and enzyme activity) were pooled and concentrated using Amicon Ultra 10,000 MW cutoff membrane tubes, and the concentration of the enzyme was adjusted to 100 μM using 20 mM Tris buffer (pH 8.5). The purity of the protein at each stage of purification was assessed by Coomassie blue stained SDS-PAGE gels (Fig. 2) along with the enzyme activity. Concentration of AR was quantified by the Bradford method [28].

2.2. Enzyme kinetics of aldehyde reduction activity by AR

Aldehyde reduction activity by AR was determined at 25 °C by the rate of decrease in the absorption of NADPH at 340 nm using a Beckman DU600 model spectrophotometer [29]. The activity of the enzyme is represented as μmol of NADPH oxidized/min/l. The assay mixture contained 0.2 M potassium phosphate buffer (pH 6.2), 0.05–10 mM DL-glyceraldehyde, 0–100 μM fibrates (bezafibrate, gemfibrozil, clofibric acid, ciprofibrate and fenofibrate) (Fig. 1), 0.15 mM NADPH and human AR (0.5 μM, 18 μg/ml).

The experimental design included nine inhibitor concentrations of the fibrates, zopolrestat and sorbinil as positive controls, starting from the maximum concentration of $[I]_0$ = 100 μM. It also included seven 2- and 10-fold serial dilutions (50, 10, 5, 1, 0.5, 0.1, and 0.05 μM) as well as the negative control $[I]_0$ = 0 μM, in the presence of six concentrations of the substrate DL-glyceraldehyde (0.05, 0.1. 0.5, 1, 5, and 10 mM). Each individual rate measurement was evaluated in duplicate. At least three independent determinations were performed for each kinetic constant.

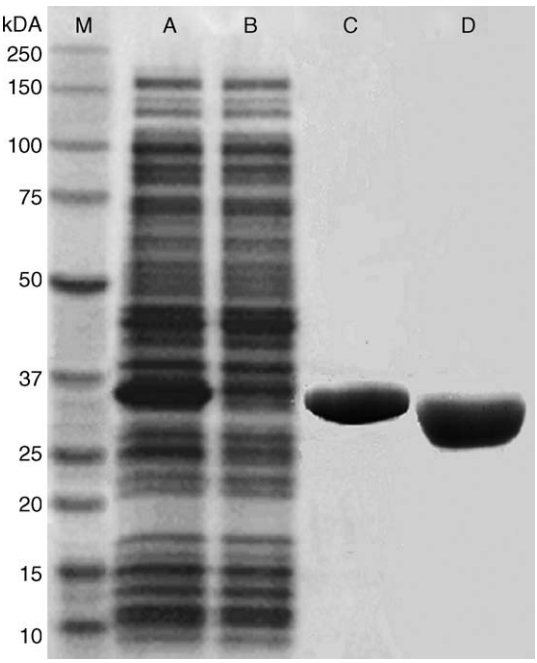

Fig. 2. Purification of recombinant human placental aldose reductase. (M) SDS-PAGE molecular weight marker, (A) bacterial lysate expressing His-tagged hAR, (B) flow through lysate after passing through BD TALON column, (C) eluted His-tagged hAR from BD TALON column and (D) DEAE Sephadex purified His-tag removed hAR.

$$v = V_{max} {}^{*}S / (K_m + S) \quad \text{(Eq.1)}$$

$$v = V_{max} {}^{*}S / [K_m (1 + I / K_{is}) + S] \quad \text{(Eq.2)}$$

$$v = V_{max} {}^{*}S / [K_m (1 + I / K_{is}) + S (1 + I / K_{ii})] \quad \text{(Eq.3)}$$

$$v = V_{max} {}^{*}S / [K_m + S (1 + I / K_{ii})] \quad \text{(Eq.4)}$$

$$log\ Y = log [c / (1 + K_b / [H^+])] \quad \text{(Eq.5)}$$

$$log\ Y = log [c / (1 + [H^+] / K_a)] \quad \text{(Eq.6)}$$

$$log\ Y = log\ [c / (1 + [H^+] / K_a + K_b / [H^+])] \quad \text{(Eq.7)}$$

Fig. 3. Equations used in the kinetic studies. Competitive, non-competitive and uncompetitive models of inhibition were investigated using Eqs. (2)–(4), respectively, where v is the rate of reaction, V_{max} the maximum initial velocity for the uninhibited reaction, K_m the Michaelis constant, S the substrate concentration, I the inhibitor concentration, K_{is} the slope inhibition constant and K_{ii} is the intercept inhibition constant. The pH profile data were fitted to Eqs. (5)–(7), where K_a and K_b are the dissociation constants for the enzyme–substrate complex. Y is the value of the observed parameter, and c is the pH independent value of Y.

2.3. Enzyme kinetics data analysis in the forward reaction

The inhibition constant K_i for the inhibitors was calculated from the untransformed kinetic data (mean of duplicates) were fitted to a one substrate Michaelis–Menten equation (1) (Fig. 3). To determine the K_i corresponding to the fibrates, competitive, non-competitive and uncompetitive models of inhibition were investigated using Eqs. (2)–(4) (Fig. 3), respectively. The K_i of the inhibitors were calculated from the secondary plot of slope values of the double-reciprocal plot versus inhibitor concentration. Goodness of fit was assessed using the r^2-test.

2.4. Effect of pH on the inhibition of AR activity

The pH dependence study assumes importance because the substrate and cofactor binding, oxidation state of the cofactor, the proton transfer and inhibitor binding are pH dependent. Most importantly an effective inhibitor is anticipated to bind to the enzyme at the physiological pH in which the enzyme is very active. Under disease condition, the cellular pH most likely is different from the optimum effectiveness of each component. Thus pH dependence studies will help understand the role of pH in the overall function of the inhibitors in their inhibition property more clearly.

The effect of pH on human AR activity in the forward direction in the presence of fibrates was determined by varying the concentration of 1–10 mM DL-glyceraldehyde, 0.1, 1, and 10 μM fibrates and 0.15 mM NADPH as the coenzyme in 0.125 M MES–Tris–HEPES buffer [30] between 5.0 and 10.0 pH levels with 0.5 pH unit intervals. For the reverse reaction, the effect of pH on human AR activity in the presence of fibrates was determined by varying the concentration of 1–10 mM benzyl alcohol, 0.1, 1, and 10 μM fibrates, and 0.1 mM 3-APADP$^+$ as the coenzyme in 0.125 M MES–Tris–HEPES buffer between 5.0 and 10.0 pH levels with 0.5 pH unit intervals. The pH profile data that showed a decrease in $\log(V_{max}/K_m)$ with a slope of 1 with increase in pH were fitted to Eq. (5) and if this parameter decreased at low pH, then the data were fitted to Eq. (6) (Fig. 3). Data for pH profiles showing a decrease in pK_i at both low and high pH-values were fitted to Eq. (7) (Fig. 3) [31]. The best fit to the data was chosen on the basis of the standard error of the fitted parameter and the lowest value of σ, which is the residual sum of squares divided by the degrees of freedom. Similar experiments were performed to evaluate the pH dependence of AR in the forward and reverse directions without inhibitors.

2.5. IC_{50} of AR inhibitors

The IC_{50}-value of the inhibitors were determined using the assay mixture containing 0.2 M potassium phosphate buffer (pH 6.2), 10 mM DL-glyceraldehyde, 0–100 μM fibrates, zopolrestat and sorbinil, 0.15 mM NADPH and 0.5 μM human AR. Fibrates that were used for these studies are bezafibrate, gemfibrozil, clofibric acid, ciprofibrate and fenofibrate. The IC_{50}-values were determined by nonlinear regression analysis of the percent inhibition plotted versus the log of the inhibitor concentration. Values were expressed as the mean ± standard error for three replicate experiments. Statistically significant differences of the observations were determined by analysis of variance using a one-way ANOVA analysis. The Tukey–Kramer multiple comparison test with a P-value less than 0.05 was considered statistically significant.

2.6. Enzyme kinetics of alcohol oxidation activity by human AR

To determine the alcohol oxidation activity of human AR, benzyl alcohol and 3-APADP$^+$ served as the substrate and the cofactor, respectively. The oxidation of benzyl alcohol was monitored at 25 °C as the rate of change in the absorption of 3-APADP$^+$ at 363 nm [30] using a Beckman DU600 model spectrophotometer in an assay mixture containing 0.125 M MES–Tris–Hepes buffer (pH 8.5), 0.1–10 mM benzyl alcohol, 0–100 μM fibrates, 0.10 mM 3-APADP$^+$ and 0.5 μM human AR. The experimental design for the reverse reaction was similar to the forward reaction which includes nine inhibitor concentrations of the fibrates, zopolrestat and sorbinil where zopolrestat and sorbinil served as positive controls, starting from the maximum concentration of $[I]_0$ = 100 μM. Each individual rate measurement was evaluated in duplicate. At least three independent determinations were performed for each kinetic constant. The K_m and the V_{max} for benzyl alcohol oxidation, the K_i and the mode of inhibition for

Table 1
Effects of inhibitors on the activity of human AR in aldehyde reduction

Compound	Kinetic parameters (μM)			Mode of inhibition
	K_i	K_{ii}	K_{is}	
Bezafibrate	$K_{ii} \neq K_{is}$	2.62 ± 0.03	1.09 ± 0.16	mNC
Gemfibrozil	$K_{ii} \neq K_{is}$	3.7 ± 0.8	1.2 ± 0.3	mNC
Clofibric acid	1.24 ± 0.05	$K_{ii} = K_{is}$	$K_{ii} = K_{is}$	pNC
Ciprofibrate	0.88 ± 0.1	$K_{ii} = K_{is}$	$K_{ii} = K_{is}$	pNC
Fenofibrate	0.56 ± 0.1	$K_{ii} = K_{is}$	$K_{ii} = K_{is}$	pNC
Sorbinil	0.45 ± 0.04	$K_{ii} = K_{is}$	$K_{ii} = K_{is}$	pNC
Zopolrestat	0.043 ± 0.01	$K_{ii} = K_{is}$	$K_{ii} = K_{is}$	pNC

$(K_{ii} = K_{is}) = K_i$; mNC: mixed non-competitive; pNC: pure non-competitive.

Table 2
Inhibition potency of ARIs in the forward reaction

Compound	pK_a	pK_b	IC_{50} (μM)
Bezafibrate	5.75 ± 0.45[a]	8.47 ± 0.52[a]	3.0 ± 0.2
Gemfibrozil	5.9 ± 0.2[a]	8.1 ± 0.3[a]	6.5 ± 02
Clofibric acid	6.15 ± 0.3	7.4 ± 0.1	1.2 ± 0.1
Ciprofibrate	6.1 ± 0.2	8.05 ± 0.2	0.86 ± 0.04
Fenofibrate	6.15 ± 0.3	8.05 ± 0.2	0.56 ± 0.06
Sorbinil	6.55 ± 0.36	9.35 ± 0.27	2.1 ± 0.05
Zopolrestat	6.15 ± 0.15	8.45 ± 0.34	0.062 ± 0.002

[a] K_{ii} was used in the calculations; comparisons of IC_{50}-values considered by ANOVA analysis reveal a statistically significant P-value of 0.05 or less.

fibrates were calculated following the methods described for the forward direction.

3. Results

3.1. Inactivation kinetics of AR

The $K_{m(NADPH,DL\text{-glyceraldehyde})}$ and $k_{cat\ DL\text{-glyceraldehyde}}$ values for uninhibited forward reaction (aldehyde reduction) of human AR were 0.092 ± 0.01 mM and $45.5 \pm 7\ min^{-1}$, respectively. In the uninhibited reverse reaction (alcohol oxidation) of human AR the $K_{m(3\text{-}APADP^+,benzyl\ alcohol)}$ was 1.72 ± 0.14 mM and the $k_{cat\ -\ benzyl\ alcohol}$ was $14.4 \pm 0.5\ min^{-1}\ mM^{-1}$. The kinetic constants for the aldehyde reduction and alcohol oxidation activity of human AR in the presence of fibrates are given in Tables 1–4. All the five fibrates showed various levels of inhibition of aldehyde reduction activity of human AR when DL-glyceraldehyde and NADPH were used as the substrate and the coenzyme, respectively. Bezafibrate and gemfibrozil displayed a mixed non-competitive inhibition pattern in the forward reaction and pure non-competitive inhibition pattern in the reverse reaction (Figs. 4a and b and 5a and b). Clofibric acid, ciprofibrate and fenofibrate showed pure non-competitive inhibition pattern in the forward reaction (Figs. 4c–e and 5c–e). However, in the reverse reaction clofibric acid displayed a non-competitive inhibition pattern while ciprofibrate and fenofibrate displayed competitive inhibition patterns. The fractional inhibition was almost identical at all the substrate concentrations and was not markedly altered by increasing substrate concentration due to the reduction in V_{max}. Of all the fibrates, fenofibrate followed by ciprofibrate and clofibric acid were comparatively more effective in inhibiting human AR. In vivo studies are underway to evaluate the potential use of fibrates as AR inhibitors.

3.2. pH dependence

The V_{max} of hAR was found to be pH-dependent. It decreased at high pH when NADPH and DL-glyceraldehyde were used as the substrates (Fig. 6), and decreased at low pH when 3-APADP$^+$ and benzyl alcohol were used as the substrates (Fig. 6). According to the $V/K_{DL\text{-glyceraldehyde}}$ and $V/K_{benzyl\ alcohol}$ plots, the recombinant human AR displayed a pK of 8.35 ± 0.12 for the forward reaction, and a pK of 7.54 ± 0.1 for the reverse reaction. In the

Table 3
Effect of inhibitors on the activity of human AR in alcohol oxidation

Compound	Kinetic parameters (μM)			Mode of inhibition
	K_i	K_{ii}	K_{is}	
Bezafibrate	1.26 ± 0.38	$K_{ii} = K_{is}$[a]	$K_{ii} = K_{is}$[a]	NC
Gemfibrozil	8.79 ± 0.88	$K_{ii} = K_{is}$[a]	$K_{ii} = K_{is}$[a]	NC
Clofibric acid	0.34 ± 0.05	$K_{ii} = K_{is}$[a]	$K_{ii} = K_{is}$[a]	NC
Ciprofibrate	$K_{ii} \neq K_{is}$	$K_{ii} \neq K_{is}$	0.30 ± 0.0222	C
Fenofibrate	$K_i \neq K_{is}$	$K_{ii} \neq K_{is}$	0.246 ± 0.37	C
Sorbinil	$K_{ii} \neq K_{is}$	$K_{ii} \neq K_{is}$	0.16 ± 0.04	C
Zopolrestat	$K_{ii} \neq K_{is}$	$K_{ii} \neq K_{is}$	0.049 ± 0.03	C

NC: non-competitive; C: competitive.
[a] $(K_{ii} = K_{is}) = K_i$.

Table 4
Inhibition potency of ARIs in the reverse reaction

Compound	pK_a	pK_b	IC_{50} (μM)
Bezafibrate	7.16 ± 0.04[a]	9.1 ± 0.08[a]	3.8 ± 0.5
Gemfibrozil	7.35 ± 0.1[a]	9.04 ± 0.1[a]	12.9 ± 0.4
Clofibric acid	7.46 ± 0.15[a]	8.95 ± 0.15[a]	0.58 ± 0.06
Ciprofibrate	7.35 ± 0.2[b]	8.7 ± 0.15[b]	0.49 ± 0.03
Fenofibrate	7.42 ± 0.1[b]	8.95 ± 0.04[b]	0.35 ± 0.026
Sorbinil	7.5 ± 0.1[b]	8.85 ± 0.1[b]	0.28 ± 0.025
Zopolrestat	7.45 ± 0.1[b]	8.75 ± 0.04[b]	0.066 ± 0.004

Comparisons of IC_{50}-values considered by ANOVA analysis reveal a statistically significant P value of 0.05 or less.
[a] pK_i was used for calculations.
[b] pK_{is} was used for calculations.

forward direction, the V/K_{NADPH} plot revealed a bell-shaped pH dependence (Fig. 6). The pK was 5.4 ± 0.1 and 7.4 ± 0.5 at low pH and high pH, respectively. In the reverse direction, the $V/K_{3\text{-APADP}^+}$ that is dependent on the rate constant of binding 3-APADP$^+$ to human AR, decreased at low pH with a pK of 6.7 ± 0.4. When the pH is equal to 7.5 or higher, the binding of 3-APADP$^+$ becomes pH-independent and hence there is no appreciable change in $V/K_{3\text{-APADP}^+}$ at pH-values equal to 7.5 or higher.

3.3. pH profile of fibrates

The variation of pK_i-values as a function of pH were fitted to Eq. (5) (Fig. 3) when DL-glyceraldehyde was used as the varied substrate (where K_a (acid) and K_b (base) are dissociation constants for the groups in the enzyme, H^+ is the hydrogen ion concentration, Y is the value of the observed parameter, and c is the pH-independent value of Y) showed that the value of the measured parameter decreased at both high and low pH-values (Fig. 7). The pK_a- and pK_b-values estimated upon fitting Eq. (5) [32] to the data for sorbinil, zopolrestat and fibrates are given in Table 2. Bezafibrate exhibited a mixed non-competitive mode of inhibition in the physiological pH range but the activity of bezafibrate diminished rapidly below pH 6.5 and above pH 7.5. In addition, bezafibrate was found to be very effective in the pH range between 6.5 and 7.5, with the maximum activity being at pH 7.5. Furthermore, inhibition of AR by zopolrestat diminished rapidly below pH 6.0 and above pH 8.5, respectively, with the optimum pH for AR inhibition activity between pH 7.0 and 8.0. Sorbinil inhibited AR activity very effectively in the pH range between 7.5 and 9.0, however, the inhibition diminished rapidly below pH 6.5 and above pH 8.5. Remarkably fenofibrate like sorbinil shows a similar pattern of pK_i profile over the pH range between 5.0 and 10.0.

4. Discussion

The critical role AR plays in diabetic complications is the rationale for the development of AR inhibitors (ARIs) that might prevent or delay these complications [33,34]. AR participates in osmoregulation and glucose metabolism is the first enzyme of the polyol pathway, that converts glucose to its sugar alcohol sorbitol. SDH, the second and final enzyme of the pathway, catalyzes sorbitol to fructose. Under hyperglycemic conditions, the second step of the polyol pathway produces an excess of NADH, resulting in a chronically elevated cytosolic NADH/NAD$^+$ ratio. This imbalance cannot be corrected by mitochondrial respiration, and leads to physiological consequences like ischemia [35]. Thus polyol pathway is implicated in increased oxidative stress, which is thought to play an important role in the pathogenesis of various diabetic complications [36,37]. However, AR also plays a protective role against toxic aldehydes derived from lipid peroxidation and steroidogenesis that could affect cell growth and differentiation when accumulated [35,38–40]. While searching for potent AR modulators through fragment-based design-like approach, we found that fibrates and fibrate like compounds with various substitutions at the position adjacent to carboxylic acid moiety and modification could inhibit AR.

Fibrates display a mixed and pure non-competitive inhibition pattern with hAR in the forward direction (aldehyde reduction) (Table 1) and display a pure non-competitive and competitive inhibition pattern in the reverse direction (alcohol oxidation) (Table 3). According to the $V/K_{DL\text{-glyceraldehyde}}$ and $V/K_{benzyl\ alcohol}$ plots, hAR display a pK of 8.35 ± 0.12 and 7.54 ± 0.1 for the forward and reverse reactions, respectively. The pK_a- and pK_b-values of fibrates show that these compounds are effective in the pH range that is favorable for both the aldehyde reduction (Fig. 7) as well as alcohol oxidation (Fig. 7) by hAR.

The decrease in the pK_i-values of zopolrestat, sorbinil, and fibrates at low and high pH may be due to several factors, such as that the inhibitors may bind to different functional groups, where changes in the protonation of these functional groups may alter the binding affinities of these inhibitors. On the other hand, differences in the protonation and/or deprotonation of the polar atoms in the inhibitors themselves may prevent them from binding to AR strongly because Inskeep et al. [41] found the pK_a to be 5.46 for zopolrestat. Therefore the protonation/deprotonation of the inhibitors may be partly responsible for the overall pH dependent phenomenon observed in these studies.

In the reverse reaction the fibrates were generally effective in the alkaline pH range between 8.0 and 9.0 (Fig. 7). All the fibrates showed similar trends in the pK_a- and pK_b-values. Part of the differences in the pH dependent properties seen between the aldehyde reduction and alcohol oxidation can be attributed to the use of benzyl alcohol and 3-APADP$^+$ compared to the glyceraldehyde and NADPH that were used in the reactions. This is because the substrates and the cofactors for the oxidation as opposed to reduction reactions may have different properties at different pH-values.

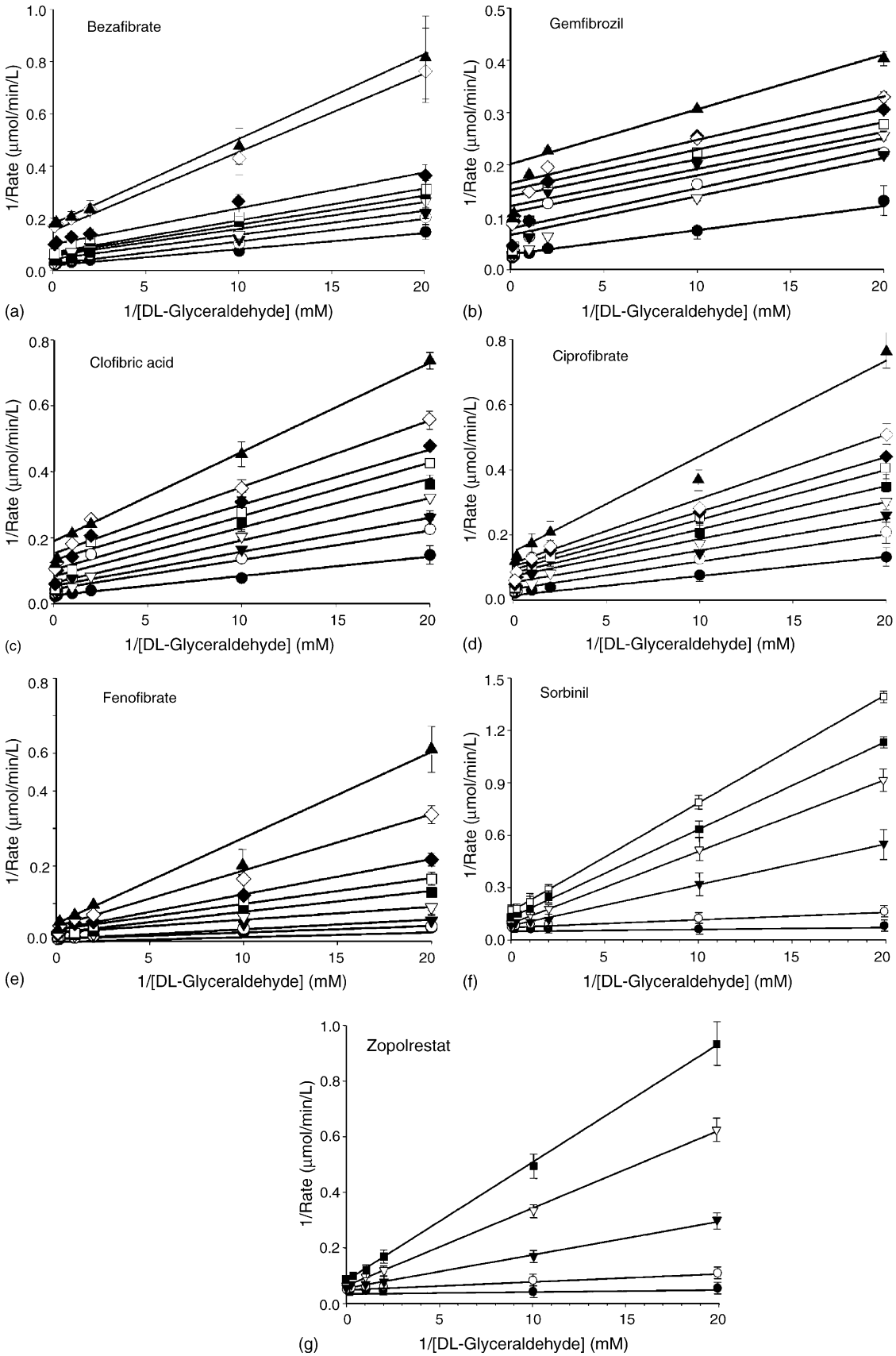

Fig. 4. (a–g) Double reciprocal plot of the rate of reduction of aldehyde by AR. Lineweaver–Burk plots of rate of reduction of DL-glyceraldehyde in the presence of various concentrations ((●) 0 μM; (○) 0.05 μM; (▼) 0.1 μM; (▽) 0.5 μM; (■) 1.0 μM; (□) 5.0 μM; (◆) 10.0 μM; (◇) 50.0 μM; (▲) 100.0 μM) of bezafibrate (a), gemfibrozil (b), clofibric acid (c), ciprofibrate (d), fenofibrate (e), sorbinil (f) and zopolrestat (g).

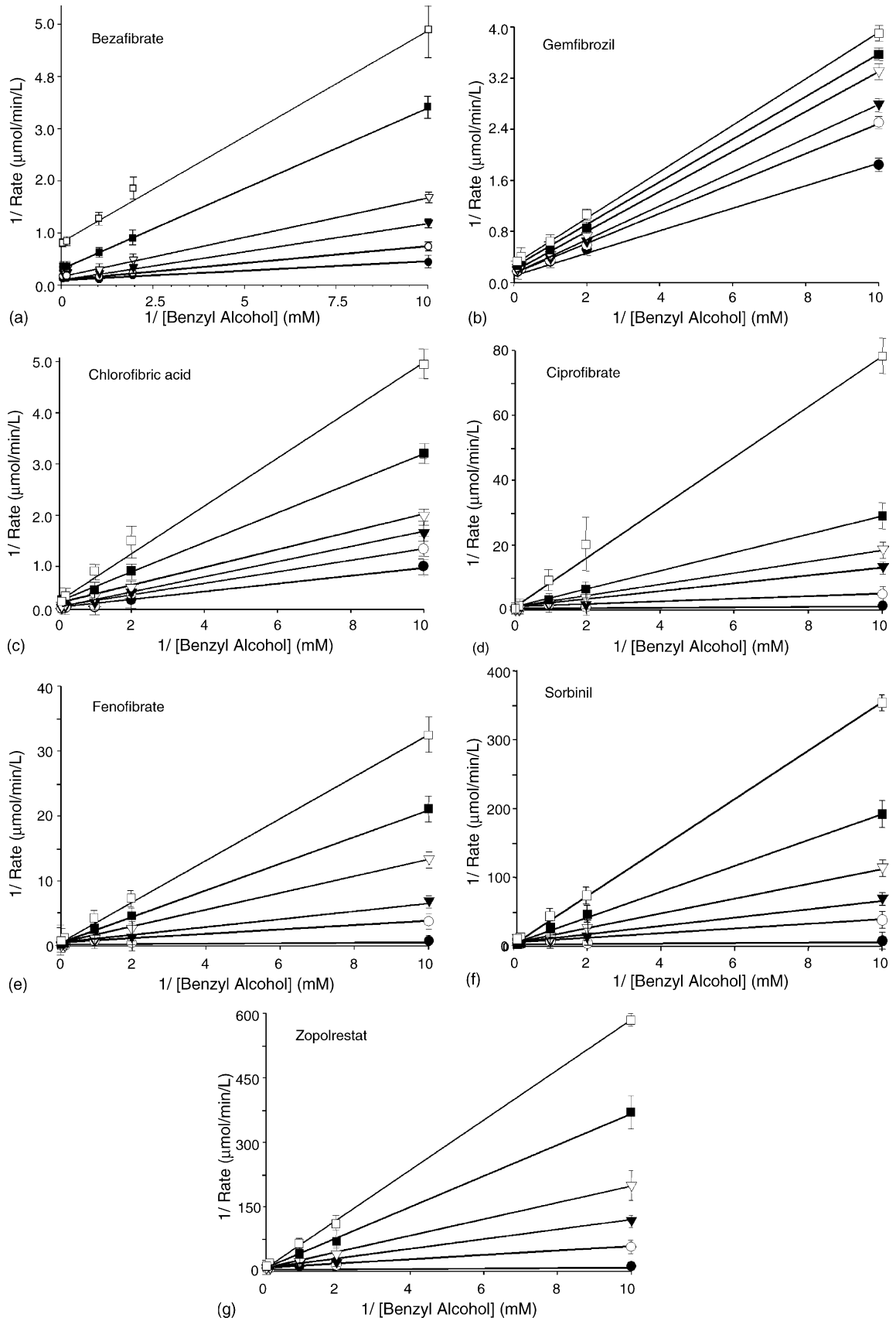

Fig. 5. (a–g) Lineweaver–Burk plot for the benzyl alcohol oxidation reaction of AR in the presence of fibrates. Double reciprocal plot of the rate of oxidation of benzyl alcohol in the presence of various concentrations ((●) no inhibitor; (○) 0.1 μM; (▼) 0.5 μM; (▽) 1.0 μM; (■) 5.0 μM; (□) 10.0 μM) of fibrates. At 50 and 100 μM concentrations, the enzyme activity of alcohol oxidation was found completely inhibited by the fibrates and hence, data not included.

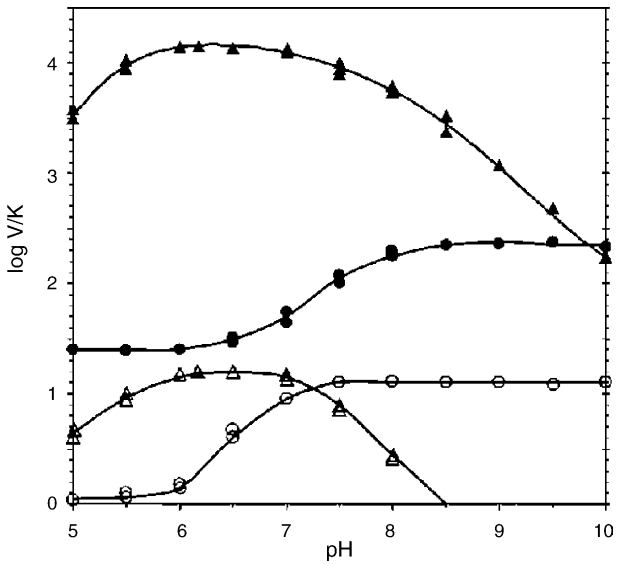

Fig. 6. pH profile of aldehyde reduction and alcohol oxidation by hAR. $V/K_{\text{DL-glyceraldehyde}}$ (▲) and $V/K_{\text{benzyl alcohol}}$ (●) were determined between pH 5.0 and 10.0 using DL-glyceraldehyde and benzyl alcohol as the variable substrates respectively, while concentrations of NADPH (150 μM) and 3-APADP$^+$ (100 μM) were kept constant. V/K_{NADPH} (△) and $V/K_{\text{3-APADP}}$ (○) were determined between pH 5.0 and 10.0 keeping the concentration of DL-glyceraldehyde (10 mM) and benzyl alcohol (10 mM) constant but varying NADPH and 3-APADP$^+$ concentration.

The non-competitive inhibition pattern exhibited by ciprofibrate and fenofibrate in the reduction of glyceraldehyde as well as the competitive inhibition pattern exhibited with benzyl alcohol as varied substrate are perhaps due to the isomerization of AR after glyceraldehyde binding and before benzyl alcohol binding. This isomerization signifies the binding of these two fibrates to AR-glyceraldehyde-NADPH ternary complex and to AR-3-APADP$^+$ binary complex. The inhibitors may not bind to the free AR as product release steps of AR are ordered with NADPH getting oxidized in the beginning before the substrate is reduced and NADP$^+$ being reduced at the end after the substrate is oxidized.

Among the fibrates, fenofibrate has the lowest IC_{50}-value of 0.56 ± 0.06 μM in inhibiting aldehyde reduction activity of AR (Tables 3 and 4). Also, fenofibrate has the best IC_{50}-value of 0.35 ± 0.026 μM in inhibiting the alcohol oxidation activity of AR. Gemfibrozil has the highest IC_{50}-value of 6.5 ± .02 μM for the forward reaction and 12.9 ± 0.4 μM for the reverse reaction among all the fibrates. IC_{50}-values of bezafibrate, clofibric acid and ciprofibrate are about 2-, 5- and 7-fold better than gemfibrozil in the forward reaction whereas they are 3-, 22- and 26-fold superior than gemfibrozil in the reverse reaction, respectively.

4.1. Significance of polyol pathway to hyperlipidemia

Under normal conditions, cellular glucose is phosphorylated into glucose 6-phosphate by hexokinase, causing only about 3% of nonphosphorylated glucose to enter the polyol pathway [42,43], convert to sorbitol and further to fructose. However, under hyperglycemia, the polyol pathway accounts for as much as one-third of the total glucose turnover [7,44,45] because of the saturation of hexokinase with ambient glucose, the increased flux of glucose, leading to overflow of the polyol pathway products, sorbitol and fructose. This causes oxidative stress due to the depletion of NADPH and NAD$^+$ and raising the NADH/NAD$^+$ ratio, modifying the redox state of the cells, and leading to the production of superoxide anions [46]. The formation and accumulation of sorbitol in the cells through the action of AR that catalyzes the reduction of glucose to sorbitol, the first step of the polyol pathway [6,7] causes major diabetic complications like cardiovascular diseases, retinopathy, neuropathy and nephropathy. Fructose formed from the oxidation of sorbitol by SDH via the second step of the polyol pathway is regarded as more lipogenic than glucose as it stimulates a greater conversion of acetate to fatty acids [47] and increased rates of de novo lipogenesis [48,49]. Fructose provides carbon atoms for both the glycerol and the acyl portions of acylglycerol formation [50] and is implicated as a major contributor of hyperlipidemia [51–57]. Thus, the inhibition of the polyol pathway and prevention of fructose formation could possibly be an additional mechanism apart from the five known mechanisms fibrates are known to employ in order to control triglycerides level because fructose is the precursor to triglycerides synthesis.

4.2. Fibrates as ARIs

None of the many potent human ARIs tested displayed competitive inhibition in aldehyde reduction. Rather, these inhibitors follow non-competitive inhibition. This is likely a reflection of the sequential reaction mechanism of AR with NADPH binding first [58], followed by the conformational changes associated with the binding and release of coenzyme, inhibitor and substrate partly. In addition, it may be due to the tight binding of the inhibitors, with affinities greater than the K_m-value of glucose [59].

Simmons et al. suggested that the control of very low-density lipoprotein (VLDL) metabolism by fibrates could offer a method for reducing the progression of diabetic neuropathy [60]. However, Corcia et al., believe that fenofibrate and generally fibrates can be responsible for neuropathies even when given in approved doses and in the absence of renal failure [61]. Furthermore, various studies have indicated potential development of neuropathy with lipodaemic agents [62,63]. To make any conclusion on the suitability of using fibrates clinically further investigations are required to evaluate the causal role of lipid lowering drugs on the onset of peripheral neuropathy. Clinical trials have demonstrated that reduction of low-density lipoprotein cholesterol (LDL-C) using fibrates reduces myocardial ischemia in patients with coronary heart disease [64–66]. Ramasamy et al. demonstrated that ischemia increases myocardial AR activity and that pharmacological inhibi-

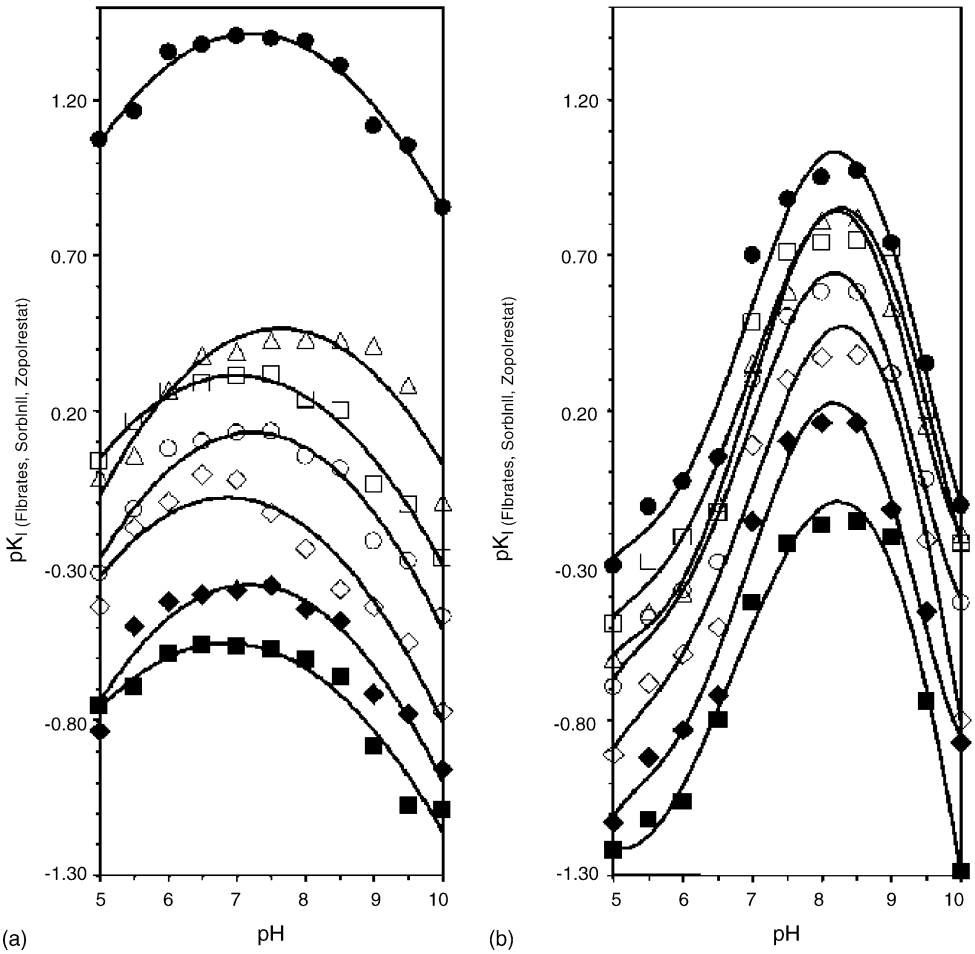

Fig. 7. (a) Effect of pH on the aldehyde reduction activity of AR in the presence of fibrates. The pK_i-values were plotted from the K_{ii}-values of bezafibrate (◆) and gemfibrozil (■), K_i-values for clofibric acid (◇), ciprofibrate (○), fenfibrate (□), sorbinil (▲) and zopolrestat (●). The K_i (or K_{ii})-values were determined at 0.15 mM NADPH, by varying the DL-glyceraldehyde concentration (0.1–10 mM) in 0.125 M MES–Tris–HEPES at the indicated pH-values. (b) Effect of pH on the alcohol oxidation activity of AR in the presence of fibrates. The pK_i-values were plotted from the K_i-values of bezafibrate (◆), gemfibrozil (■), clofibric acid (◇), and the K_{is}-values of ciprofibrate (○), fenofibrate (□), sorbinil (△) and zopolrestat (●). The K_i-values were determined with 0.1 mM 3-APADP$^+$, by varying the benzyl alcohol concentration (0.1–10 mM) in 0.125 M MES–Tris–HEPES at the indicated pH-values. The pK_a- and pK_b-values were estimated by fitting Eq. (5) to the data for the inhibitors in Tables 3 and 4.

tion of AR is cardioprotective by increasing myocardial glycolysis and glucose oxidation, as well as by conserving ATP during ischemia [67]. Conversely, Shinmura et al. suggested that AR plays an obligatory role in mediating the protective effects of the late phase of ischemic preconditioning [68]. Thus it is possible that fibrates decrease mycocardial ischemia by reducing LDL-C by activating PPAR, as well as by inhibiting AR. This could be further evidence that AR inhibition protects ischemic myocardium.

Inhibition of AR or any AKR family members by fibrates was revealed for the first time in this study. This is an important attribute and an interesting phenomenon exhibited by all the five fibrate molecules with immense significance as they are already in use as effective hyperlipidemic therapeutic agents and are generally well absorbed from the gastrointestinal tract. This observation also indicates that AR plays a role in fibrate mechanism of action. This phenomenon can be utilized in the design of next generation molecules based on fibrate-like compounds for a variety of health conditions requiring modulation of AR, AKR and AR-like targets.

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