Structure-based enzyme inhibition mechanism studies of kaempferol and its prenylated derivatives as aldose reductase inhibitors using kinetics and molecular docking modeling

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Abstract

**Background:** Aldose reductase inhibitors (ARIs) suppressing the hyperglycemia-induced polyol pathway have been provided as potential therapeutic candidates in the treatment and prevention of diabetic complications. It was previously reported that prenylated flavonols such as desmethylanhydroicaritin (1) and sophoflavescenol (2) from *Sophora flavescens* are promising inhibitors of rat lens aldose reductase (RLAR) and human recombinant aldose reductase (HRAR). Based upon structure–activity relationships, 3,4′-dihydroxy flavonols with a prenyl or lavandulyl group at the C-8 position and a hydroxyl or methoxy group at the C-5 position are important for AR inhibition. In order to prove the above results, a combination of computational prediction and enzyme kinetics has begun to emerge as an effective screening technique for the potential.

**Results:** In the present study, we predicted the 3D structure of AR in human using a docking algorithm to simulate binding between AR and prenylated flavonoids (1 and 2) and kaempferol (3) and scrutinized the reversible inhibition of AR by these proteins. Docking simulation results of 1 ~ 3 demonstrated negative binding energies (Autodock 4.0 = −9.11 to −7.64 kcal/mol; Fred 2.0 = −79.54 to −51.84 kcal/mol) and an additional hydrogen bond through Phe122 and Trp219, in addition to the previously proposed interaction of AR and phenolics through Trp20, Tyr48, His110, and Trp111 residues, indicating that the presence of 8-prenyl and 5-methyl groups might potentiate tighter binding to the active site of the enzyme and more effective AR inhibitors. Moreover, types of RLAR inhibition were different depending on the presence or absence of the 8-prenyl group, in that 1 and 2 are mixed inhibitors with
respective $K_i$ values of 0.69 μM and 0.94 μM, while 3 showed noncompetitive inhibition with a $K_i$ value of 4.65 μM when analyzed with Dixon plots.

**Conclusion:** The present study suggests that an effective strategy for screening potential AR inhibitors could be established by predicting 3D structural conformation of prenyl flavonoids and the orientation within the enzyme as well as by simultaneously determining the mode of enzyme inhibition.
Background

Long-term hyperglycemia in diabetes mellitus is considered to be the primary instigator of the pathogenesis of long term diabetic complications, including retinopathy, cataractogenesis, nephropathy, and neuropathy. To date, the pathogenesis of diabetic complication has been explained by several possible mechanisms, including increased aldose reductase (ALR2; EC 1.1.1.21)-related polyol pathway, increased advanced glycation endproduct (AGE) formation, and excessive oxidative stress [1]. ALR2 and aldehyde reductase (ALR1; EC 1.1.1.2), members of the aldo-keto reductase superfamily, are NADPH-dependent oxidoreductases that catalyze the reduction of a wide variety of aldehydes and ketones to their corresponding alcohols. In particular, ALR2 is a key enzyme in the polyol pathway that catalyzes the conversion of glucose to sorbitol in a hyperglycemic state [2]. ALR2 is found in almost all mammalian cells and at high levels in some organs, such as the lens, retina, and sciatic nerves, which are easily damaged by increased polyol pathway flux-related diabetic complications and can cause cataract [3]. Cataractogenesis is the leading cause of blindness in worldwide diabetic patients [4, 5]. Moreover, sorbitol and its metabolites accumulate in the nerves, retina, and kidneys due to their poor penetration across membranes and inefficient metabolism, resulting in the development of diabetic complications, including retinopathy, neuropathy, and nephropathy [6]. Thus, suppression of the hyperglycemia-induced polyol pathway flux by ALR2 inhibitors (ARIs) may be a potential therapeutic opportunity in the treatment and prevention of diabetic complications [7,8]. Additionally, ALR1 belongs to aldo-keto superfamily that closely resemble to ALR2 but significantly different in the C-terminal loop (297-315, 306-313) responsible for substrate and inhibitor specificity. ALR1 preferentially
metabolizes 3-deoxyglucosone and methylglyoxal, which are both reactive intermediates for AGE formation and may account for some undesirable side effects [9]. Therefore, it is necessary to exploit the specificity and selectivity of these two homologues. In particular, the ability of ALR2 to reduce excess glucose to sorbitol in diabetes mellitus has implicated the enzyme in the pathogenesis of diabetic complications affecting the eyes, kidneys, and nervous system [10].

Structure-based enzyme mechanism studies have been prominently used to elucidate the mechanism of inhibition. With respect to structural information of enzymes and inhibitors, various 3D-molecular docking programs have been developed in recent years. However, limitations have been imposed on the explanation of the enzyme/inhibitor complex, including the binding affinity of enzyme-inhibitors and enzyme-substrates, as well as reaction velocity. Therefore, kinetic studies will take advantage of supporting evidence for the predicted mechanism from molecular docking models. Two kinetic methods have been widely used to determine the type of inhibition: Lineweaver–Burk plot and the Dixon plot. The former is the double reciprocal plot [plot of 1/enzyme velocity (1/V) against 1/substrate concentration (1/[S])] drawn to distinguish the inhibition pattern, including competitive, non-competitive, and uncompetitive inhibition and the enzyme kinetic parameters, including $K_m$ and $V_{max}$ values according to the Michaelis–Menten kinetics equation. The $y$-intercept of such a graph is equivalent to $1/V_{max}$; the $x$-intercept of the graph represents $-1/K_m$. Competitive inhibitors have the same $y$-intercept ($1/V_{max}$) but different slopes ($K_m/V_{max}$) and $x$-intercepts ($K_m$) with increasing concentrations of inhibitors. In the case of mixed inhibition, the inhibitor is capable of binding to both the free enzyme and to the enzyme-substrate complex. This inhibition type is different from noncompetitive inhibition in that the dissociation constant ($K_{ia}$) for binding the
free enzyme may differ from the dissociation constant ($K_{ib}$) for binding the enzyme-substrate complex. Since the mixed inhibitor binds to the enzyme at a distinguished location from the substrate binding site, the binding of the inhibitor will either alter $K_m$ or $V_{max}$ or both. In other words, the apparent $K_m$ may decrease, depending on the relative values of $K_{ia}$ and $K_{ib}$. In a situation where the two $K_i$ values are the same, the apparent $K_m$ will be unchanged. This is called non-competitive inhibition and shows the point on the x-intercept representing $−1/K_m$ [11]. Compared with the Lineweaver-Burk plot, the Dixon plot is a single reciprocal graphical method (plot of $1/enzyme$ velocity ($1/V$) against inhibitor concentration [I]) used to determine the type of enzyme inhibition and is used to easily calculate the dissociation or inhibition constant ($K_i$) of the enzyme/inhibitor complex [12,13]. The $K_i$ value is an indication of inhibitor potency and how tightly an inhibitor binds to enzymes; it is the concentration required to produce half of the maximum inhibition as well as the affinity between enzymes and inhibitors. Plotting $1/V$ against concentration of inhibitor [I] at various concentrations of substrate produces a group of intersecting lines. The corresponding concentration at the intersection point on the x-y plane (x-axis value) is equal to $−K_i$ value in mixed inhibition, while the value of the x-intercept implies $−K_i$ in noncompetitive inhibition.

In order to confirm RLAR inhibitory activity, prediction of the protein-ligand confirmation was carried out with two predicting programs, Autodock 4.0 and Fast Rigid Exhaustive Docking (Fred) 2.0. The programs were used to dock the inhibitors into the binding sites of the crystallographic structures of enzymes defined with all residues located 5~6 Å from the original enzyme/inhibitor complex. Currently, automated docking is widely used as an effective means of quickly and accurately predicting biomolecular conformations and binding energies of protein-ligands.
complexes in molecular design. In particular, Autodock 4.0 uses a semi-empirical free energy force field to predict binding free energies of protein–ligand complexes of a known structure and binding energy for both the bound and unbound states [14]. Apart from Autodock 4.0, Fred 2.0 was also employed due to consolidated evidence including the rigid rotations and translations of each conformer within the binding site. The approach of the Fred software is to thoroughly dock the scores of all possible positions of each ligand in the binding site, exhaustively test all poses of the ligand within the defined binding site, and maintain the protein-ligand complex as rigid during most of the docking process, leading to compensation for target flexibility [15].

The highly hydrophobic active site pocket of ALR2 is formed by aromatic residues (Trp20, Tyr48, Trp79, Trp111, Phe121, Phe122 and Trp219); nonpolar residues (Val47, Pro218, Leu300 and Leu301) and polar residues (Gln49, Cys298 and His110) [1,9]. ARIs generally make use of both polar and nonpolar interactions to establish complementarity with the extended enzyme binding pocket, which is best described as comprising two regions: (1) a polar site with residues Trp20, Tyr48 (the proton donor), and His110 and the positively-charged nicotinamide moiety of NADP+ and (2) a nonpolar site with residues Trp111, Thr113, Phe115, Phe122, Leu300 [8].

Prenyl-substituted kaempferols, including desmethylanhydroicaritin (1) and sophoflavescenol (2), were isolated from dried roots of Sophora flavescens AIT (Leguminosae, Sophorae Radix), which are well known in traditional Chinese medicine (TCM). In our previous study, Sophora flavescens and its prenylated flavonoids were reported to possess antioxidant [16,17], anti-diabetic, and anti-diabetic complication activities [18,19]. In particular, two prenylated flavonoids 1 and 2 exhibited RLAR inhibitory activities; however, there is no detailed information on the mode of inhibition or the enzyme-inhibitor molecular interactions. Therefore, the
aims of this study were to identify an approach to develop potent anti-diabetic complication drugs by scrutinizing molecular docking predictions and enzyme kinetics of prenylated flavonoids 1 and 2.

Results

Enzyme kinetics in RLAR inhibition

The RLAR inhibitory activity of test flavonoids 1 ~ 3 was evaluated. Among them, sophoflavescenol (2) ranked with the most potent inhibitory activity with an IC\textsubscript{50} value of 0.76 ± 0.04 μM, and desmethylanhydroicaritin (1) came in second with an IC\textsubscript{50} value of 1.03 ± 0.13 μM, followed by kaempferol (3) with an IC\textsubscript{50} value of 5.13 ± 0.05 μM. Considering individual structures of 1 ~ 3, compound 1 harbors a prenyl group at the 8 position of the A-ring in the kaempferol skeleton; 2 possesses the additional 5-methoxy group in the A-ring of 1 (Figure 1). Under interpretation using Dixon plotting, compounds 1 and 2 showed mixed type inhibition with respective $K_i$ values of 0.94 μM and 0.69 μM, and 3 showed noncompetitive inhibition with a $K_i$ value of 4.65 μM (Table 1; Figure 2). Similar to the results of the Dixon plots, the lines of both 1 and 2 intersected in the left side, indicating mixed type inhibitors, while the lines of 3 show the same point on the x-intercept representing noncompetitive inhibitors in Lineweaver-Burk plots (Figure 3). The respective kinetic parameters of 1 ~ 3 were also calculated using the Lineweaver-Burk equation:

$$\frac{1}{V} = \left(1 + \frac{[I]}{K_i}\right) \frac{K_m}{V_{\text{max}} [S]} + \frac{1}{V_{\text{max}}}$$

and an altered equation: $V_{\text{max app}} = V_{\text{max}} / \left(1 + \frac{[I]}{K_i}\right)$. The $K_m$ and $V_{\text{max}}$ values of 1~3 depending on the concentrations of inhibitors are presented in Table 2. In the presence of different concentrations of 1~3, respective $V_{\text{max}}$ values were decreased, but the $K_m$ values between prenylated flavonoids (1 and 2) were

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distinguished from those of non-prenylated flavonoid (3). There was no change in the $K_m$ (40.11 mM) or $K_i$ value (6.30 and 6.20 μM) of 3 with different concentrations of substrate, a characteristic of noncompetitive RLAR inhibition. On the other hand, the respective $K_m$ values of 2 and 3 increased with higher substrate concentrations. As for 1, the $K_m$ value changed from 9.85 mM at a concentration of 0.11 μM to 12.32 mM at a concentration of 0.56 μM and the $V_{max}$ value also decreased from 0.016 μmole/mL/min to 0.010 μmole/mL/min, depending on the inhibitor concentration.

The similar enzymatic parameter mode was also detected in 2, showing the increasing $K_m$ values from 12.14 mM to 13.38 mM and the decreasing $V_{max}$ values from 0.016 μmole/mL/min to 0.010 μmole/mL/min with increasing inhibitor concentrations. The above enzymatic parameters for 2 and 3 confirm a mixed RLAR inhibition. In the case of mixed type inhibition, two kinds of $K_i$ values representing the affinity between the enzyme and inhibitor can be calculated if the inhibitors bind to enzyme alone ($K_{ia}$) or enzyme-substrate complexes ($K_{ib}$). Interestingly, the $K_{ia}$ and $K_{ib}$ of 1 were 0.29 and 0.74 μM, respectively, and those of 2 were 0.35 and 0.46 μM at a concentration of 0.11 μM, indicating that 2 has much greater potential for binding to not only enzyme but also enzyme/substrate complexes than does 1.

**Molecular docking model of 1~3 in RLAR inhibition**

As illustrated in Figure 4, the AR-inhibitor complexes were formed with compounds 1~3 stably posed in the pocket of the AR in Autodock 4.0 (pink) and Fred 2.0 (blue). As for 1, the binding site predicted by Autodock 4.0 was formed by residues Leu30; Leu301; Trp20; His110; Cys298; Phe122; Val297; Ala299; Trp111; Trp219; Val47; Pro218; Trp79, while that predicted by Fred 2.0 was formed by residues Leu300; Leu301; Trp20; His110; Ser302; Cys298; Phe122; Val297; Ala299; Trp111; Trp219; Pro218; Val47; Tyr48; Trp79. For 2, the binding site predicted by Autodock 4.0 was
formed by residues Ser210; Cys298; Trp20; Tyr209; Phe121; Trp79; Lys21; Phe122; Gln49; Trp111; Trp219; His110; Val47; Tyr48; Ser22; Asn50, while that predicted by Fred 2.0 was formed by residues Leu300; Ser210; Cys298; Trp20; Tyr209; His110; Ser302; Asn160; Phe122; Cys303; Glu185; Gln49; Leu301; Trp111; Trp219; Val47; Tyr48; Trp79. In the case of 3, the binding site predicted by Autodock 4.0 was formed by residues Leu17; Ser210; Cys298; Asp43; His110; Asp216; Thr19; Trp111; Lys77; Ser214; Trp20; Tyr209; Gly18; Asn160; Pro261; Gln183; Lys262; Pro211; Pro215; Ser159; Tyr48; Ile260; Gly213; Lys21, while that predicted by Fred 2.0 was formed by residues Leu17; Ser210; Cys298; Asp43; His110; Asp216; Thr19; Trp111; Lys77; Ser214; Trp20; Tyr209; Gly18; Asn160; Pro261; Gln183; Lys262; Pro211; Pro215; Ser159; Tyr48; Ile260; Lys21. Due to the similarity in flavonoid structure, it was observed that test compounds 1~3 interacted with AR through well-known active sites, such as hydrophobic and polar residues [1,8]. Due to the presence of a prenyl group, 1 and 2 might interact with ALR2 via a specific and additional nonpolar site, including that of the Leu300 and Phe122 residues which are not present in 3 (Figure 4). In addition to the active site residue, the docking analysis also showed that the respective docking energies of 1~3 were −7.94, −7.64, −9.11 kcal/mol according to Autodock 4.0 and −51.84, −57.27, −79.50 kcal/mol according to Fred 2.0, when accounting for the lowest energy conformation of the most predicted complex. This result indicated that 1~3 bound tightly at the active site.

**Discussion**

The International Diabetes Federation has recently noted that a staggering 366 million people around the world are struggling with either type I or II diabetes. In particular,
type II diabetes is a leading health concern due to its escalating prevalence rate throughout the world and its associated serious diabetic complications. Moreover, high morbidity and mortality rates associated with chronic diabetic complications make the disease the third largest killer after cancer and cardiovascular disease [20]. The disease is taking a deadly toll, causing 4.6 million deaths each year. There has been a growing demand for the treatment and prevention of diabetes and diabetic complications. In particular, ALR2, a key enzyme in the polyol pathway is reported to be highly implicated in the pathogenesis of diabetic complications. Thus, AR reduction of the hyperglycemia-induced polyol pathway flux by AR inhibitors (ARIs) could be a potential therapeutic opportunity [7,8]. In order to select and develop therapeutic drugs for diabetes and diabetic complications, structure-based enzyme mechanism studies have been undertaken as an effective approach. Considering enzymes and inhibitors, mutual studies of molecular docking and enzyme kinetic mechanism of RLAR can predict the potentials of ARIs as anti-diabetic complication agents.

Flavonoids are secondary metabolites that are distributed in member of the high plant kingdom such as fruits and vegetables. Due to relatively lower toxicity and a stronger bioactive potential for increase human health, especially antioxidants, there have been numerous studies based on the development of pharmaceutical drugs. Starting from the basic structure (C6-C3-C6) which is present in chalcones, flavones, flavanones, flavonols, and aurones, the structures of flavonoids are diverse and are determined by the number and arrangement of the substituents and glycosylations [21,22]. Since many flavonoids have been implicated in the alleviation of diabetic complications, many researchers have scrutinized their relevance in AR inhibition [7,23-25]. In particular, prenylated flavonoids possess additional hydrophobic and
anionic characteristic moieties (prenyl groups) on their flavonoid skeletons which may play important roles in enzyme inhibition [18]. In our previous study, the inhibitory activity of 3, lacking a prenyl group at the C-8 position, was drastically decreased compared to those of prenylated flavonols 1 and 2, indicating that the hydrophobic aliphatic groups may, at least in part, be associated with increased inhibitory activity [18,19]. Furthermore, the type of RLAR inhibition depended on the absence or presence of prenyl groups, in that 3 showed noncompetitive inhibition, whereas 1 and 2 are mixed inhibitors to RLAR in the present study (Figures 2 and 3). In other words, 2 and 3 can bind to both the allostatic site of the free enzyme and to the enzyme/substrate complex; 3 binds to the free enzyme and inhibits the formation of the enzyme/substrate complex. Depending on the relative values of the $K_i$ (the dissociation constant of inhibitors), the $V_{\text{max}}$ results of 2 and 3 possessing an additional prenyl group increased regardless of whether the substrate was bound to the enzyme.

Based on molecular docking studies, flavonoids have been shown to be widely disseminated as naturally occurring ALR2 inhibitors. Recently, there has been staggering supporting research on structure-activity relationship of flavonoids, including enzyme kinetics and molecular docking studies [26,27]. With respect to the docking modeling, the findings have unveiled that the dissociated anionic hydroxyl group at C-7 interacts with Tyr48, His110, Trp111, and the positively-charged nicotinamide ring of the NADP$^+$ cofactor in the active site cavity of ALR2. Moreover, the additional hydrophobic pocket located in the active site (Leu300 and Trp111) interacts with the C-2 benzyl substituent. The presence of a 4′-hydroxyl group on the B-ring can also increase the affinity and inhibitory potency against ALR2 via interaction with Thr113 at the active site [23,28]. Through modeling studies, the
phenoxyl group has been shown to provide a good structural replacement for the carboxylate group (the most well known ARIs are tolrestat, sorbinil), which can account for the possibility of flavonoids as potent ALR2 inhibitors [8]. In fact, the specificity and selectivity of ALRs are closely related to the hydrophobic pocket of ALRs, including Leu300 and Trp111. In particular, Leu300 is the short segment of the enzyme susceptible to conformational changes, followed by the determination of the ligand specificity toward ALR2 compared to that of ALR1 [28]. Comparative molecular modeling studies of 1–3 revealed that all three compounds can bind tightly to the active site through Trp20, Tyr48, His110, and Trp111 residues. Unlike compound 3, the two prenylated kaempferols 1 and 2 interact with Leu300 and Phe122 residues at a specific nonpolar site of ALR2 (Figure 4). Considering this result, the presence of the 8-prenyl group might have an important role in the selectivity and potency of ALR inhibition via strong anionic tendency at the adjacent 7-hydroxyl group as well as the 4’-hydroxyl group.

**Conclusions**

The docking simulation results of 1 ~ 3 demonstrating the negative binding energies (Autodock 4.0 = –9.11 to –7.64 kcal/mol; Fred 2.0 = –79.54 to –51.84 kcal/mol) and the additional hydrogen bonds through Phe122 and Trp219, in addition to the previously proposed interaction of AR and phenolics through Trp20, Tyr48, His110, and Trp111 residues, have highlighted that the presence of 8-prenyl and 5-methyl groups might potentiate tighter binding to the active site of an enzyme, making them much more effective as AR inhibitors. The present study suggested that an effective strategy for screening the potential of AR inhibitors could be established by predicting
the 3D structural conformation of prenylated flavonoids and the orientation within the enzyme, as well as by simultaneously determining the mode of enzyme inhibition. This simultaneous approach might be underlined as a potential guideline for the design of AR-selective inhibitors.

Methods

General
The $^1$H- and $^{13}$C-NMR spectra were determined using a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for $^1$H and 100 MHz for $^{13}$C in deuterated dimethylsulfoxide (DMSO)-$d_6$. Column chromatography was conducted using silica (Si) gel 60 (70–230 mesh, Merck, Darmstadt, Germany) and Sephadex LH-20 (20–100 μm, Sigma, St. Louis, MO, USA). All TLC was conducted on pre-coated Merck Kieselgel 60 F$_{254}$ plates (20 × 20 cm, 0.25 mm, Merck) or RP-18 F$_{254}$s plates (5 × 10 cm, Merck), using 50% H$_2$SO$_4$ as a spray reagent.

Chemicals and reagents
β-Nicotinamide adenine dinucleotide phosphate (NADPH), DL-glyceraldehyde dimer, kaempferol, and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents were purchased from Merck, Fluka, Duksan Pure Chemical Co., or Sigma Aldrich Co., unless stated otherwise.
Isolation of desmethylanhydroicaritin and sophoflavescenol

The dried roots of *S. flavescens* (10 kg) were refluxed with methanol (MeOH) for 3 h (3 × 10 L). The total filtrate was then concentrated to dryness in vacuo at 40 °C in order to render the MeOH extract (2.2 kg). This extract was successively partitioned with methylene chloride (CH$_2$Cl$_2$), ethyl acetate, *n*-butanol and water. The CH$_2$Cl$_2$-soluble fraction was chromatographed over a Si gel column with a CH$_2$Cl$_2$–MeOH mixture (gradient) and further separated by Sephadex LH-20 (solvent: MeOH) to yield desmethylanhydroicaritin (1, 45 mg) and sophoflavescenol (2, 600 mg). These compounds were characterized and identified by spectroscopic methods, including $^1$H- and $^{13}$C-NMR, as well as through comparison with published data [16,19]. The structures are shown in Figure 1, and their spectral data are as follows.

**Desmethylanhydroicaritin (1)** $^1$H-NMR (400 MHz, DMSO-$d_6$) δ : 12.40 (1H, s, OH-5), 10.73 (1H, s, OH-7), 10.12 (1H, s, OH-4′), 9.36 (1H, s, OH-3), 8.03 (2H, d, $J$ = 8.87 Hz, H-2′, 6′), 6.93 (2H, d, $J$ = 8.87 Hz, H-3′, 5′), 6.29 (1H, s, OH-6), 5.17 (1H, t, $J$ = 6.85 Hz, H-2″), 3.42 (2H, d, $J$ = 6.45 Hz, H-1″), 1.74 (3H, br s, H-4″), 1.62 (3H, br s, H-5″); $^{13}$C-NMR (100 MHz, DMSO-$d_6$) δ : 176.11 (C-4), 161.13 (C-7), 159.14 (C-4′), 158.25 (C-5), 153.44 (C-9), 146.72 (C-2), 135.49 (C-3), 130.91 (C-3″), 129.34 (C-2′, 6′), 122.54 (C-2″), 121.96 (C-1′), 115.44 (C-3′, 5′), 105.56 (C-8), 102.98 (C-10), 97.76 (C-6), 25.42 (C-5″), 21.19 (C-1″), 17.80 (C-4″).

**Sophoflavescenol (2)** $^1$H-NMR (400 MHz, DMSO-$d_6$) δ : 10.58 (1H, s, 7-OH), 9.99 (1H, s, 4′-OH), 7.98 (2H, d, $J$ = 8.8 Hz, H-2′/H-6′), 6.91 (2H, d, $J$ = 8.8 Hz, H-3′/H-5′), 6.44 (1H, s, H-6), 5.15 (1H, t-like, H-2″), 3.80 (3H, s, 5-OCH$_3$), 3.46 (2H, brd, $J$ = 6.7 Hz, H-1″), 1.75 (3H, s, H-5″), 1.62 (3H, s, H-4″). $^{13}$C-NMR (100 MHz, DMSO-$d_6$) δ: 171.2 (C-4), 159.5 (C-7), 158.5 (C-4′), 157.9 (C-5), 155.4 (C-9), 141.8 (C-2), 136.8 (C-3), 130.9 (C-3″), 128.6 (C-2′/C-6′), 122.7 (C-2″), 122.3 (C-1′), 115.4 (C-3′/C-5′),
Assay for RLAR inhibitory activity

Rat lens homogenates were used as AR sources. In the experiment, we followed the Guidelines for Care and Use of Laboratory Animals as approved by Pukyong National University (Busan, Republic of Korea). Rat lens homogenate was prepared according to the modified method of Hayman and Kinoshita [29]. Briefly, the lenses were removed from the eyes of Sprague-Dawley rats weighing 250 ~ 280 g (Samtako BioKorea, Inc.) and homogenized in sodium phosphate buffer (pH 6.2). The supernatant was obtained by centrifugation of the homogenate at 10,000 rpm at 4 °C for 20 min and was frozen until use. A crude AR, with a specific activity of 6.5 U/mg, was used in the evaluations of enzyme inhibition. The partially purified material was separated into 1.0 ml aliquots, and stored at -80 °C. Each 1.0 ml cuvette contained equal units of enzyme, 100 mM sodium phosphate buffer (pH 6.2), and 1.6 mM NADPH, both with and without 50 mM of the substrate, DL-glyceraldehyde, and an inhibitor (f.c. 100 μM for the isolated compounds, dissolved in 100% DMSO) with a final concentration of 1% DMSO. The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 4 min period on a Ultrospec® 2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, New Jersey, USA). The inhibition percentage (%) was calculated as \[\left\{(1 - (\Delta A \text{ sample/min} - \Delta A \text{ blank/min}) / (\Delta A \text{ control/min} - \Delta A \text{ blank/min})) \times 100\right\}\], where \(\Delta A \text{ sample/min}\) represents the reduction of absorbance for
4 min with the test sample and substrate, respectively, and ΔA control/min represents the same but with 100% DMSO instead of a sample.

**Kinetic parameters in RLAR inhibition – Dixon and Lineweaver–Burk plots**

In order to determine the kinetic mechanism, two kinetic methods using Lineweaver–Burk plots and the Dixon plots were complementarily used [11-13]. Each enzymatic inhibition at various concentrations of three test compounds was evaluated by monitoring the effects of different concentrations of the substrates in Dixon plots (single reciprocal plot). Dixon plots for inhibition of RLAR were obtained in the presence of different concentrations of DL-glyceraldehyde substrate: 25 mM (●); 50 mM (○); and 100 mM (▼). The test concentrations of test flavonoids in the RLAR kinetic analysis were as follows: 0.56 and 0.11 μM for 1; 2.78, 2.71, 0.54, and 0.11 μM for 2; and 34.94, 17.47, and 3.49 μM for 3. The enzymatic procedures consisted of the same, aforementioned RLAR assay methods. The inhibition constants (K_i) were determined by interpretation of the Dixon plots. By means of Lineweaver–Burk double reciprocal plots, K_m and V_max values of RLAR were determined at various concentrations of DL-glyceraldehyde (25, 50, and 100 mM) substrate in the absence and presence of different concentrations of test compounds (0.56 and 0.11 μM for 1; 2.78, 2.71, 0.54, and 0.11 μM for 2; and 34.94, 17.47, and 3.49 μM for 3). The K_i value was also derived by plotting slopes obtained from Lineweaver–Burk plots and Dixon plots.
Molecular docking simulation in RLAR inhibition – Autodock 4.0 and Fred 2.0

In order to estimate the conformation of the protein-ligand complex and to increase accuracy, repeatability, and reliability of the docking results, two programs: Autodock 4.0 (AutoDock4 and AutoDockTools4) and Fred 2.0 (OpenEye Scientific Software, Santa Fe, NM, USA) were utilized. Twelve ligand structures were constructed and minimized using Chemsketch 3.5 and Omega 2.0 software (OpenEye Scientific Software, USA), for 2D and 3D conformation, respectively [30]. For docking studies, the crystal structures of the protein targets (NCBI protein ID : NP_001619.1) were allocated from the protein sequence alignment [Brookhaven Protein Data Bank (PDB ID: 2acr chain A)]. The 3D structures of test compounds are as follows: desmethylanhydroicaritin (PUBCHEM ID: 5318624); sophoflavescenol (PUBCHEM ID : 9929189); and kaempferol (PUBCHEM ID: 5280863). 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. The docking of the aldose reductase-flavonoid molecule was successful, as indicated by statistically significant scores.

Statistics

Statistical significance was analyzed by one-way ANOVA and Student’s t-test (Systat Inc., Evanston, IL, USA) and considered significant at $p < 0.01$. All results are presented as mean ± SEM.

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**Authors' contributions**

HAJ and JSC carried out the design, coordination, and analysis of overall bioactive assays, enzyme kinetics, and molecular docking studies, and drafted and corrected the manuscript. HEM and SHO participated in the performance and analysis of the individual study. BWK, HSS, SBK conceived of the study, and participated in its coordination and helped to review the manuscript. All authors read and approved the final manuscript.

**References**


Figure legends

Figure 1  Structures of test compounds 1~3

Figure 2  Dixon plots for RLAR inhibition of compounds 1~3

Desmethylanhydroicaritin (A), sophoflavescenol (B), and kaempferol (C) were tested in the presence of different concentrations of substrate (DL-glyceradehyde): 25 mM (●); 50 mM (○); and 100 mM (▼).

Figure 3  Lineweaver-Burk plots for RLAR inhibition of compounds 1~3

RLAR inhibition was analyzed in the presence of different concentrations of sample as follows: 0 µM (●), 0.11 µM (○), and 0.56 µM (▼) for desmethylanhydroicaritin (A); 0 µM (●), 0.108 µM (○), 0.54 µM (▼), and 2.71 µM (▽) for sophoflavescenol (B); 0 µM (●), 17.47 µM (○), and 34.94 µM (▼) for kaempferol (C).

Figure 4  Molecular docking models for RLAR inhibition of compounds 1~3

desmethylanhydroicaritin (A), sophoflavescenol (B), and kaempferol (C)
Table 1  IC<sub>50</sub> values and dissociation constants (K<sub>i</sub>) of compounds 1~3 for RLAR activity using the Dixon plot

<table>
<thead>
<tr>
<th>Test compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (µM)</th>
<th>Inhibition type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.03 ± 0.13</td>
<td>0.94</td>
<td>mixed</td>
</tr>
<tr>
<td>2</td>
<td>0.76 ± 0.04</td>
<td>0.69</td>
<td>mixed</td>
</tr>
<tr>
<td>3</td>
<td>5.13 ± 0.05</td>
<td>4.65</td>
<td>noncompetitive</td>
</tr>
</tbody>
</table>

Table 2  Kinetic parameters of compounds 1~3 regarding RLAR activity according to the Lineweaver-Burk plot

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Conc. (µM)</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (µM)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (µmole/mL/min)</th>
<th>Inhibition type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>8.20 ± 0.04</td>
<td>0.016 ± 0.002</td>
<td>mixed</td>
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</tr>
<tr>
<td></td>
<td>0.11</td>
<td>9.84 ± 0.05</td>
<td>0.014 ± 0.001</td>
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<tr>
<td></td>
<td>0.56</td>
<td>12.32 ± 0.08</td>
<td>0.010 ± 0.001</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>0.00</td>
<td>11.48 ± 0.07</td>
<td>0.016 ± 0.002</td>
<td>mixed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>12.14 ± 0.05</td>
<td>0.013 ± 0.001</td>
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</tr>
<tr>
<td></td>
<td>0.54</td>
<td>13.38 ± 0.08</td>
<td>0.010 ± 0.000</td>
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</tr>
<tr>
<td>3</td>
<td>0.00</td>
<td>40.10 ± 0.12</td>
<td>0.026 ± 0.001</td>
<td>noncompetitive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.47</td>
<td>40.11 ± 0.15</td>
<td>0.007 ± 0.000</td>
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</tr>
<tr>
<td></td>
<td>34.94</td>
<td>40.11 ± 0.14</td>
<td>0.004 ± 0.000</td>
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</tr>
</tbody>
</table>
desmethylanhydroicaritin

sophoflavescenol

kaempferol
Figure 2
Figure 4