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Inhibition of aldose reductase by dietary antioxidant curcumin: Mechanism of inhibition, specificity and significance

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ABSTRACT

Accumulation of intracellular sorbitol due to increased aldose reductase (ALR2) activity has been implicated in the development of various secondary complications of diabetes. In this study we show that curcumin inhibits ALR2 with an IC_{50} of 10 μM in a non-competitive manner, but is a poor inhibitor of closely-related members of the aldo-keto reductase superfamily, particularly aldehyde reductase. Results from molecular docking studies are consistent with the pattern of inhibition of ALR2 by curcumin and its specificity. Moreover, curcumin is able to suppress sorbitol accumulation in human erythrocytes under high glucose conditions, demonstrating an in vivo potential of curcumin to prevent sorbitol accumulation. These results suggest that curcumin holds promise as an agent to prevent or treat diabetic complications.

1. Introduction

Prolonged exposure to chronic hyperglycemia in diabetes can lead to various complications, affecting the cardiovascular, renal, neurological and visual systems [1]. Although mechanisms leading to diabetic complications are not completely understood, many biochemical pathways associated with hyperglycemia have been implicated [1]. Among these, the polyol pathway has been extensively studied [2]. Aldose reductase (ALR2[1] or AKR1B1; EC: 1.1.1.21) belongs to aldo-keto reductase (AKR) super family. It is the first and rate-limiting enzyme in the polyol pathway and reduces glucose to sorbitol utilizing NADPH as a cofactor. Sorbitol is then metabolized to fructose by sorbitol dehydrogenase [2]. Accumulation of sorbitol leads to osmotic swelling, changes in membrane permeability, and also oxidative stress culminating in tissue injury [3].

A number of studies with experimental animals suggest that inhibition of ALR2 could be effective in the prevention of some diabetic complications including cataract, retinopathy, nephropathy and neuropathy [3–5]. A number of ALR2 inhibitors (ARI), both synthetic and natural, have been found to delay or substantially prevent some diabetic complications in animal models and some have been evaluated in clinical trials [5,6]. To date, most ARIs have met with limited success, and some of the synthetic ARIs were associated with deleterious side effects and poor penetration of target tissues such as nerve and retina [5–8]. Largely, two chemical classes of ARI have been tested in phase III trials. While carboxylic acid inhibitors (zopolrestat, ponalrestat and tolerestat) have shown poor tissue permeability and are not very potent in vivo, spiroimide (spirohydantoin) inhibitors (sorbinil) penetrate tissues more efficiently but many have been associated with skin reactions and liver toxicity [3–6].

Aldehyde reductase (ALR1; EC: 1.1.1.2) is one of AKR family members that is closely related to ALR2 and known to play a role in the detoxification of reactive aldehydes [9,10]. Since many ARIs inhibit both ALR2 and ALR1 [9,10], it has been suggested that poor selectivity might have contributed to the poor outcome of ARI clinical trials [5]. Recently, other AKR members have been identified that are similar to ALR2. One such AKR is human small intestine reductase (HSIR or AKR1B10), which has 70–80% sequence similarity with ALR2 [11,12]. Similar to other members of the AKR family, AKR1B10 can reduce a variety of aldehydes and ketones [11,12]. Although, studies suggest that AKR1B10

Abbreviations: AKR, aldo-keto reductase; ALR1, aldehyde reductase; ALR2, AKR1B1, human aldose reductase; HSIR, AKR1B10, human small intestine reductase; ARI, aldose reductase inhibitor; RBC, red blood cells

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may have a potential role as a tumor marker, its physiological function remains still unclear.

We have previously reported ARI activity contained in a few spice/dietary sources prevented diabetic complications using in vitro, ex vivo and animal models [13–17]. Curcumin was effective in delaying streptozotocin (STZ)-induced diabetic cataract in rats mainly through its antioxidant property and inhibition of rat lens ALR2 [14]. Subsequently, Du et al. have also reported that curcumin and its synthetic analogues inhibit bovine lens ALR2 [18]. However, mechanism of inhibition, specificity with other AKRs and its functional significance has not been reported. In the present study we have characterized the inhibition of human recombinant ALR2 by curcumin and have provided insights into the nature of inhibition. Further, we have investigated the specificity of curcumin towards two closely-related AKRs and its effects on intracellular sorbitol accumulation in red blood cells (RBC) under ex vivo high glucose conditions.

2. Materials and methods

2.1. Materials

D-Glucose, DL-glyceraldehyde, lithium sulfate, 2-mercaptoethanol, NADPH, NADP, dimethyl sulfoxide (DMSO), sorbitol, curcumin, glycine, methyl orange, perchloric acid, ammonium sulfate, DEAE-cellulose, Tris–HCl, EDTA, sucrose and sorbitol dehydrogenase were purchased from Sigma Chemicals Company (St. Louis, MO). All other chemicals were obtained from local companies.

2.2. Expression and purification of recombinant human ALR2

Recombinant human ALR2 was over-expressed in *Escherichia coli* and purified from bacterial cultures essentially as described previously [19] with a minor modification. Chromatography over AffiGel Blue (Bio-Rad) affinity matrix was used as a final purification step.

2.3. Expression and purification of human AKR1B10

AKR1B10 was produced by over-expression from the pET23 plasmid vector containing cDNA encoding the enzyme. Purification from *E. coli* strain BL21 expression cultures was carried out as described [10]. Purification steps included column chromatography over MacroQ ion exchange and AffiGel Blue dye affinity media essentially as described previously [19].

2.4. Purification of ALR1 from bovine kidney

ALR1 was partially purified from bovine kidney following the previously described methods [17,20]. Briefly, freshly obtained bovine kidney was homogenized in 3 vol. of 10 mM sodium phosphate buffer, pH 7.2 containing 0.25 M sucrose, 2.0 mM EDTA, 2.5 mM 2-mercaptoethanol. The homogenate was centrifuged at 16 000×*g* for 20 min and the supernatant was subjected to ammonium sulfate precipitation. Precipitate obtained between 45% and 75% saturation was dissolved in the above buffer and dialyzed extensively against the same buffer. DEAE-52 resin was added to the dialyzed material and then removed by centrifugation. The supernatant was used as the source of ALR1.

2.5. ALR2 assay

The activity of ALR2 was measured as described previously [13]. The change in the absorbance at 340 nm due to NADPH oxidation was followed in a Lamda35 spectrophotometer (Perkin–Elmer, Shelton, USA).

2.6. AKR1B10 assay

The activity of AKR1B10 was measured by following the rate of oxidation of NADPH at 340 nm [10]. The assay mixture in 1 ml contained 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 20 mM glyceraldehyde, 0.15 mM NADPH and enzyme. Reaction mixture without enzyme served as blank.

2.7. ALR1 assay

The activity of ALR1was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm as a function of time at 37 °C using glyceraldehyde as substrate [17]. The assay mixture in 1 ml contained 50 mM sodium phosphate buffer of pH 7.2, 0.2 M ammonium sulfate, 10 mM DL-glyceraldehyde, 5 mM β-mercaptoethanol and 0.1 mM NADPH.

2.8. Inhibition studies

For inhibition studies concentrated stocks of curcumin prepared in DMSO were used and the final concentration of DMSO was not more than 1%. Various concentrations of curcumin were added to assay mixtures of ALR2, ALR1 or AKR1B10 and incubated for 5 min before initiating the reaction by NADPH as described above. The percentage inhibition was calculated considering the activity in the absence of curcumin as 100%. The IC_{50} values were determined by non-linear regression analysis of the plot of percent inhibition versus log inhibitor concentration.

2.9. Enzyme kinetics

K_m and V_{max} of recombinant ALR2 were determined with varying concentrations of glyceraldehyde as substrate in the absence and presence of different concentrations of curcumin by Lineweaver–Burk double reciprocal plots. Inhibitory constant (K_i) was derived by plotting slopes obtained from Lineweaver–Burk plots versus curcumin concentration.

2.10. In vitro incubation of RBC

Five milliliters of blood was collected into heparinized tubes from healthy male volunteers after an overnight fast. Red blood cells were separated by centrifugation and washed three times with isotonic saline at 4 °C. Washed RBCs were suspended in Kreb's-ringer bicarbonate buffer, pH 7.4 (pre-equilibrated with 5% CO_2). Duplicate samples were incubated at 37 °C in the presence of 5% CO_2 for 3 h under normal (5.5 mM) and high glucose (55 mM) conditions [17]. The effect of curcumin (25–100 μM) on sorbitol accumulation was evaluated by incubating the RBC with different concentrations of curcumin.

2.11. Estimation of sorbitol in RBC

At the end of the incubation period, RBCs were homogenized in 9 vol of 0.8 M perchloric acid. The homogenate was centrifuged at 5000×*g* at 4 °C for 10 min and the pH of the supernatant was adjusted to 3.5 with 0.5 M potassium carbonate. The sorbitol content of the supernatant was measured by a fluorometric method as described previously [21] using a spectrofluorometer (Jasco-FP-6500).

2.12. Molecular docking

Molecular docking studies were done by SYBYL FlexX software (Tripos). Ligand structures were constructed and minimized using the SYBYL modeling program. The FlexX module in SYBYL 7.0 was

used to dock the compounds into the active site of the crystallographic structures, which was defined as all residues within 6.5 Å away from the inhibitor in original complex by using an incremental construction algorithm. For docking studies coordinates of crystal structure of proteins (ALR1: PDB # 2Ao0 and ALR2: PDB # 1PWM,) were taken from Brookhaven Protein Data Bank (PDB). The predicted protein ligand complexes were optimized and ranked according to the empirical scoring function ScreenScore, which estimates the binding free energy of the ligand receptor complex [22,23].

3. Results and discussion

The beneficial effect of ARI in preventing or delaying the onset of diabetic complications in experimental models provides a strong support to the hypothesis that ALR2 inhibition could be an effective strategy in the prevention or delay of certain diabetic complications. However, studies with ARI have yielded inconsistent results in experimental animals and also in clinical trials to assess efficacy against various diabetic complications [5,7,24,25]. In addition to its antioxidant property, we observed a lowered activity of ALR2 in curcumin fed diabetic rat lens compared to untreated diabetic rat lens [14], indicating that possibility of inhibition of ALR2 by curcumin. In the current study we demonstrate by in vitro assays that curcumin inhibits ALR2 directly. Further, we probed the mechanism of inhibition and specificity toward ALR2 as compared with other members of the AKR family. To test for physiological significance, we measured the ability of curcumin to block ALR2 activity in freshly harvested human erythrocytes.

Curcumin inhibited human recombinant ALR2 with an IC_{50} of 10.0 ± 4.0 μM (Fig. 1A). The primary structure of ALR2 displays high similarities with aldehyde reductase (ALR1) and human small intestine reductase (HSIR, AKR1B10), closely-related members of the aldo-keto reductase superfamily. ALR1 and ALR2 both catalyze the reduction of biogenic aldehydes, and all the three AKRs catalyze the NADPH-dependent reduction of a variety of carbonyls such as glyceraldehye, glucuronate, and short chain alkanals [10–12]. Because many active site residues including Tyr-49, His-111, Cys-299, Trp-21, Phe-123 that are important in inhibitor interactions are the same in ALR2 and AKR1B10 [26], it is not surprising that many compounds known to inhibit ALR2 such as tolrestat, sorbinil and fenofibrates also inhibit AKR1B10 [27]. Therefore, we studied the specificity of curcumin with these two related AKR members. Though curcumin inhibited AKR1B10 (Fig. 1B), the IC_{50} value was three times higher than ALR2 (30.0 ± 3.0 μM), indicating its relative selectivity for ALR2. It was interesting to note that curcumin did not inhibit bovine kidney ALR1 up to 200 μM concentration under the conditions employed in the study (data not shown), signifying its marked specificity towards ALR2 over ALR1. Next we determined some kinetic parameters such as K_m and V_{max} to understand the mechanism of inhibition of ALR2 by curcumin. In the presence of different concentrations of curcumin, V_{max} was

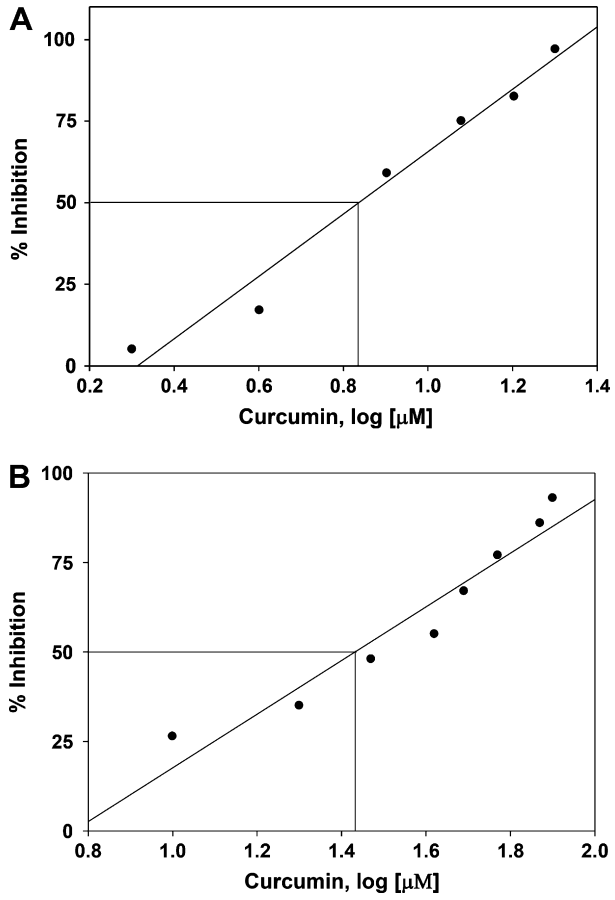

Fig. 1. Inhibition of ALR2 and AKR1B10 by curcumin. (Panel A) Representative inhibition curve of human recombinant ALR2 by curcumin. (Panel B) Representative inhibition curve of human AKR1B10 by curcumin. ALR2 and AKR1B10 activity in the absence of curcumin was considered as 100%. Data are average of three independent experiments.

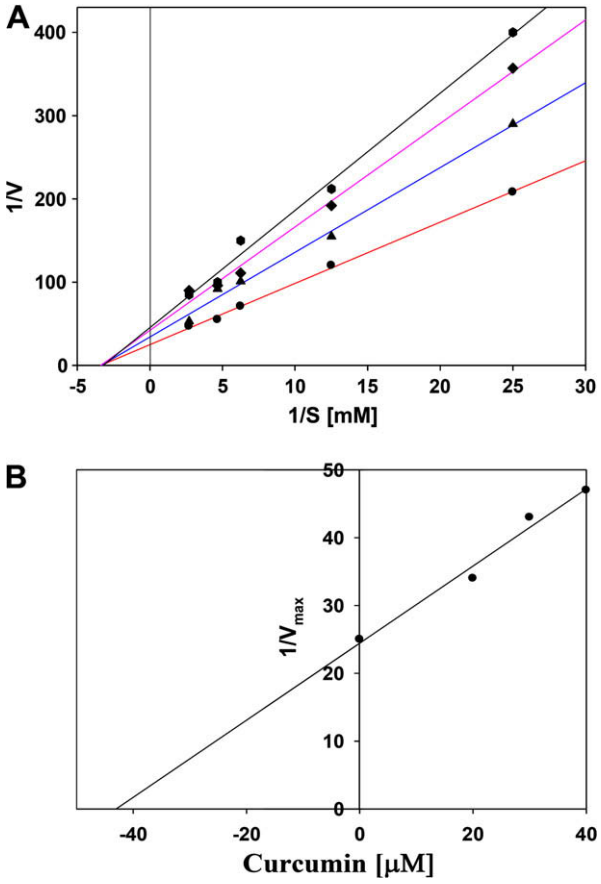

Fig. 2. Kinetics of human recombinant aldose reductase inhibition. (Panel A) Lineweaver–Burk plot of recombinant ALR2 in the absence and presence of various concentration of curcumin. Final concentrations of curcumin used in the assay system: '0' (*circles*), 10 (*triangles*), 30 (*diamond*) and 45 μM (*hexagonal*). (Panel B) Determination of inhibitory constant (K_i). Slopes of the Lineweaver–Burk plot were plotted as a function of curcumin concentration and *X*-axis intercept of this plot gives K_i. Data in Panel A and B are average of three independent experiments.

Table 1
Kinetics of human recombinant ALR2 in the absence and presence of curcumin. Data are the means ± S.E. ($n = 6$). V_{max} is reported as μmoles NADPH oxidized/min/mg protein.

Curcumin (μM)	K_m (mM)	V_{max}
'0'	0.232 ± 0.08	0.074 ± 0.019
10	0.243 ± 0.07	0.045 ± 0.011
30	0.240 ± 0.05	0.034 ± 0.014
45	0.244 ± 0.11	0.022 ± 0.013

decreased but there was no change in K_m with glyceraldehyde as substrate (Fig. 2A and Table 1). These results suggested a non-competitive inhibition of ALR2 by curcumin. Further, we have determined inhibitory constant (K_i) from the secondary plots of Lineweaver–Burk plots and K_i of curcumin for ALR2 was found to be 40×10^{-6} M (Fig. 2B). As reported by Bohren et al. [28], although many ionic inhibitors bind to active site, still show non-competitive to uncompetitive pattern inhibition because under steady-state conditions most of the enzyme will be present as enzyme–nucleotide binary complex. Hence, compounds that selectively bind to the enzyme–nucleotide complex are more effective than those bind to free enzyme.

Thus, molecular docking studies were conducted to substantiate the binding pattern and selective inhibition of ALR2 by curcumin. It was observed that curcumin likely interacts with ALR2 at active site residues Tyr-48, Lys-21, Thr-19 and Gln-183 (hydrogen bond distance 2.90, 2.03, 2.96 and 2.43 Å). Further, there was hydrogen bonding with Leu-300 and Trp-111 (distance 2.70 and 2.22 Å, respectively). Hence, it appears that curcumin might bind to ALR2 in a closed type of conformation (Fig. 3A). In case of ALR1, hydrogen bonding was observed between curcumin and

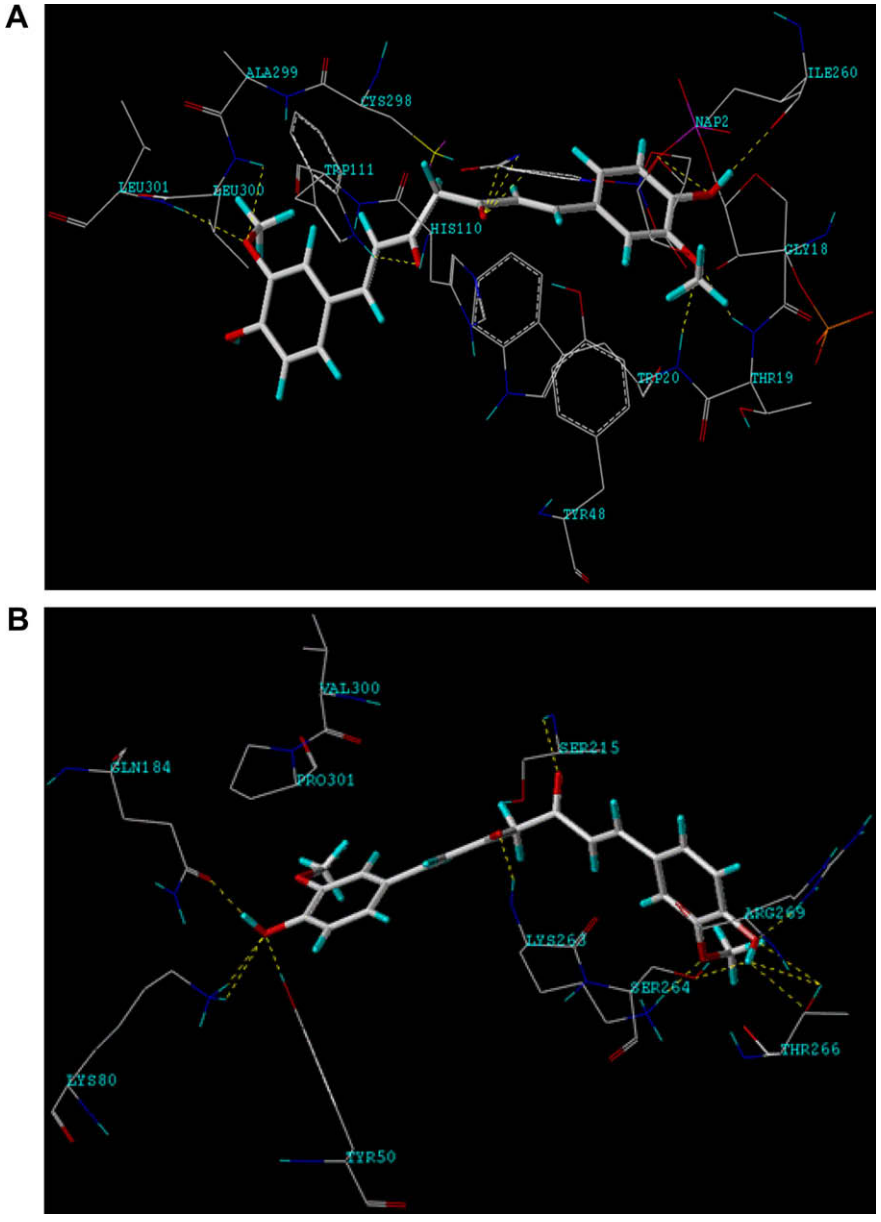

Fig. 3. Stereoviews of ALR2 and ALR1 docked with curcumin. (Panel A) Curcumin (keto form) docked into active site of ALR2 and depicts its interaction with residues Thr-19, Trp-20, His-110, Trp-111, Leu-300, Leu-301, Ile-260 and nicotinamide ring. (Panel B) Curcumin (keto form) docked into active site of ALR1. In this case curcumin interacted mainly with residues Tyr-50, Lys-80, Gln-184, Ser-215, Lys-263, Ser-264, Thr-266 and Arg-269 but no interaction with Val-300 and Pro-301. Hydrogen bonds shown in dashed yellow lines.

amino acid residues Tyr-50, Gln-184 and Lys-80 (bond distance 2.01, 2.02 and 2.50 Å) (Fig. 3B). Since Leu-300 and Leu-301 are replaced by Pro-300 and Val-301 in ALR1, curcumin did not interact with Pro-300 and Val-301. It is interesting to note that unlike with ALR1, curcumin interacted with Leu-300 and Leu-301 in ALR2 that are involved in imparting plasticity to ALR2. Because of these specific interactions curcumin could inhibit ALR2 but not ALR1. Similar interaction of fidarestat with ALR2 was implicated for its specificity towards ALR2 over ALR1 [29]. These observations indicate that curcumin could be a specific inhibitor of ALR2. Aromatic phenol rings of curcumin are connected by two α,β-unsaturated carbonyl groups and exhibits keto-enol tautomerization through an enolate intermediate (Fig. 4). Curcumin predominantly exists as a keto form under the neutral pH conditions employed in this study for inhibition assays. Though, we have performed molecular docking studies with both keto and enol forms and the results were similar with both the forms, for simplicity, we showed molecular docking data with keto form (Fig. 3).

Compared with some potent ARI (fidarestat), the IC_{50} value obtained with curcumin (10 μM) was modest. However, the relative specificity shown by curcumin towards human ALR2 over other AKRs, particularly ALR1, underscores its importance in terms of achieving good inhibition of ALR2 without side effects related to off-target inhibition of ALR1. In addition, the data also suggest that curcumin might aid in guiding the development or identification of highly specific ARIs.

Among human AKRs, ALR2 is unique in its ability to catalyze the NADPH-dependent conversion of glucose to sorbitol [12]. In addition to lens, retina, nerve and kidney, activation of ALR2 in RBC leads to the accumulation of sorbitol [30]. We have also found a direct correlation between erythrocyte ALR2 and sorbitol levels [31]. Therefore, we assessed accumulation of sorbitol in RBC under high glucose conditions (ex vivo) to understand the significance of in vitro inhibition of ALR2 by curcumin, particularly the effect of curcumin on osmotic stress. In vitro incubation of RBC with 55 mM glucose resulted in the accumulation of sorbitol 3–4-fold higher than the control (ANOVA $P < 0.05$) (Fig. 5). Incubation of RBC in the presence of curcumin under high glucose conditions lead to reduction in the accumulation of intracellular sorbitol in a dose dependent manner (ANOVA $P < 0.05$) (Fig. 5). While there was 50% reduction of sorbitol accumulation with 50 μM curcumin, complete inhibition was observed with 100 μM curcumin. Further, we found similar results with rat retinal explants cultured under high glucose conditions in the absence and presence of 50 μM curcumin (data not shown). These results not only substantiate the inhibition of ALR2 by curcumin but also indicate the significance of curcumin in terms of preventing the accumulation of intracellular sorbitol.

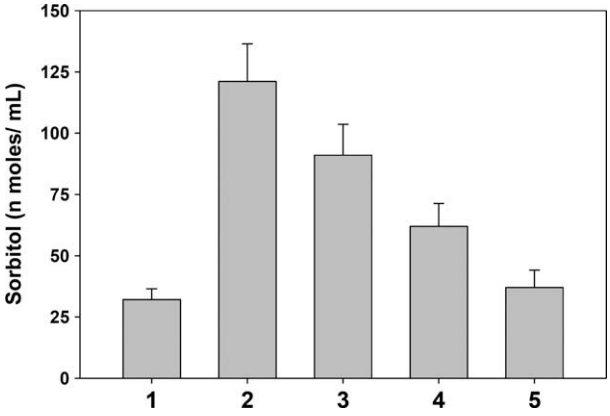

Fig. 5. Effect of curcumin on sorbitol accumulation in RBC. Sorbitol levels in RBC under normal glucose concentration (5.5 mM) (bar 1) under high glucose (55 mM) conditions in the absence (bar 2) and presence of 25 μM, 50 μM and 100 μM curcumin (bars 3–5, respectively). Data are means ± S.E. ($n = 6$).

Although the beneficial impact of strict glycemic control on prevention of diabetic complications has been well established, most individuals with diabetes rarely achieve consistent euglycemia. Hence, agents that can substantially delay or prevent the onset and development of diabetic complications, irrespective of glycemic control, would offer many advantages. In principle, ARI can be included in this category. Thus, intensive research continues to identify and test both synthetic as well as natural products for their therapeutic value to prevent the onset and/or delay progression of diabetic complications.

In conclusion, results of the present study indicate that curcumin inhibits human recombinant ALR2 in a non-competitive manner and this inhibition appears to be relatively specific towards to ALR2 over ALR1. Suppression of sorbitol accumulation in human erythrocytes under high glucose conditions by curcumin is suggestive of translating its impact to in vivo conditions which are supported by our previous studies that curcumin delayed the progression of cataract and inhibited retinal VEGF expression in STZ-induced diabetic rats [14,16]. Others also have reported the clinical significance curcumin on retina under diabetic/hyperglycemic conditions [32,33]. Finally, these observations suggest that curcumin or its synthetic analogues could be explored for alleviating complications of diabetes.

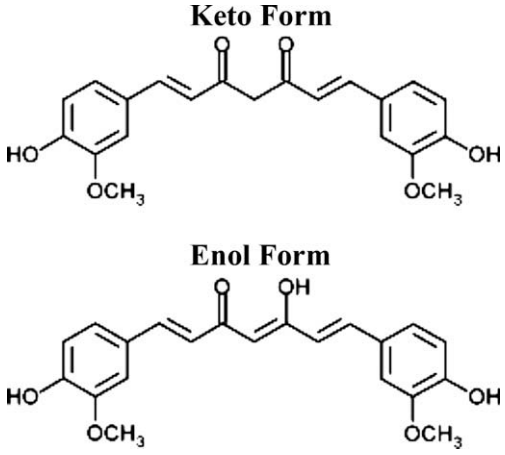

Fig. 4. Two-dimensional structures of keto and enol forms of curcumin.

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