Protective role of the curcumin derivative, FM0807, in regulating JAK2/STAT3 and TGF-β1/SMAD2/3 signaling pathways in glomerular mesangial cells and renal function in db/db mice

Graphical abstract

Highlights

- FM0807 significantly improved renal function in db/db mice and inhibited the abnormal proliferation in high glucose-induced HBZY-1 cells.

- FM0807 effectively prevented renal injury and attenuated JAK2/STAT3 signaling pathway.

- FM0807 effectively prevented the continuous progress of renal fibrosis via TGF-β1/Smad2/3 signaling pathway.

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In brief

This study identified a key role for FM0807, a curcumin derivative, in renal injury and renal fibrosis based on the results of in vivo, in vitro, and pharmacological experiments.
Protective role of the curcumin derivative, FM0807, in regulating JAK2/STAT3 and TGF-β1/SMAD2/3 signaling pathways in glomerular mesangial cells and renal function in db/db mice

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ABSTRACT

To determine the protective effects of FM0807 against diabetes-induced renal inflammation and fibrosis and the underlying mechanisms \textit{in vivo} and \textit{in vitro}. FM0807 was administered to db/db mice. Glomerular mesangial cells (HBZY-1) were cultured under high glucose conditions with or without FM0807. Gene and protein expression was assessed by quantitative real-time PCR, western blotting, and immunofluorescence. Mitochondrial reactive oxygen species were detected with MitoSOX Red. FM0807 markedly reduced blood glucose, glycosylated hemoglobin, triglycerides, and low-density lipoprotein-cholesterol levels and improved the liver organ index, the high-density lipoprotein-cholesterol level, and renal function, as evidenced by decreased 24-h urinary protein excretion and the creatinine and blood urea nitrogen levels. FM0807 ameliorated pathologic renal changes in diabetic mice (reduced glomerulosclerosis, diminished interstitial cellular inflammation, and less tubular luminal narrowing). Treatment with FM0807 also led to a significant reduction in the expression of inflammatory markers, including JAK2, STAT3, TNF-α, IL-1β, IL-6, TGF-β1, and Smad2/3, in addition to alterations in the expression of proteins associated with kidney injury. These data suggest that FM0807 alleviates diabetes-induced renal inflammation and fibrosis by modulating the JAK2/STAT3 and TGF-β1/SMAD2/3 signaling pathways.

Keywords: diabetic kidney disease, FM0807, inflammation, renal function, renal fibrosis, type 2 diabetes mellitus murine model

1. INTRODUCTION

Diabetic kidney disease (DKD) is a principal cause of end-stage kidney disease (ESKD) worldwide \cite{1-3}. Epidemiologic data indicate that approximately 40% of individuals with diabetes will develop DKD \cite{4,5}. DKD is a pathologic spectrum from simple oxidative stress to irreversible fibrosis with varying degrees of inflammation and oxidative stress damage \cite{6}. However, the pathogenesis of early DKD is complex. Although pharmacologic treatments effectively control blood glucose levels, pharmacologic treatments often do not prevent the long-term multi-organ complications associated with diabetes \cite{3,7}. Angiotensin-converting enzyme (ACE) inhibitors (e.g., benazepril) are well-established in the treatment of arterial hypertension, heart failure, and diabetic nephropathy with albuminuria. The most common adverse effects of ACE inhibitors include
hypotension, cough, hyperkalemia, and renal failure [8]. Currently there are several therapeutic options for managing DKD, including mineralocorticoid receptor antagonists (MRAs), glucagon-like peptide-1 receptor agonists (GLP-1RAs), dipeptidyl peptidase-4 (DPP4) inhibitors, and sodium-glucose cotransporter-2 (SGLT2) inhibitors [9, 10]. However, these medications are associated with adverse effects. For example, there are concerns regarding MRA-associated adverse effects, such as renal function impairment, hyperkalemia, and hypotension [11]. Studies have found that GLP-1RAs increase the risk of gastrointestinal adverse events (biliary disease, pancreatitis, bowel obstruction, and gastroparesis) in patients with diabetes [12]. DPP-4is were shown to be associated with an increased risk of retinopathy in clinical trials and a meta-analysis [13]. SGLT2 inhibitors have been linked to an increased risk of genital fungal infections, diabetic ketoacidosis, and lower limb amputations [14, 15]. Given these challenges, it is crucial to enhance our understanding of the pathogenesis underlying DKD and the development of new therapeutic interventions is warranted.

Curcumin, a diketone compound extracted from the rhizomes of the Zingiberaceae family, exhibits a range of pharmacologic properties, including anti-inflammatory, antioxidant, anti-fibrotic, and anti-cancer activities. Curcumin also has a role in lipid regulation, has low toxicity, and causes minimal adverse reactions [16, 17]. Despite the significant medicinal potential of curcumin, the clinical application of curcumin is hindered by low solubility, poor stability, and a limited absorption rate, which restricts the use of curcumin in food and pharmaceutical products [18]. To address these challenges, we synthesized a derivative of curcumin (FM0807) by conjugating curcumin with an aspirin ester. Previous studies by our group have demonstrated that FM0807 has potent anti-inflammatory properties but the protective effects against diabetic kidney injury have not been validated [19, 20]. To assess the efficacy of FM0807 in treating DKD, the db/db mouse model was selected. The db/db mouse model is characterized by spontaneous diabetes, making the db/db mouse model an ideal candidate for studying DKD complications. Mice were randomly divided into groups and administered different doses of FM0807. The therapeutic effects of the studied drug were evaluated by monitoring blood glucose and glycated hemoglobin (HbA1c) levels, and renal function indicators, including 24-h urinary protein excretion, and serum creatinine (CRE) and blood urea nitrogen (BUN) levels.

The Janus kinase/signal transducer and activator of the transcription (JAK/STAT) signaling pathway, which is unbalanced in DKD, functions through a range of metabolism-related cytokines, including interleukin 6 (IL-6) and transforming growth factor beta (TGF-β) [21]. Studies have shown that JAK2 expression is significantly increased in the glomeruli of patients with early DKD and JAK2 expression is significantly upregulated in the tubulointerstitial region of patients with progressive DKD, which corresponds to the natural progression of pathologic changes in glomerular damage [22]. Increased expression of JAK2 activates STAT3 and leads to STAT3 phosphorylation. Phosphorylated STAT3 forms dimers, which are then transferred to the nucleus, where the dimers directly bind to the DNA sequence and regulate target gene expression [23]. Furthermore, increased expression of cytokines, such as IL-6 and tumor necrosis factor alpha (TNF-α), has been reported in DKD. Critical pathways, like the JAK/STAT pathway, is essential for regulation of cytokine production in humans and mouse models of DKD [24-28]. Our prior study suggested that an anti-IL-6R fusion protein mitigated diabetic kidney injury by targeting the IL-6R/JAK2/STAT3 signaling cascade [29]. This activation facilitates the expression of the anti-apoptotic protein, Bcl-2, and suppresses the pro-apoptotic protein, Bax, which together lead to excessive proliferation and pathologic growth of glomerular mesangial cells and exacerbation of renal injury [30]. Additionally, JAK2 inhibitors have shown efficacy in reducing levels of TGF-β1 and fibronectin (FN), thus diminishing inflammation and fibrosis in the kidneys through inhibition of the JAK2/STAT3 pathway [2, 31, 32].

Kidney tissues progressively undergo transformation into renal fibrosis in patients with DKD, a process predominantly driven by the accumulation of extracellular matrix (ECM) components [33]. An imbalance between the synthesis and degradation of ECM elements accelerates the progression of fibrosis in DKD [34, 35]. Previous studies have shown that TGF-β1 is upregulated in the glomeruli and interstitium of fibrotic kidneys, which in turn further promotes the development of fibrosis [36]. TGF-β1, which contributes to fibrosis, has a role in every stage of kidney fibrosis. Because the classical TGF-β1 pathway mediates fibrosis, the TGF-β1/SMAD2/3 pathway has an important role in renal fibrosis [37]. Therefore, targeting these signaling pathways is vital for treating renal fibrosis with a focus on how FM0807 modulates the JAK2/STAT3 and TGF-β1/SMAD2/3 signaling pathways and the impact on inflammatory responses and renal pathologic processes. This study aimed to deepen our understanding of the molecular mechanisms underlying DKD and establish a foundation for developing novel therapeutic strategies.

2. MATERIAL AND METHODS

2.1 Animals

Thirty 8-week-old male C57BL/6N mice procured from the Beijing Vitonglihua Experimental Animal Technology Co., Ltd. (Beijing, China) were used in the current study. The cohort included 5 healthy db/m mice (control group, average weight approximately 25 ± 5 g) and 25 db/db mice (diabetic model, average weight approximately 40 ± 5 g). Mice were individually housed in an SPF-grade animal laboratory, maintained at 50%–60% humidity
and a temperature of 25–27°C, under a 12-h light/dark cycle. Animals had access to water and feed ad libitum with daily bedding changes. Following a week of acclimatization, which included a health assessment, the experiments commenced. The db/m mice were designated as the control group (n = 5). The db/db mice were randomly distributed into 5 groups (n = 5 per group): 1) model group; 2) benazepril group (YT71576, 20 mg·kg⁻¹; Beijing Yitai Biotechnology Co., Ltd., Beijing, China); 3) FM0807 (50 mg·kg⁻¹); 4) FM0807 (100 mg·kg⁻¹); and 5) FM0807 (200 mg·kg⁻¹). FM0807 was synthesized and purified in our laboratory, identified by HPLC and thin-layer chromatography with a purity >95%. ¹H NMR and ¹³C NMR data are provided in Figures S1 and S2. The benazepril and FM0807 groups were administered orally every other day for 8 weeks. The control and model groups received an equal volume of saline. At the conclusion of the experiments, the mice were euthanized via intraperitoneal injection of 2% sodium pentobarbital, after which venous blood was collected and centrifuged (2500 × g for 20 min), and the kidneys were excised and weighed, and the organ indices were calculated.

The experimental protocol and use of animals were approved by the Animal Ethics Committee of Fujian Medical University (approval number: FJMU IACUC 2019-0067). All animals were managed and supervised by the Experimental Animal Center of Fujian Medical University (license number SYXX [Fujian] 2016-0006). Throughout the study, all efforts were made to minimize animal pain and discomfort while respecting welfare rights. This research aimed to advance the treatment of DKD and ultimately improve the quality of life for patients.

### 2.2 Biochemical marker assessment

Mice were weighed biweekly and the fasting blood glucose levels were measured after a 12-h fast using an Accu-Chek Performa blood glucose meter (Roche Diagnostic, Shanghai Yuyan Scientific Instrument Co., Ltd., Shanghai, China). Hba1c levels were determined using a biochemical assay kit (XB1650; Seebio Biotech (Shanghai) Co., Ltd., Shanghai, China). The biochemical assay kit was also used to measure triglycerides (TG), 24-h urinary protein excretion, high- and low-density lipoprotein-cholesterol (HDL-C and LDL-C, respectively) levels, and the serum CRE and BU concentrations (to provide a comprehensive assessment of renal function).

### 2.3 Histopathologic examination

Renal tissues were subjected to hematoxylin and eosin (HE), Masson’s trichrome, periodic acid-Schiff (PAS), and periodic acid–silver methenamine (PASM) staining to visualize glomerular morphology and collagen deposition. Tissue morphology was assessed under an optical microscope at 400x magnification. The mesangial matrix index was quantitatively evaluated in PAS-stained tissues using Image-Pro Plus software (version 6.0; Media Cybernetics, Silver Spring, MD, USA) using methodologies described in prior research [38]. Glomerulosclerosis was quantified using Masson’s trichrome staining by randomly selecting 20 glomeruli per slide and calculating the percentage of the positively stained area relative to the total glomerular area.

### 2.4 Real-time quantitative polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from kidney samples using an RNA extraction kit (12259E508; Shanghai Yisheng Biotechnology Co., Ltd., Shanghai, China) and subsequently reverse-transcribed to cDNA. RT-PCRs were performed on a Roche LightCycler 96 system utilizing SYBR Green Master Mix (Q111; Nanjing Novozan Biotechnology Co., Ltd., Nanjing, China). Relative mRNA expression was quantified using the 2−ΔΔCt method with GAPDH serving as the internal reference. Primers for the mouse experiments are detailed in Table 1 and primers for the HBZY-1 cells are presented in Table 2.

### 2.5 Western blot analysis

Mouse renal tissues and HBZY-1 cells were lysed in RIPA buffer, denatured, and separated on PVDF membranes. The membranes were blocked with 5% non-fat milk for 2 h and incubated overnight at 4°C with primary antibodies, including antibodies against JAK2 (3230, Cell Signaling Technology, Danvers, MA, USA), phosphorylated JAK2 (3230, Cell Signaling Technology, Danvers, MA, USA), phosphorylated JAK2 (3771, Cell Signaling Technology, Danvers, MA, USA), STAT3 (12640, Cell Signaling Technology, Danvers, MA, USA), STAT3 (12640, Cell Signaling Technology, Danvers, MA, USA), and GAPDH (mouse) (5174S, Cell Signaling Technology, Danvers, MA, USA). The membranes were blocked with 5% non-fat milk for 2 h and incubated overnight at 4°C with primary antibodies, including antibodies against JAK2 (3230, Cell Signaling Technology, Danvers, MA, USA), phosphorylated JAK2 (3771, Cell Signaling Technology, Danvers, MA, USA), STAT3 (12640, Cell Signaling Technology, Danvers, MA, USA), and GAPDH (mouse) (5174S, Cell Signaling Technology, Danvers, MA, USA). The membranes were blocked with 5% non-fat milk for 2 h and incubated overnight at 4°C with primary antibodies, including antibodies against JAK2 (3230, Cell Signaling Technology, Danvers, MA, USA), phosphorylated JAK2 (3771, Cell Signaling Technology, Danvers, MA, USA), STAT3 (12640, Cell Signaling Technology, Danvers, MA, USA), and GAPDH (mouse) (5174S, Cell Signaling Technology, Danvers, MA, USA). The membranes were blocked with 5% non-fat milk for 2 h and incubated overnight at 4°C with primary antibodies, including antibodies against JAK2 (3230, Cell Signaling Technology, Danvers, MA, USA), phosphorylated JAK2 (3771, Cell Signaling Technology, Danvers, MA, USA), STAT3 (12640, Cell Signaling Technology, Danvers, MA, USA), and GAPDH (mouse) (5174S, Cell Signaling Technology, Danvers, MA, USA).

### Table 1 | RT-qPCR primer sequences (mouse).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK2 (mouse)</td>
<td>Forward: 5′-TCAATCTGAGGAGGAAGCAA-3′&lt;br&gt;Reverse: 5′-ATCATTGCCATAAATTCAGGG-3′</td>
</tr>
<tr>
<td>STAT3 (mouse)</td>
<td>Forward: 5′-GGCTAATCTGTGCAAGAAAACA-3′&lt;br&gt;Reverse: 5′-TCTTGCAGGAACCCATGAT-3′</td>
</tr>
<tr>
<td>TNF-α (mouse)</td>
<td>Forward: 5′-GGCTGCTGTAAGGAGAACAG-3′&lt;br&gt;Reverse: 5′-CAGCGGTGTCAGTCTGCT-3′</td>
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<tr>
<td>IL-1β (mouse)</td>
<td>Forward: 5′-TGGAGCTTCCAGGATGAGAACA-3′&lt;br&gt;Reverse: 5′-GTTCATCTGCAGGAGGCTG-3′</td>
</tr>
<tr>
<td>CTGF (mouse)</td>
<td>Forward: 5′-CCAGATTACCAATGAACTACCTT-3′&lt;br&gt;Reverse: 5′-CGCAGAATTTGCCAGCTGAT-3′</td>
</tr>
<tr>
<td>TGF-β1 (mouse)</td>
<td>Forward: 5′-TGACAGCCTGGTGCTGTTCT-3′&lt;br&gt;Reverse: 5′-CACCAAGAAGCAGTGCGCTA-3′</td>
</tr>
<tr>
<td>GAPDH (mouse)</td>
<td>Forward: 5′-TGGAAAAGCTGGAGCAGT-3′&lt;br&gt;Reverse: 5′-TACTTGCAAGTTCCTCCAGG-3′</td>
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Table 2 | RT-qPCR primer sequences (rat).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
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<td>JAK2 (rat)</td>
<td>Forward: 5′-AGTCCCATATTCTGATGCTCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GCCGATCATCGCATAAAATTC-3′</td>
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<tr>
<td>STAT3 (rat)</td>
<td>Forward: 5′-GCTGACCAATACCCCAAGAAC-3′</td>
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<tr>
<td></td>
<td>Reverse: 5′-ATGTGATCTGACACCCTGAGTAG-3′</td>
</tr>
<tr>
<td>IL-6 (rat)</td>
<td>Forward: 5′-CTTACAAGCTTCCGAGAGGAGG-3′</td>
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<tr>
<td></td>
<td>Reverse: 5′-TGCCATGCAACTCTTTTTC-3′</td>
</tr>
<tr>
<td>TGF-β1 (rat)</td>
<td>Forward: 5′-GCTGAACCAAGAGACGGAAA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CCTGACGTTGGAGACTGAT-3′</td>
</tr>
</tbody>
</table>

MA, USA), phosphorylated STAT3 (9134, Cell Signaling Technology, Danvers, MA, USA), Bcl-2 (ab182858, Abcam, Cambridge, UK), Bax (ab32503, Abcam, Cambridge, UK), Caspase-3 (ab184787, Abcam, Cambridge, UK), cleaved Caspase-3 (9664, Cell Signaling Technology, Danvers, MA, USA), phosphorylated Smad2/3 (88285, Cell Signaling Technology, Danvers, MA, USA), Smad2/3 (86855, Cell Signaling Technology, Danvers, MA, USA), β-Tubulin (21465, Cell Signaling Technology, Danvers, MA, USA), and β-Actin (4967, Cell Signaling Technology, Danvers, MA, USA). Signal detection was performed using an enhanced chemiluminescence system (ChemiScope series600, Qinxiang Scientific Instrument Co., Ltd., Shanghai, China).

2.6 Cell culture experiments
The rat glomerular mesangial cell line, HBZY-1 (HT-X1809; Shenzhen Haodi Huatao Biotechnology Co., Ltd., Guangdong, China), was cultured in DMEM supplemented with 5.5 mM glucose (C0162-811; Beijing Wobisen Technology Co., Ltd., Beijing, China). Cells were maintained at 37°C in a 5% CO₂ atmosphere until the cells reached 80% confluence and were then passaged using trypsin. For experimental assays, cells were plated in 96-well plates at a density of 1 × 10⁵ cells/mL and cultured for 48 h under the following conditions: control group (NG, 5.5 mM D-glucose); model group (HG, 25 mM D-glucose); AG490 group (25 mM D-glucose + 50 μM AG490); and FM0807 groups with 25, 50, and 100 μM concentrations (25 mM D-glucose + respective concentrations of FM0807). Cell proliferation was assessed using the MTT assay.

2.7 Cell apoptosis and oxidative stress assessment
Apoptosis was detected using an API/Hoechst kit (SY1206; Beijing Yitai Biotechnology Co., Ltd., Beijing, China) and analyzed with a high-content imaging system. An ROS assay kit (460913-100T; Shanghai Byun Tian Biotechnology Co., Ltd., Shanghai, China) and Mitotracker Red CMXROS kit (C1035; Shanghai Yaji Biotechnology Co., Ltd., Shanghai, China) were used to evaluate intracellular oxidative levels and mitochondrial membrane potential. Fluorescence microscopy was used to capture fluorescence images. Flow cytometry was performed using the Annexin FITC/PI kit (FY-PLS5835; Shanghai Fuyu Biotechnology Co., Ltd., Shanghai, China) to determine the cell apoptosis rate.

2.8 Statistical analysis
Data were analyzed using GraphPad Prism software (version 8.0; GraphPad Software, Inc., San Diego, CA, USA) and expressed as the mean ± standard deviation (SD). Initially, data were subjected to tests for normality and homogeneity of variances to confirm distribution characteristics. For data conforming to a normal distribution and homogeneity of variances, analysis of variance (ANOVA) was used to compare differences between groups. Bonferroni’s post hoc test was used for detailed intergroup analysis for multiple group comparisons. Repeated measures ANOVA was applied to the data on repeated measures, such as body weight and fasting blood glucose. A P < 0.05 was considered statistically significant in all statistical tests.

3. RESULTS
3.1 Significant reduction in blood glucose and Hba1c levels, and improvement in diabetes-related biochemical markers by FM0807 in a diabetic mouse model
The structure of FM0807 is shown in Figure 1A. In evaluating the efficacy of FM0807 or benazepril in diabetic mice, blood glucose and Hba1c levels served as primary indicators. The db/db mouse model had significantly elevated blood glucose levels relative to the control group (Figure 1B). By the 8th week of treatment with a high dose of FM0807, there was a marked reduction in the blood glucose levels compared to the model group, showcasing the robust glucose-lowering capabilities of FM0807. Additionally, the levels of Hba1c, TG, and LDL-C in the model group were significantly elevated compared to the control group (Figure 1C, E, F), and these levels decreased after FM0807 treatment. Conversely, the liver organ index and the level of HDL-C in the model group were decreased compared to the control group (Figure 1D, G). FM0807 treatment increased the liver organ index and HDL-C level. These findings indicated that FM0807 effectively reduces the blood glucose concentration and improves hepatic metabolism.

3.2 Dose-dependent Improvement in renal function indicators by FM0807 in diabetic mice
The impact of FM0807 on renal function in diabetic mice was another focus of this study. Serum indicators related to renal function and blood lipids were measured using commercial kits. Compared to the control group, mice in the model group exhibited significantly increased 24-h
urinary protein excretion, and CRE and BUN levels (Figure 2A-C). These indicators showed significant decreases following FM0807 or benazepril treatment. These results demonstrated the effectiveness of FM0807 in a dose-dependent manner mitigating renal injury associated with a high blood glucose level and underscore the protective role of FM0807 in maintaining renal function.

3.3 Significant improvement in renal pathologic features and inhibition of renal inflammation by FM0807 in diabetic mice
A key focus of this study was to investigate the mechanism underlying FM0807 and the effect of FM0807 on renal inflammatory factor expression. Renal tissues from mice were analyzed using HE, Masson’s trichrome, PAS,
and PASM staining. The control group showed no significant signs of interstitial inflammatory cell infiltration, interstitial fibrosis, or collagen fiber deposition in the glomeruli and tubules. There was also minimal glycogen accumulation and no thickening of the basement membrane. In contrast, the model group displayed typical DKD features, such as glomerulosclerosis, interstitial cell inflammation, and tubular luminal narrowing (Figure 3). These pathologic changes were significantly alleviated following treatment with FM0807 or benazepril. This improvement indicates the substantial role of FM0807 in reducing renal inflammation in diabetic mice, highlighting a potential anti-inflammatory mechanism.

3.4 FM0807 attenuates renal injury in diabetic mice by modulating the JAK2/STAT3 and TGF-β1/Smad2/3 signaling pathways and reducing key inflammatory and apoptotic protein expression

To elucidate the therapeutic effects of FM0807, the impact on renal inflammation markers and kidney injury-related expression was investigated. The pathogenesis of DKD is multifaceted, often involving activation of the JAK2/STAT3 and TGF-β1/Smad2/3 signaling pathways by high blood glucose levels. The diabetic model showed significant activation of the JAK2/STAT3 pathway with elevated levels of inflammatory factor mRNA (JAK2, STAT3, TNF-α, and IL-1β), growth factors (connective tissue growth factor [CTGF], which is upregulated during renal fibrosis), and altered levels of JAK2, STAT3, Bcl-2, and Bax protein expression, thus increasing the p-JAK2:JAK2, p-STAT3:STAT3, and Bcl-2:Bax ratios. However, FM0807 or benazepril treatment decreased the expression of JAK2 and STAT3 (Figure 4A-D, G-I) with a corresponding decrease in the levels of inflammatory factor mRNA. FM0807 adjusted the Bcl-2:Bax ratio and restored expression of the pro-apoptotic protein, Bax, indicating the potential of FM0807 to mitigate renal injury by modulating cell apoptosis. These findings collectively demonstrated that FM0807 protects against kidney injury in diabetic mice by inhibiting the JAK2/STAT3 pathway and regulating apoptosis markers. To further confirm the effects of FM0807 on renal fibrosis and the TGF-β1/Smad2/3 pathway, PCR and western blot analysis were performed. Subsequently, the expression of collagen I and a-SMA was determined by western blot analysis. The expression of TGF-β1, and Smad2/3 and its phosphorylated form (Figure 4F, J) were inhibited, suggesting a potential inhibitory effect on renal fibrosis and providing substantial experimental evidence for FM0807 as a promising therapeutic agent for DKD.

3.5 FM0807 inhibits high glucose-induced proliferation of HBZY-1 cells and modulates mitochondrial membrane potential alleviates pathologic changes in DKD

The impact of FM0807 on renal cell proliferation was then assessed. Using an in vitro model, glomerular mesangial cells (HBZY-1 cells) were exposed to 25 mM D-glucose,
which significantly induced proliferation within 48 h, as demonstrated by MTT assay results (Figure 5A). This glucose concentration and induction period were thus utilized as standard conditions for the experiments. The IC₅₀ of FM0807 for high glucose-induced HBZY-1 cells was established at 38.76 μM (Figure 5B,C). Subsequent experiments used 25 μM, 50 μM, and 100 μM FM0807 concentrations over 48 h and showed no cytotoxic effects on HBZY-1 cells (Figure 5D). FM0807 effectively curtailed the excessive proliferation induced by high glucose, mitigating the associated pathologic changes in the kidneys.

Additionally, MitoTracker Red CMXROS, a red fluorescent dye that accumulates in living cell mitochondria in proportion to the membrane potential, was used to assess mitochondrial dynamics. Compared to the control group, cells in the high glucose model group displayed a significant increase in mitochondrial membrane potential (Figure 7C). With increasing concentrations of FM0807, the mitochondrial membrane potential decreased, indicating the ability of the compound to inhibit the pathologic proliferation of HBZY-1 cells under high glucose conditions. This result underscores the potential of FM0807 in suppressing the pathologic proliferation of renal cells driven by high blood glucose, potentially ameliorating renal pathologic changes in DKD.

3.6 FM0807 reverses pathologic proliferation and promotes apoptosis in HBZY-1 cells under high blood glucose conditions to mitigate diabetic kidney injury

Cell apoptosis has a critical role in the progression of DKD, highlighting the importance of understanding how FM0807 influences this process. Our studies...
demonstrated that high glucose levels induced pathologic proliferation in HBZY-1 cells, contributing to renal tissue damage. We utilized the Hoechst 33342/PI dual-staining kit to assess cell apoptosis. Relative to the control group, the number of apoptotic HBZY-1 cells significantly decreased in the model group (Figure 6A, C), indicating that apoptosis is suppressed in high glucose conditions. Both AG490 and FM0807 curtailed the pathologic proliferation induced by high glucose in HBZY-1 cells with FM0807 exerting a dose-dependent effect. Importantly, FM0807 treatment significantly enhanced the proportion of apoptotic cells, suggesting the capacity of FM0807 to reverse suppression of apoptosis under high glucose conditions, thereby inhibiting excessive cell proliferation and alleviating renal pathologic changes. The proportion of apoptotic cells was notably lower in the model group compared to the control group in flow cytometry experiments, supporting the hypothesis that high glucose suppresses apoptosis, leading to unchecked cell growth (Figure 6B). FM0807 treatment led to a significant increase in the proportion of apoptotic cells compared to the model group, suggesting that FM0807 counteracts the adverse effects of high blood glucose by promoting apoptosis. This response potentially aids in mitigating the renal injury associated with diabetes, underscoring the therapeutic potential of FM0807 in reversing detrimental cellular processes in DKD.

3.7 FM0807 reduces high glucose-induced oxidative stress, protecting HBZY-1 cells from diabetic kidney injury by lowering ROS levels

Oxidative stress has a pivotal role in the pathogenesis of DKD, making oxidative stress imperative to understand the impact of FM0807 on this process. The levels of ROS in HBZY-1 cells under high glucose conditions were assessed using an ROS assay kit. Relative to the control group, the model group showed a marked increase in the number of green fluorescent cells, indicating elevated ROS levels in HBZY-1 cells (Figure 7A-B). Treatment with AG490 and FM0807 resulted in a noticeable reduction in green fluorescence, demonstrating a significant decrease in intracellular ROS levels in a dose-dependent manner with FM0807. These results suggest that FM0807 effectively mitigates oxidative stress in cells under diabetic conditions by reducing ROS production, thereby offering protective effects against renal injury.

Figure 6 | Effect of FM0807 on apoptosis in HBZY-1 cells in a high-glucose environment.

Note: (A) Effects of FM0807 on apoptosis in HBZY-1 cells induced by high glucose using Hoechst 33342/PI dual staining; (B) Flow cytometry analysis results. The control group was treated with 5.5 mM D-glucose, the model group was treated with 25 mM D-glucose, the AG490 group with 25 mM D-glucose combined with 50 μM AG490, and the FM0807 treatment groups with 25 mM D-glucose combined with different concentrations of FM0807 (25/50/100 μM). The magnification of microscope images is 200×. All cell experiments were repeated three times.
3.8 FM0807 alleviates high glucose-induced inflammatory responses and cell apoptosis by modulating the JAK2/STAT3 and TGF-β1/Smad2/3 signaling pathways

Understanding the modulation of inflammatory responses by FM0807 is vital for comprehending its therapeutic impact on DKD. The JAK2/STAT3 signaling pathway has a pivotal role in mediating the effects of numerous cytokines. Prior animal studies indicated increased expression of inflammation-related factors, including JAK2, STAT3, and IL-6, in renal tissues of mice with diabetic kidney injury. qRT-PCR results showed a significant elevation in the expression of JAK2, STAT3, IL-6, and TGF-β1 genes in the model group (Figure 8A-D). Following treatment with FM0807, there was a dose-dependent decrease in the levels of inflammation-related gene expression. In agreement with animal study findings, total and phosphorylated levels of JAK2 and STAT3, as well as the Bcl-2:Bax ratio, were elevated in the model group compared to controls, suggesting that high glucose suppresses apoptosis-related protein expression in HBZY-1 cells (Figure 8E-K). Post-treatment with FM0807, there was a reduction in the total and phosphorylated levels of JAK2 and STAT3, the Bcl-2:Bax ratio, TGF-β1, and Smad2, while the ratio of cleaved caspase-3 to caspase-3 increased. This finding implied that FM0807 enhances apoptosis in HBZY-1 cells under high glucose conditions, which is a reversal from the reduced apoptosis observed in the model group. These findings suggested that FM0807 effectively mitigates inflammatory responses and protects against kidney injury induced by high glucose via modulation of the JAK2/STAT3 and TGF-β1/Smad2/3 signaling pathways.

4. DISCUSSION

This study identified a key role for the curcumin derivative, FM0807, in renal injury and renal fibrosis based on the results of in vivo, in vitro, and pharmacologic experiments. The major findings of this study include the following: (i) FM0807 significantly improved renal function in db/db mice and inhibited the abnormal proliferation in high glucose-induced HBZY-1 cells; (ii) FM0807 was characterized as a terminator of renal fibrosis using spontaneous type 2 diabetes mice and glomerular mesangial cellular models; (iii) mechanistically, FM0807 showed effectively prevented renal injury and attenuated JAK2/STAT3 signaling pathway; and (iv) the role of TGF-β1/Smad2/3 in organ fibrosis is widely recognized, especially in renal fibrosis. FM0807 effectively prevented the continuous progress of renal fibrosis via the TGF-β1/Smad2/3 signaling pathway.

Traditionally viewed as a non-inflammatory glomerular disease, recent genome-wide transcriptome analyses have unveiled the activation of inflammatory signaling pathways in DKD [5, 35, 39, 40]. This study contributes new insights into the complex pathologic mechanisms of DKD by evaluating the therapeutic role of FM0807 in db/db mice and high glucose-induced HBZY-1 cells, marking a departure from traditional treatments that primarily focus on slowing DKD progression.

Unique among similar studies, our research demonstrated the ability of FM0807 to reduce blood glucose levels in db/db mice at high doses. However, more pronounced effects were evident in its anti-inflammatory, antioxidant, and anti-proliferative actions. This shift in treatment strategy not only underscores the multifaceted efficacy of FM0807 but also offers a fresh perspective on the complex DKD pathology.

The use of db/db mice and high glucose-induced HBZY-1 cell models was pivotal in the current study. These models effectively replicate fundamental DKD pathologic changes, such as glomerulosclerosis, tubular atrophy, and interstitial fibrosis, making models invaluable tools for the current investigation. Nevertheless, limitations are inherent in any animal model, particularly
in the capacity to mimic the full complexity of human diseases. Therefore, while these models provide significant insight, the findings necessitate further validation in comprehensive clinical studies.

The evaluation of renal function indicators, including urinary protein, CRE, and BUN, has underscored the therapeutic efficacy of FM0807. Notably, FM0807 improved key biochemical markers in db/db mice, such as TG, LDL-C, CRE, and BUN. These markers are essential for assessing the progression and treatment effects of DKD [41]. This comprehensive evaluation method highlights the significant impact of treatment on renal pathology and suggests the potential of FM0807 in improving lipid abnormalities, offering a more holistic view of its therapeutic effects compared to prior studies.

The significant outcomes of this study included the effectiveness of FM0807 in ameliorating the pathologic features of DKD. Histologic enhancements observed through HE, PAS, PASM, and Masson trichrome staining demonstrated that FM0807 mitigates glomerular structural damage induced by high glucose levels, thereby reducing glomerulosclerosis and inflammatory cell infiltration. These pathologic improvements substantiate the critical role of FM0807 in treating DKD and suggest the potential to prevent the progression to renal fibrosis.

While discussing any new treatment method, it is imperative to consider the safety and potential side effects of a drug. Although this study primarily focused on the efficacy of FM0807, a thorough investigation of the long-term safety is required. In forthcoming studies, especially human clinical trials, the safety profile and spectrum of side effects associated with FM0807 will be critical areas of focus. This further investigation will help us to more comprehensively evaluate the viability of FM0807 as a therapeutic agent for DKD.

This study highlights the significant therapeutic efficacy of FM0807 in treating DKD, underscoring its substantial clinical potential. First, FM0807 introduces a novel strategy by targeting specific biomarkers, such as the JAK2/STAT3 and TGF-β1/Smad2/3 signaling pathways (Figure 9). This targeted approach may offer advantages over traditional treatments by directly influencing the molecular mechanisms underlying the disease.
Additionally, the capability of FM0807 to improve lipid abnormalities and reduce renal inflammation and fibrosis underscores the potential to prevent the progression of renal disease. These properties are crucial for alleviating symptoms in DKD patients and enhancing the quality of life. Moreover, the potential of FM0807 to reduce proteinuria and improve renal function opens new avenues for early treatment strategies in DKD. However, despite these promising results, the effectiveness and safety of FM0807 need further validation in comprehensive human clinical trials.

Despite the promising outcomes of treating DKD with FM0807, the current study had limitations. First, the use of animal models may not fully replicate the complexity and physiologic responses of human diseases, which could limit the direct applicability of the findings. Moreover, data on the long-term safety and potential side effects of FM0807 are currently insufficient, presenting a significant hurdle for clinical application. Additionally, while this study has begun to explore the mechanisms of FM0807, further in-depth research is required to fully elucidate molecular interactions with FM0807. Given the complexity and multifactorial nature of DKD, the effects of FM0807 could vary among individuals or different DKD types, suggesting a need for personalized treatment approaches.

Future research on FM0807 in DKD treatment should focus on several key areas. Conducting large-scale human clinical trials is imperative to verify the therapeutic effects observed in animal models and assess long-term safety and potential side effects. Moreover, future studies should deepen the understanding of FM0807 in its molecular mechanisms, particularly its differential effects across various DKD types or stages and explore ways to optimize its efficacy through personalized treatment strategies. Additionally, it is essential to examine how FM0807 interacts with existing DKD treatments and its potential role in comprehensive treatment plans. Considering the complexity of DKD, further research should also identify additional biomarkers and therapeutic targets to develop more comprehensive and effective treatment strategies. Given the complexity and multifactorial nature of DKD, the effects of FM0807 could vary among individuals or different DKD types, suggesting a need for personalized treatment approaches.

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