A chalcone derivative AD-021 inhibits kidney fibrosis in a mouse model of high fat diet/streptozotocin-induced diabetic nephropathy

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Abstract

Diabetic nephropathy (DN) is the most prevalent microvascular disorder in diabetes mellitus and plays an integral role in the aggravation of renal injury and fibrosis, which currently lacks effective treatments. The chalcone derivative AD-021 is a small synthetic molecule recently identified for treating kidney disease. In this study, we evaluated the effects of AD-021 on kidney function and kidney fibrosis in a high-fat diet (HFD) combined with streptozotocin (STZ)-induced DN in mice. Mice were treated with intraperitoneal administration of AD-021 (50 mg/kg/day) for 12 weeks. Determination of fasting blood glucose (FBG), renal function-related albumin/creatinine ratio, and urine volume in mice and histopathological section analysis of collagen deposition in kidney tissue were performed. Moreover, western blot analysis was used to investigate the expressions of TGF-β1 and profibrotic-related proteins. We found that administration of AD-021 (50 mg/kg/day) reduced FBG, albumin/creatinine ratio, urine volume and collagen deposition in kidney tissue of DN mice. Furthermore, AD-021 significantly inhibited expression of TGF-β1 and fibronectin, and alleviated loss of E-cadherin protein expression in HFD/STZ-induced DN mice. These data suggest that AD-021 has the potential as a nephroprotective agent in DN patients.

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Introduction

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes, accounting for approximately 40% of patients with type 1 or type 2 diabetes and nearly 50% of DN patients progressing to end-stage renal disease (ESRD).[1,2] Additionally, the 2020 Annual Report Thailand Renal Replacement Therapy indicates that diabetic nephropathy is the leading cause of ESRD patients with kidney transplant therapy in 41.5% in Thailand. The effects of DN on kidney dysfunction include an increase in microalbuminuria along with a decrease in the glomerular filtration rate (GFR). There are also kidney injuries including tubulo-interstitial fibrosis, extracellular matrix (ECM) deposition, glomerular basement membrane thickening and podocyte foot destruction processes. All these factors cause chronic loss of kidney function leading to ESRD.[3] Under diabetic conditions, renal fibrosis is the final common pathway in the DN pathophysiology resulted from renal ischemia, hemodynamic changes, glucose metabolism abnormalities, increasing of oxidative stress, inflammation, and overactive renin-angiotensin-aldosterone system (RAAS).[4] There are several molecules involved in the fibrogenesis of DN, including transforming growth factor-β1 (TGF-β1), a major player of tubulointerstitial fibrosis induction. Through Smad and non-Smad signaling pathways, TGF-β1 promotes the production of ECM and deposition of collagen leading to tissue fibrosis in the diabetic kidney.[5] Anti-fibrotic agents targeting TGF-β1 have been shown to have high efficacy in inhibiting fibrosis progression in experimental models.[6] Natural compounds and their derivatives represent a valuable source for developing novel anti-fibrotic agents due to their wide ranges of biological activities, accessibility and well-known safety profiles.

Chalcone derivatives have previously been shown to hold promise as anti-diabetic treatment due to their beneficial biological activities including anti-inflammation[7] and anti-diabetic effect.[8] Our research group has recently identified a novel chalcone derivative AD-021 as an anti-fibrotic agent that inhibited renal fibrosis through inhibition of TGF-β-induced both Smad and non-Smad signaling pathways via suppression of TGFβRII phosphorylation in an animal model of renal fibrosis induced by unilateral ureteral obstruction (UUO).[9] However, the beneficial effect of this chalcone derivative on DN is

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still unknown. Therefore, the present study aimed to investigate the therapeutic effects and safety profiles of AD-021 in the treatment of DN. Using an *in vivo* model, we demonstrated that AD-021 was effective in ameliorating DN progression with reduced fasting blood glucose, improved kidney function and attenuated kidney fibrosis in HFD/STZ-induced DN mice model.

Materials and Methods

Compound and reagents

The chalcone derivative, AD-021 (>95% purity by NMR analysis) was synthesized according to the established method. Reagents used for western blot analysis in this research, anti-fibronectin (ab2413), anti-TGF-β1 antibody (ab92486) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (ab6721) were purchased from Abcam (Waltham, MA, USA). Anti-E-Cadherin (cat no. 3195) and anti-GAPDH (cat no. 5174) were purchased from Cell Signaling Technology (Danvers, MA, USA). Streptozotocin (cat no. 18883664) was purchased from Cayman Chemicals (Ann Arbor, MI, USA).

Animal and experimental design

Eight-week-old male C57BL/6 mice (body weight of 22–25 g) were purchased from Nomura Siam International, Thailand. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University (permit number MUSC63-006-514). All animals were acclimatized for a week before the experiment with a 12 h light/dark cycle as well as free access to water and food. After acclimation, mice were divided into three groups. The first group received intraperitoneal (i.p.) injections with sodium citrate buffer (pH 4.5) for three consecutive days and fed with a standard (STD) chow (D12450Ji) as a control. The second and the third groups received i.p. injections with low-dose streptozotocin (STZ) in sodium citrate buffer (pH 4.5) for five consecutive days (50 mg/kg/day) combined with a high fat diet (HFD) (D12492i) to establish an HFD/STZ-induced DN model without or with administration of AD-021 daily at dosages of 50 mg/kg/day, respectively. The contents of fat percentage in the diets were 10 kcal% fat and 60 kcal% fat in no DN and DN models, respectively. AD-021 was dissolved in a mixture of 1% DMSO, 20% propylene glycol and 79% PBS. Body weight and fasting blood glucose were measured every two and three weeks, respectively. After AD-021 treatment for 12 weeks, urine was collected. The mice were euthanized, and blood was collected through cardiac puncture and kidney were collected. The harvesting of the kidney was under a surgical level of anesthesia followed by rinsing with 0.1% phosphate buffered solution. The kidneys were wrapped in aluminum foil, then snapped frozen in liquid nitrogen, and stored at −80 °C prior to further investigation.[10]

Measurement of fasting blood glucose

Fasting blood glucose levels in mice were measured at 0, 3, 6, and 12 weeks after treatment using an Accu-Chek meter for diabetic monitoring (Roche Diagnostics, Indianapolis, IN, USA) via the tail vein. The mice were fasted for 6 hours before the measurement.

Measurement of albumin/creatinine

After 12 weeks of treatment, spot urine from all mice was collected via a metabolic cage. The level of albumin/creatinine ratio was measured using an albumin-to-creatinine ratio assay kit (cat no. K551, BioVision, Milpitas, CA, USA). The assay and data analysis were done in accordance with the manufacturer's instructions.

Measurement of serum creatinine and alanine transaminase (ALT)

At the end of the experiment, serum creatinine and serum ALT levels from the mice were measured using the serum creatinine commercially available kits (cat no. ab65340, Abcam) and ALT assay kits (Cayman Chemical, cat no. 700260), respectively. These assays and data analysis were done according to the manufacturer's instructions.

Picro-sirius red (PSR) staining of kidney tissues

Picro-Sirius red (PSR) staining was conducted to detect collagen accumulation in the kidney tissues of the mice. Kidneys were instantly fixed in 4% paraformaldehyde and dehydrated in ethanol. The tissues were embedded in 4 μm thick paraffin sections. 0.1% PSR staining solution (ab246832; Abcam) was added to the tissue sections and incubated for an hour. After the PSR solution was removed, the tissues were washed three times with 0.5% acetic acid, and the collagen in the extracellular matrix was assessed under a microscope. Quantification of the red-stained fibrotic area was done to assess tubulointerstitial fibrosis.

Western blot analysis

The kidney tissue from each mouse in the control group (n = 4), HFD/STZ-induced DN group (n = 5), and HFD/STZ-induced DN with AD-021 treatment group (n = 4) was extracted and used for protein expression measurement using western blotting analysis separately in each well. Moreover, the renal cortex was the part of the kidney tissue taken for protein extraction. The whole protein was extracted using RIPA lysis buffers (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitors. Extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.45 μm nitrocellulose membranes. The membranes were then blocked with 5% nonfat milk in Tris-buffered saline and 0.1% Tween 20 for an hour at room temperature. Then, overnight incubation at 4 °C with primary antibodies specific to

the proteins of interest including TGF-β1, fibronectin, E-cadherin, and GAPDH was done. After incubation, the membrane was washed and further incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies for an hour at room temperature. Membrane protein expression was measured by adding HRP substrate to produce chemiluminescence. The intensity of protein bands was measured using a ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

All experimental results were expressed as mean ± SEM. Statistical differences between groups were tested using student's t-test or one-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis. *P* value < 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results

Effect of AD-021 on fasting blood glucose in HFD/STZ-induced DN mice

To evaluate the anti-diabetic effect of AD-021 in DN mice, AD-021 was intraperitoneally administered (50 mg/kg/day) to an HFD/STZ-induced DN mouse model for 12 weeks. Mice in the HFD/STZ-induction group had significantly lower body weight throughout the duration of HFD/STZ-induced DN compared with the STD group (Figure 1A). Mice in the AD-021 treatment group had no effect on body weight compared with the vehicle-treated HFD/STZ-induced DN group (Figure 1A). Importantly, mice with HFD/STZ-induction had increased fasting blood glucose (FBG), indicating the development of diabetes mellitus (Figure 1B). Interestingly, AD-021 treatment significantly reduced FBG in the HFD/STZ mice, indicating anti-diabetic effect of AD-021 (Figure 1B).

Effect of AD-021 on kidney and liver functions in HFD/STZ-induced DN mice

We next investigated the effect of AD-021 on kidney functions in HFD/STZ-induced DN mice. Mice with HFD/STZ-induction had increased serum creatinine, albumin/creatinine ratio and urine volume, indicating an impaired kidney function (Figure 2A-2C). These two parameters were significantly reduced by AD-021

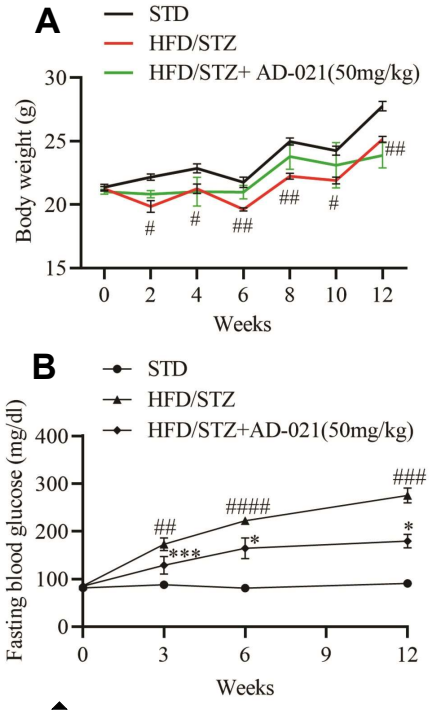

Figure 1 Effect of AD-021 on body weight and fasting blood glucose level in DN mice. (*A*) Body weight and (*B*) fasting blood glucose levels in each group. (n = 4-5 per group). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, and $^{\#\#\#\#}P < 0.0001$ versus STD group; $^{*}P < 0.05$ and $^{***}P < 0.001$ versus HFD/STZ group. Data are expressed as mean ± SEM (two-way ANOVA).

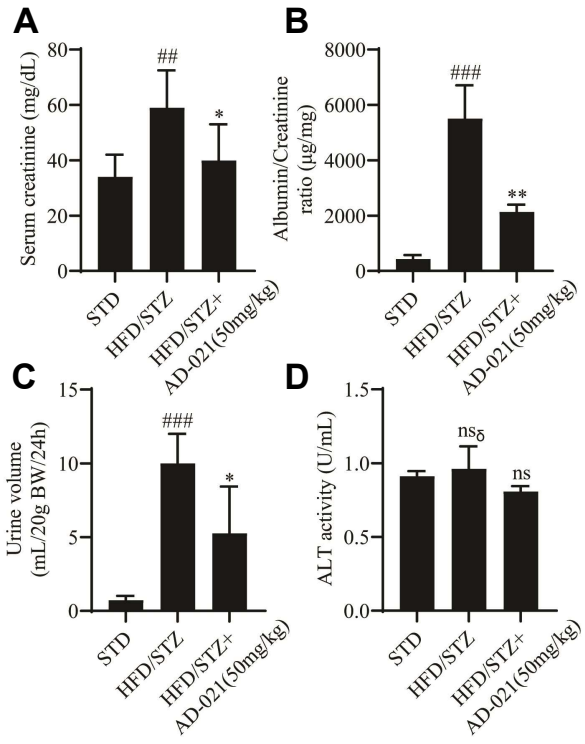

Figure 2 Effect of AD-021 on serum creatinine, albumin/creatinine ratio, urine volume and serum ALT level in DN mice. (*A*) Serum creatinine, (*B*) albumin/creatinine ratio in urine, (*C*) urine volume in each group, and (*D*) serum ALT levels in mice. (n = 4-5 per group). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus STD group; $^{*}P < 0.05$, and $^{**}P < 0.01$ versus HFD/STZ group; ns_{δ}, not significant versus STD group; ns, not significant versus HFD/STZ group. Data are expressed as mean ± SEM (one-way ANOVA).

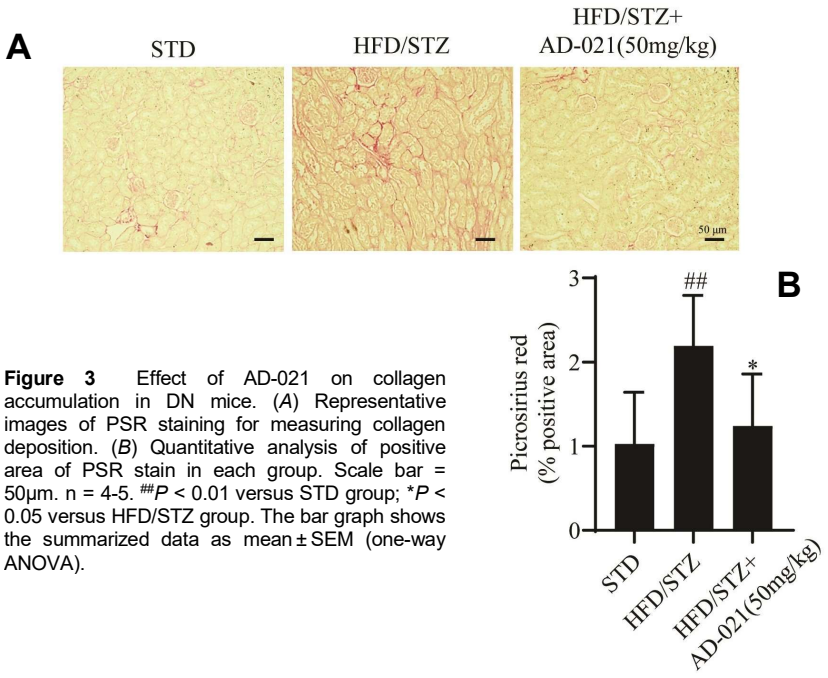

Figure 3 Effect of AD-021 on collagen accumulation in DN mice. (*A*) Representative images of PSR staining for measuring collagen deposition. (*B*) Quantitative analysis of positive area of PSR stain in each group. Scale bar = 50µm. n = 4-5. $^{\#\#}P < 0.01$ versus STD group; $^{*}P < 0.05$ versus HFD/STZ group. The bar graph shows the summarized data as mean ± SEM (one-way ANOVA).

treatment (Figure 2A-2C). To initial evaluate hepatotoxic effect of AD-021, serum ALT levels in all groups were measured. We found that there was no significant difference among groups of mice indicating that AD-021 did not produce hepatotoxicity in mice after treatment for 12 weeks (Figure 2D).

Effect of AD-021 on collagen accumulation in HFD/STZ-induced DN mice

To investigate the anti-fibrotic effects of AD-021, we determined collagen accumulation, which was a primary pathologic feature in fibrotic processes, in DN mice by PSR staining of kidney tissues. Quantitative analysis of the positive area of PSR stain using ImageJ software by National Institutes of Health. The kidney samples from the control group (n = 4), HFD/STZ-induced DN group (n = 5), and HFD/STZ-induced DN with the AD-021 treatment group (n = 4) were analyzed using five images of

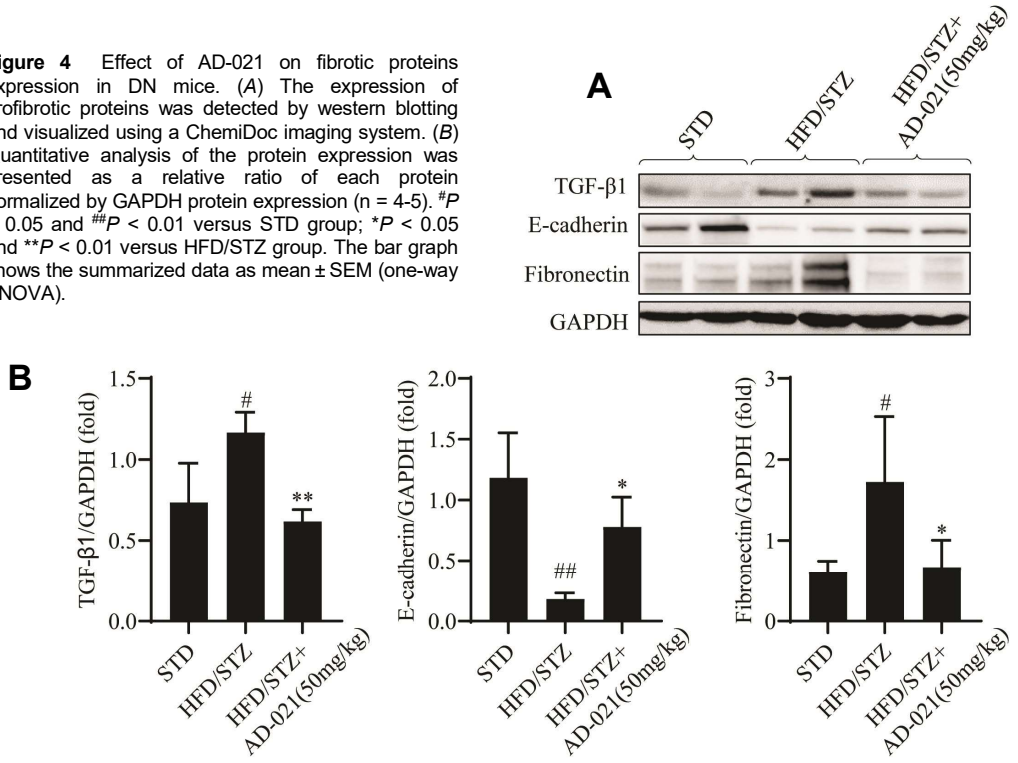

Figure 4 Effect of AD-021 on fibrotic proteins expression in DN mice. (*A*) The expression of profibrotic proteins was detected by western blotting and visualized using a ChemiDoc imaging system. (*B*) Quantitative analysis of the protein expression was presented as a relative ratio of each protein normalized by GAPDH protein expression (n = 4-5). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus STD group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus HFD/STZ group. The bar graph shows the summarized data as mean ± SEM (one-way ANOVA).

PSR staining per kidney sample for intensity measurement (% positive areas). The results showed that DN mice had increased collagen deposition compared to the STD group as analyzed by the percentage of the positive area of the PSR staining (Figure 3). AD-021 treatment significantly attenuated collagen deposition in the HFD/STZ-induced DN mice (Figure 3). These results indicated that AD-021 were effective in preventing development of renal fibrosis in the DN mouse model.

Effect of AD-021 on biomarkers of kidney injuries in HFD/STZ-induced DN mice

To further investigate potential mechanisms underlying the anti-fibrotic effect of AD-021 at cellular levels, effects of AD-021 on biomarkers related to kidney injuries and renal fibrogenesis including TGF-β1, E-cadherin, and fibronectin were determined in the mouse kidney tissues. It was found TGF-β1 was elevated in DN group, which was restored by AD-021 treatment, indicating that AD-021 ameliorate kidney injuries by reducing expression/production of TGF-β1 in kidney tissues (Figure 4). Furthermore, we found that E-cadherin protein expression was decreased in DN mice, and AD-021 treatment restored the loss of E-cadherin, indicating improved viability of epithelial cells by AD-021. The DN group also had increased expression of fibronectins, a profibrotic markers, compared to the STD group, which was attenuated by AD-021 treatment. These findings suggest that AD-021 attenuated kidney fibrosis at least in part by reducing kidney injuries.

Discussion

A growing number of DN prevalence has recently been a concern in developing countries. It can affect kidney functions and lead to kidney fibrosis via promoting epithelial-mesenchymal transition (EMT) by the important inducer, including TGF-β1. It has been reported that the DN condition had a high level of TGF-β1 consequent to induced fibronectin and loss of E-cadherin developing into kidney fibrosis. Previous studies have found that natural products can inhibit TGF-β1 and reduce the incidence of kidney fibrosis and the development of DN. For example, echinacoside, a natural phenylethanol isolated, a commonly used drug in traditional Chinese medicine, was found that it can reduce the occurrence of kidney fibrosis and reduce the development of DN in the db/db mice model of DN by inhibiting TGF-β1 signaling pathway.[11] Therefore, it is interesting to find a novel compound that can treat diabetes and reduce kidney fibrosis in patients with DN. The present study was aimed to find a novel anti-fibrotic agent in DN mice model. We identified AD-021, a chalcone derivative, to inhibit the HFD/STZ-induced DN mice via reduced fasting blood glucose, improved kidney function including decreased albumin/creatinine ratio and urine volume. Furthermore, AD-021 inhibited expression of TGF-β1, fibronectin and loss of E-cadherin. Thus, in a mouse model of HFD/STZ-induced DN, AD-021 alleviated renal fibrosis and preserved renal functions without causing overt systemic toxicity.

Chalcone is a beneficial plant's secondary metabolite and precursor to flavonoid synthesis. It has been used in traditional medicine for a long time due to its wide range of biological effects.[12] One of those effects has been reported to have excellent anti-diabetic effects.[13] Chalcone is a group of open-chain flavonoids that are not only biosynthesized by plants but can also be prepared synthetically. The chalcones have several types of reactive hydrogen in their basic structure. This property allows structural modification and synthesis of various chalcone derivatives.[14] Previous studies have demonstrated that chalcone derivatives have shown manifold biological activities, including anti-diabetic effects. Chalcone-1-deoxynojirimycin heterozygote, a new compound designed and synthesized from chalcone's basic structure, has shown therapeutic effect and reduces blood glucose levels in diabetic rats.[15] The anti-diabetic effect on HFD/STZ-induced DN of AD-021 is associated with a decline in FBG. Moreover, newly synthesized chalcone derivative Cpd-20 has been reported to improve kidney function in cisplatin-induced acute kidney injury mouse model.[16] In this study, AD-021 administration significantly improved renal function. In HFD/STZ-induced DN mice, albumin/creatinine ratio and urine volume were elevated, whereas administration of AD-021 significantly inhibited the increase of these parameters. However, proportions of alanine aminotransferase (ALT), an enzyme found in the liver, were performed at the end of the experiment as it indicates whether the liver has been damaged. ALT is released and increases in the bloodstream.[17] Therefore, we used the ALT test to detect toxicity and liver damage. ALT was determined, and AD-021 treatment did not affect liver function. Recently, novel chalcone derivatives are increasingly recognized as an alternative source of antioxidant and anti-fibrotic agents. Previous studies have reported that novel chalcone biphenyl diester derivative-39 inhibited biomarkers of kidney fibrosis (collagen IV and laminin) via activates Nrf2/ARE signaling pathway in high-fat diet feeding combined with streptozotocin-induced type 2 diabetes model with intraperitoneal administration.[18] Our present study showed that AD-021 inhibited HFD/STZ-induced kidney fibrosis in DN mice through inhibition of collagen deposition, TGF-β1, fibronectin and loss of E-cadherin protein expression. According to these results, AD-021 treatment can be regarded as a renoprotective functional component that can protect kidney fibrosis against diabetic kidney disease.

Conclusion

AD-021 represents a chalcone derivative possessing anti-fibrotic effects in HFD/STZ-induced DN mice through mechanisms involving reduction of FBG and kidney injuries. Further development of AD-021 may provide an effective nephroprotective and therapeutic agent for the treatment of diabetic nephropathy.

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Conflict of Interest

None to declare.

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