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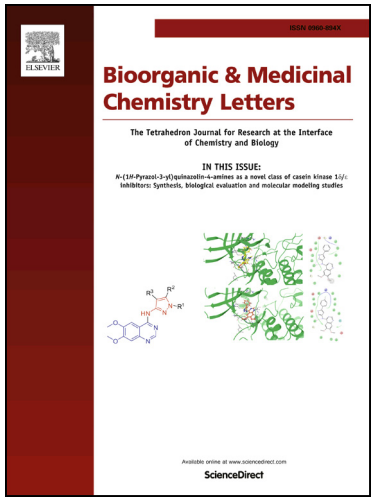

1-Acetyl-5-phenyl-1H-pyrrol-3-ylacetate: an aldose reductase inhibitor for the treatment of diabetic nephropathy

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1-Acetyl-5-phenyl-1H-pyrrol-3-ylacetate: an aldose reductase inhibitor for the treatment of diabetic nephropathy

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Abstract

Diabetic nephropathy (DN) is the most common and serious complication in diabetes mellitus, but the efficacy of available strategies for preventing this disorder remains poor. The aim of this study was to investigate the possible beneficial effects of 1-acetyl-5-phenyl-1H-pyrrol-3-ylacetate (APPA), an aldose reductase inhibitor, on DN. In the present study, a model of rat glomerular mesangial cells (HBZY-1) damaged by high glucose was used to confirm the protective effects of APPA in vitro. Then, a rat model of streptozotocin-induced diabetes was used to assess the effects of APPA in vivo. APPA increased viability and reduced apoptosis in HBZY-1 cells. In vivo, APPA improved the signs of DN as determined by measurements of blood glucose, urinary microalbumin, serum total antioxidant capacity, serum catalase activity, serum glutathione levels, and serum total superoxide dismutase activity. Hematoxylin and eosin staining of kidney tissue confirmed the protective effect. Moreover, APPA reduced the levels of transforming growth factor-β1, collagen IV, and laminin in HBZY-1cells incubated in high glucose, and in serum in DN rats. In summary, APPA can effectively prevent apoptosis and the symptoms of streptozotocin-induced diabetes by inhibiting the polyol pathway in rats. This suggests that APPA could be a potential drug in treating DN.

Keywords: 1-acetyl-5-phenyl-1H-pyrrol-3-ylacetate; diabetic nephropathy; aldose reductase inhibitor; apoptosis

Diabetic nephropathy (DN) is a major vascular complication associated with diabetes and a leading cause of end-stage renal disease.[1] Extensive research indicates that the pathogenesis of DN includes hyperglycemia, hemodynamic and metabolic factors, and oxidative stress, as well as inflammatory reactions. However, the exact mechanism is unclear.[2, 3] Researchers have found that abnormal glucose metabolism and advanced glycosylation anomalies induced by hyperglycemia maybe the most important factors contributing to the progression of DN.[4-6] Treatment of DN with agents such as angiotensin-receptor blockers, angiotensin-converting enzyme inhibitors, and antihypertensives has been tried in clinical practice.[7] Unfortunately, currently available medical interventions are unable to effectively reverse or even delay the progression of DN.[8, 9]

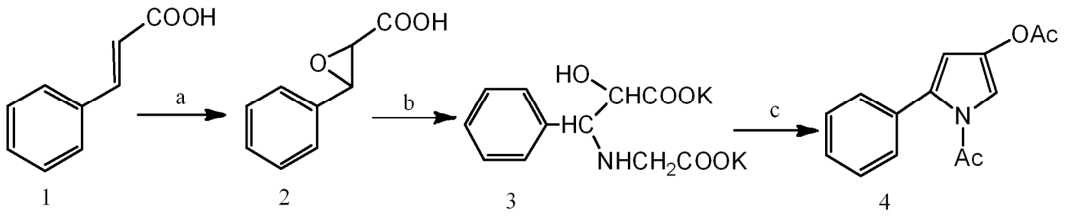
Aldose reductase (AR) is the principal enzyme of the polyol pathway that catalyzes the NADH-dependent reduction of glucose to sorbitol.[10] The polyol pathway plays an important role in the development of degenerative complications of diabetes, such as nephropathy, retinopathy, neuropathy, and cataract diseases.[11, 12] AR inhibitors (ARIs) offer the possibility of preventing or arresting the progression of these long-term diabetic complications.[13, 14] Therefore, developing pharmacological inhibitors that inhibit AR might provide a therapeutic approach for delaying and preventing diabetic complications.

1-Acetyl-5-phenyl-1H-pyrrol-3-ylacetate (APPA) (Fig. 1) is a novel and very important compound synthesized in our laboratory (the synthesis route is shown in Scheme 1). Through ananalysis of structure-activity relationships, we found that APPA may have ARI activity and might provide a therapeutic approach for delaying and preventing diabetic complications.[11] To confirm this speculation, the current study was designed to evaluate the effects of APPA on DN. In this study, rat glomerular mesangial cell (HBZY-1) damage was induced by high glucose (HG, 33 mM) to establish a model of DN. APPA significantly prevented HG-induced damage to HBZY-1 cells and improved the symptoms of streptozotocin (STZ)-induced DN by inhibiting the polyol pathway and apoptosis. These results provide evidence supporting the development of a novel DN treatment that may be useful in clinical practice.

Rat glomerular mesangial HBZY-1 cells were purchased from Tong PaiBiological (Shanghai, China). Cells were cultured in HyClone Dulbecco's modified Eagles low glucose medium (5.56mM glucose) (Thermo Fisher Scientific, Beijing, China) supplemented with 10% fetal bovine serum. Cells were maintained at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Cells were divided into six groups: normal saline (NS), HG (33 mM), APPA (0.3, 0.6, and 0.9 μM), and epalrestat (EPS, 10 μM).

APPA (4) was prepared following the synthesis pathway depicted in Scheme 1 from

commercially available trans-cinnamic acid (1). The compound 2 was successfully prepared by dissolving compound 1 in 50% volume of alkaline aqueous acetone via a mildly reaction processes with potassium monopersulfate. Compound 3 was synthesized by the condensation of compound 2 with suitable alkaline aqueous solution in the presence of glycine. Compound 3 was added acetic anhydride and pyridine for catalytic oxidation to synthesis APPA (MP 60-61□). The AR enzyme was prepared as described previously.[15] AR inhibitory activity was determined according to the method of Kim et al.[13] AR activity in the absence of inhibitor was considered as 100%. Epalrestat[16] (IC_{50}, 0.0939 μM) was used as the standard AR inhibitor.



Cell proliferation and viability were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well microplates at a density of 2×10^3 cells/well. The cells were incubated and allowed to adhere prior to exposure to test substances for 48 h. MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well at a final concentration of 0.2 mg/mL and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 4 h. The supernatant was removed and formazan crystals were dissolved in 200 μL dimethyl sulfoxide (Sigma-Aldrich) for 15 min. The absorbance was determined at 490 nm using a microplate reader (ELX-800; BioTek, Winooski, VT, USA).

A Hoechst staining kit (Beyotime Institute of Biotechnology, Haimen, China) was used to detect apoptotic cells according to the manufacturer's instructions. Cells (1×10^4) were seeded in 12-well plates, incubated at 37°C in a humidified atmosphere with 5% CO_2, and allowed to adhere. Ethanol (0.5 mL/well, 70%) was used to fix the cells for 20 min at room temperature. After washing with phosphate-buffered saline, cells were stained with 0.5 mL Hoechst staining liquid for 5 min. The cells were observed under a fluorescence microscope (BX53; Olympus, Tokyo, Japan) and photographed at 400× magnification after the addition of an anti-fluorescence quencher.

Apoptosis was also assessed by flow cytometry using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) and an apoptosis detection kit (Bioworld Technology, Nanjing,

China) according to the manufacturers' instructions. Cells were harvested, centrifuged, and washed prior to resuspension in 400 μL binding buffer. Annexin V-FITC (5 μL) was added to the cell suspensions and incubated at 28°C for 15 min in the dark. Propidium iodide (10 μL) was then added and the cell suspensions incubated at 28°C for 5 min in the dark, followed by flow cytometry analysis within 1 h.

Healthy male Wistar rats weighing 300±50g were purchased from the Laboratory Animal Center of Jilin University. Rats were maintained under specific pathogen-free conditions during the adaptation period when they had *ad libitum* access to food and water. The animal experiment protocol was approved by the Animal Ethics Committee of Jilin University. The DN model and measurements of blood glucose were performed as described previously.[3] Rats with blood glucose levels of 13.88 mM or higher, and elevated urinary microalbumin levels, were considered diabetic.

Rats were divided randomly into six groups: normal saline (NS), DN, APPA at doses of 40, 80, and 160mg/kg, and epalrestat (100 mg/kg). Rats were treated daily for eight weeks. Changes in body weight of the various groups were recorded weekly. Urine and blood were collected at the end of weeks 2, 4, 6, and 8. At these times, the rats were weighed, euthanized, and a minimal amount of blood was withdrawn from the heart for the biochemical determinations. Kidney tissues were removed and some were frozen in liquid nitrogen and stored at -70°C for future analyses. Other tissues were fixed immediately in 4% paraformaldehyde and embedded in paraffin for subsequent analyses. The paraffin-embedded kidney tissue was sliced into 5-μm thick sections, followed by conventional hematoxylin and eosin staining as described previously.[17]

Serum samples were used for measurements of total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD) and catalase activities, and glutathione (GSH) levels. Detection was carried out according to the manufacturer's protocol from each diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Enzyme-linked immunosorbent assay kits (Cloud-Clone, Wuhan, China) were used to detect the levels of serum collagen IV and laminin, blood glucose, and urinary microalbumin. The experimental procedures were conducted in strict accordance with the kit instructions. Absorbance values at 450 nm were measured using a microplate reader (ELX-800,BioTek). Curve expert 1.3 software was used to process the resulting data.

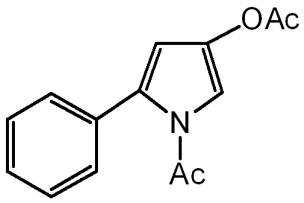
Cells were lysed by NP-40 Lysate (Beyotime Institute of Biotechnology, Haimen, China). Total protein from the serum of each group was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime). Protein concentrations were determined using the bicinchoninic acid method (Beyotime). Forty micrograms of total

protein from each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with primary antibodies against transforming growth factor (TGF)-β1 (1:1000, Bioss, Beijing, China) overnight at 4°C. Subsequently, the membrane was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:5000, Beyotime) at room temperature for 1 h, followed by detection using enhanced chemiluminescence. The film was scanned and analyzed by Gel-ProAnalyzer6.3 software to determine the optical density of the target bands. β-actin was used as the internal control.

Data are expressed as means ± standard deviation. Comparisons between groups were performed using one-way analysis of variance followed by the Bonferroni post-hoc test. GraphPad Prism 5.0 software was used to process data and produce graphs. $P < 0.05$ indicated that a difference was statistically significant.

To confirm the inhibitory activity of APPA on AR, and for optimization, a kinetic study was conducted according to the method described earlier.[10] DL-Glyceraldehyde was used as the substrate and epalrestat used as the positive control. The results with 3 different concentrations of APPA (60, 90, and 140nM) showed that the IC_{50} was 0.0223 μM (Fig.1). As the concentration of APPA increased, the slope of the line obtained in a double-reciprocal plot increased. This indicated that the inhibition of AR by APPA was uncompetitive, and APPA was one of the effective aldose reductase inhibitors.

A



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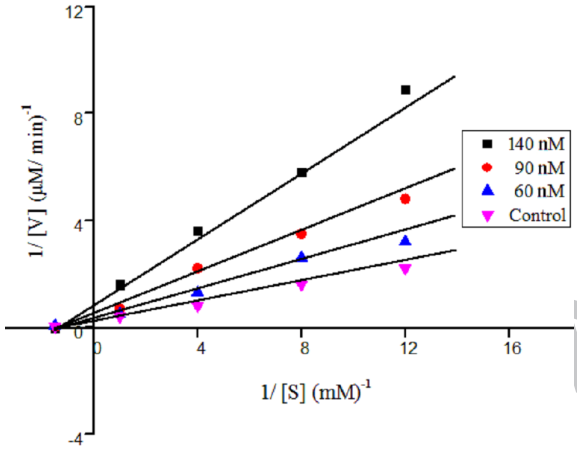
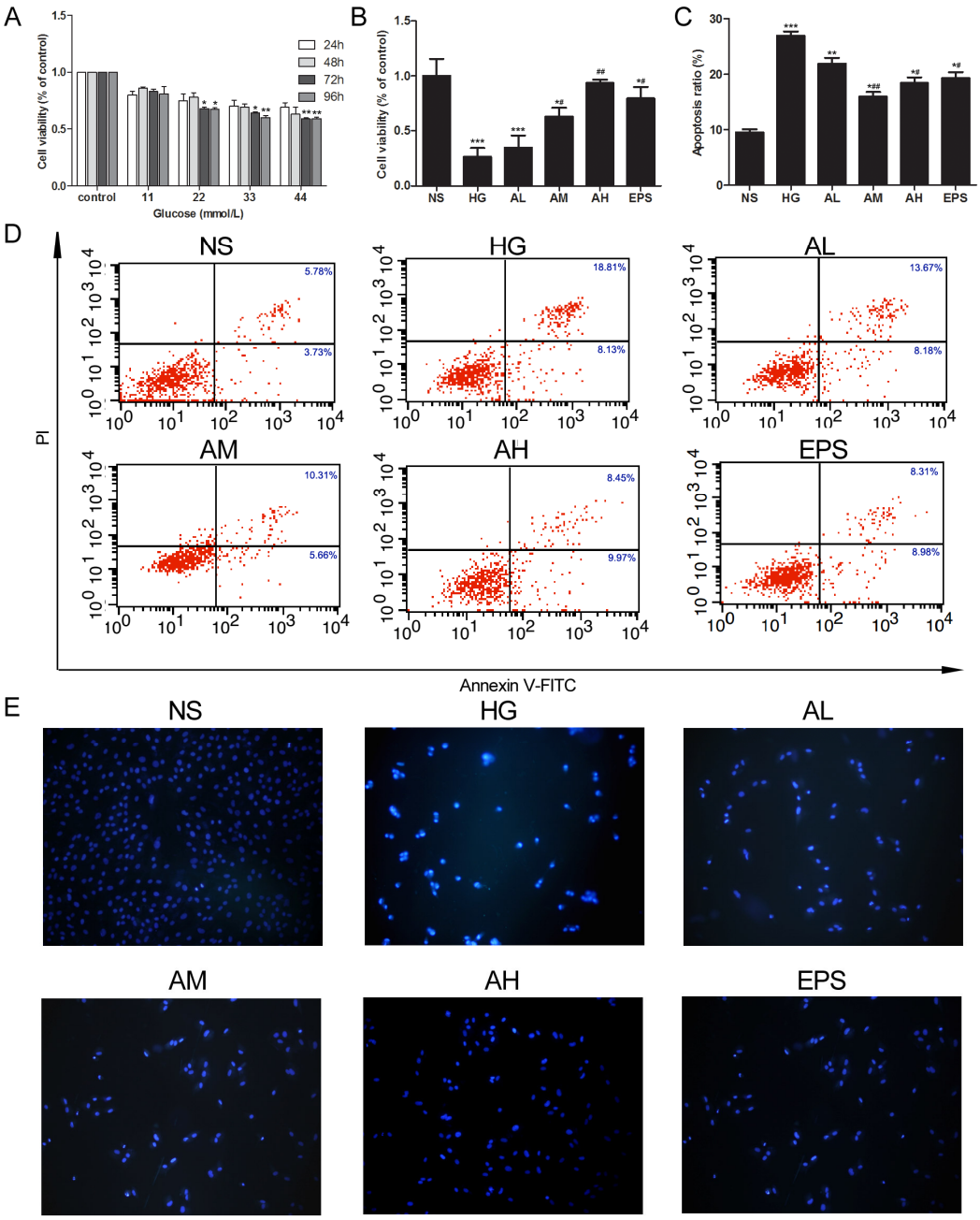


The effect of APPA in HBZY-1 cells was investigated by determining its effect on the damage induced by HG (33 mM). The results of the MTT assay demonstrated that, up to 0.9 μM, APPA alone had no obvious toxicity to the cultured cells. HG induced 73% cell death (Fig. 2). Pretreatment with APPA (0.3, 0.6, and 0.9μM) significantly prevented HG-induced cell death. The protection with 0.9 μM APPA was even better than the positive control, epalrestat.

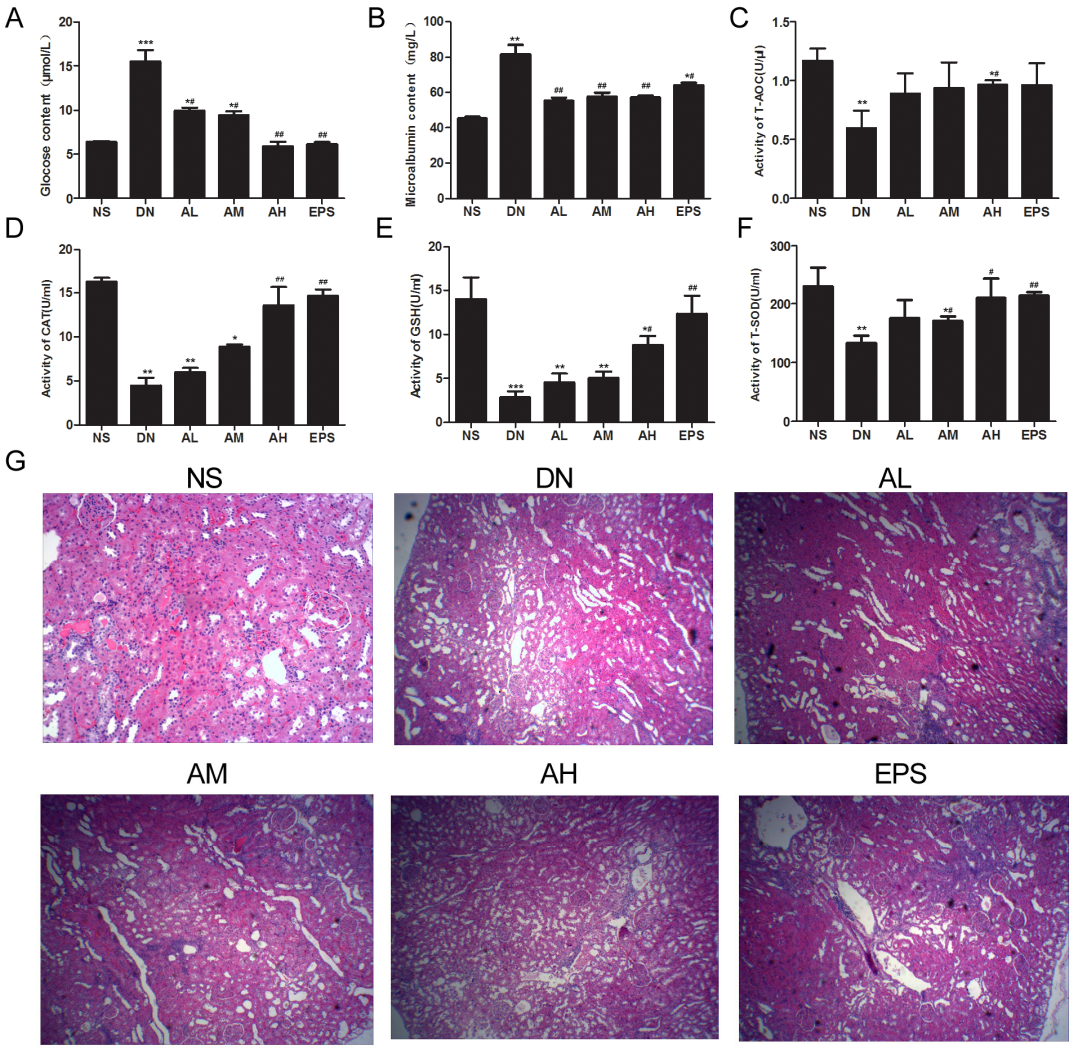
Hoechst staining and flow cytometry were used to assess apoptosis. Hoechst staining demonstrated DNA fragments and floating cells in the HG group (Fig. 2E), which were signs of cell apoptosis and necrosis. HG significantly increased apoptosis in HBZY-1 cells. The number of apoptotic cells was reduced significantly by APPA at 0.3, 0.6, and 0.9μM compared with that reported with HG alone. Apoptosis increased 2.83-fold with HG, and 2.29-, 2.07-, and 1.93-fold with APPA 0.3, 0.6, and 0.9 μM, respectively. Apoptosis increased 1.81-fold in cells exposed to HG and treated with 0.9 μM epalrestat compared with that in cells in the NS group (Fig.2C, D). These results indicated that APPA effectively inhibited apoptosis in HBZY-1 cells cultured in HG medium.



The blood glucose and urinary microalbumin levels in rats were determined to investigate the effects of APPA on STZ-induced DN. STZ increased blood glucose 207% and urinary microalbumin 184% (Fig. 3A, $P < 0.001$). Treatment of these DN rats with APPA significantly reduced these changes.

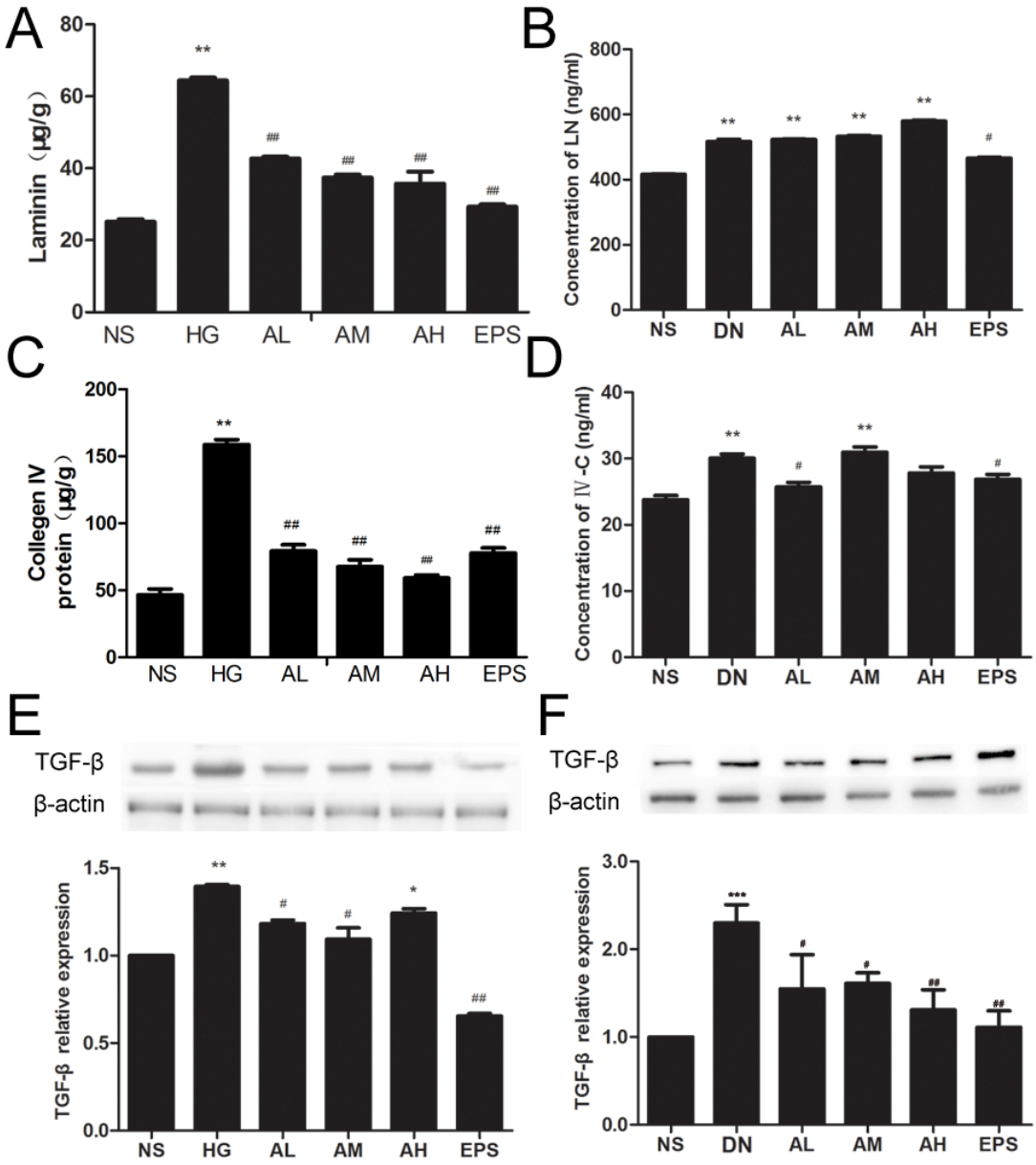
T-AOC, catalase, and T-SOD activities, and GSH levels in serum of DN rats decreased compared with that in the NS group. Treatment of these DN rats with APPA significantly increased the activity of these factors (Fig. 3). Hematoxylin and eosin staining showed that APPA effectively protected kidney tissue from DN. In summary, APPA attenuated

HG-induced DN in vivo.



To elucidate whether APPA improved the signs of DN via the TGF-β signaling pathway, levels of collagen IV and laminin in HBZY-1 cells, and rat serum in different treatment groups, were determined(Fig. 4). Compared with that in the NS group, collagen IV and laminin levels in the HG and DN groups increased significantly. APPA could efficiently prevent this change in HBZY-1cells and rat serum. Levels of the active form of TGF-β in HBZY-1 cells increased significantly in the HG compared with that in the NS group, while the levels of TGF-β in the APPA and epalrestat groups decreased compared with that in the HG group. Similar results were observed in serum from DN rats with and without APPA treatment. These results suggest that APPA decreased collagen IV and laminin levels, and inhibited activation of the TGF-β signaling pathway in HBZY-1 cells and DN rats. Overall, APPA inhibited apoptosis in HBZY-1cells cultured in HG medium and improved the

symptoms in DN rats through effects on the TGF-β signaling pathway.



Previous studies showed that ARIs were potentially useful in preventing the progression of DN.[18-20] However, changes in glycemic control, and many other confounding factors, complicate determining the expected effects of ARIs on DN. In the current study, HBZY-1 cells and Wistar rats were used to investigate the effects of APPA, an ARI, on DN, as well as the therapeutic potential of APPA in treating DN. The results showed that APPA effectively inhibited cellular apoptosis in vitro and attenuated DN-like symptoms in vivo. These findings suggest the potential for APPA to be used as a therapeutic agent for DN.

An imbalance between cell proliferation and death is considered to be an important

event in many diseases. Apoptosis is a well-orchestrated mechanism for cell death.[21] In the current study, we found that APPA could effectively prevent HG-induced apoptosis of HBZY-1 cells, and the effect was better than an existing drug, epalrestat.

Blood glucose and urinary microalbumin contents are important factors to assess the extent of DN in vivo. An increase in polyol pathway activity induced by hyperglycemia has been reported to contribute to abnormalities such as increased osmotic pressure and oxidative stress, factors that have been cited as promoters of diabetic microvascular diseases including DN.[3] The activities of SOD and catalase, and GSH and AOC levels decrease in diabetes. [22, 23]Treatment of rats with APPA at different doses significantly reduced glucose and microalbumin levels, and conversely increased AOC, GSH, SOD, and catalase activities as compared with that in the DN group.

Early DN presents with increasing glomerular volume and cell numbers.[24] These changes are visible with hematoxylin and eosin staining. In the present study, APPA could reverse these changes in the DN kidney.

Several studies have shown that TGF-β plays a critical role in many diseases including DN.[25, 26] Focal segmental glomerulosclerosis depends on a wide range of biologic functions such as regulation of the cell cycle, apoptosis, differentiation, and immunologic responses.[27] In particular, TGF-β is a key mediator of DN that increases the levels of extracellular matrix proteins such as collagen IV and laminin.[28] In addition, the levels of TGF-β increase in DN kidneys suggesting that the TGF-β signaling pathway is involved in the progression of DN. In the present study, increases of TGF-β by HG in kidney glomerular cells in vitro were considered to beclosely associated with the pathogenesis of DN. Furthermore, the levels of TGF-β significantly increased in DN rats. Importantly, the levels of collagen IV and laminin were also increased. APPA could ameliorate these changes and improve the symptoms of DN in rats. These results are similar to prior work showing that over production of extracellular matrixproteins, such as collagen IV and laminin, is prevented by ARIs.[29, 30]

In summary, APPA could inhibit apoptosis in rat glomerular mesangial cells in vitro. In addition, APPA improved the pathological symptoms of STZ-induced DN in rats by affecting antioxidant activities and reducing the levels of TGF-β, collagen IV, and laminin. These results demonstrate a positive role of APPA in the pathogenesis of DN and suggest that it might be a promising potential drug for treating DN.

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Scheme Legends

Scheme 1: Reagents and conditions: a: $KHSO_5/H_2O$, $NaHCO_3$; b: KOH/H_2O, Glycine, pH 11.5; c: Ac_2O/Pyridine, 120□.

Figure Legends

Figure 1. Inhibitory effects of 1-acetyl-5-phenyl-1H-pyrrol-3-yl acetate (APPA) on aldose reductase (AR). (A) Chemical structure of APPA. (B) Lineweaver-Burk plots showing the reciprocal of the velocity (1/V) of AR versus the reciprocal of the substrate concentration (1/S). DL-Glyceraldehyde was the substrate at concentrations of 0.083, 0.125, 0.25, and 1.0 mM.

Figure 2. Effects of 1-acetyl-5-phenyl-1H-pyrrol-3-yl acetate (APPA) on high glucose-induceddamage to HBZY-1 cells. (A, B) Cell viability by the MTT assay. (C, D) Cultured cells were double-stained with a FITC-conjugated anti-Annexin V antibody and propidium iodide, and subjected to a flow cytometric analysis of apoptotic cells. (E) Hoechst staining was used to detect apoptosis of HBZY-1 cells at different concentrations of APPA. Representative images are shown. Data are expressed as means ± standard deviation (n=3). *P < 0.05, **P < 0.01, and ***P < 0.001compared with the normal saline (NS) group. $^{\#}P < 0.05$ and$^{\#\#}P <0.01$compared with the HG group.

Figure 3. 1-Acetyl-5-phenyl-1H-pyrrol-3-yl acetate (APPA) attenuates streptozotocin-induced diabetic nephropathy (DN) in rats.(A) Blood glucose levels, (B) urinary microalbumin levels, (C) serumtotal antioxidant capacity (T-AOC), (D) serum catalase (CAT) activity, (E) serum glutathione (GSH) levels, and (F) serum total superoxide dismutase (T-SOD) activity are shown. (G) Hematoxylin and eosin staining shows the effects of different concentrations of APPA on glomerular mesangial hyperplasia. Data are expressed as means ± standard deviation (n = 8). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the normal saline (NS) group. $^{\#}P < 0.05$ and$^{\#\#}P < 0.01$compared with the DN group.

Figure 4. 1-Acetyl-5-phenyl-1H-pyrrol-3-yl acetate (APPA) inhibits the transforming growth factor (TGF)-β signaling pathway. The effect of different concentrations of APPA on levels of (A) laminin in HBZY-1cells, (B) laminin in rat serum, (C) collagen IV in HBZY-1 cells, and (D) collagen IV in rat serum. Western blotting was used to detect the effects of different concentrations of APPA on the expression of TGF-β in (E) HBZY-1 cells and (F) rat serum. Gray scaledensitometric analyses were conducted using β-actin as an internal reference. Representative results from repeated experiments are shown. Data are expressed as means ±

standard deviation (n = 6).*P < 0.05, **P < 0.01, and ***P < 0.001compared with the normal saline (NS) group. $^{\#}$P < 0.05 and$^{\#\#}$P < 0.01compared with the high glucose (HG) or diabetic nephropathy (DN) groups.