Beclin-1-derived peptide MP1 attenuates renal fibrosis by inhibiting the Wnt/β-Catenin pathway

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d) Abbreviations

α-SMA - α-Smooth muscle actin

FN - Fibronectin

Col I - Collagen I

MMP2 - Matrix metallopeptidase 2

Vim - Vimentin

Scr - Serum creatinine
BUN - Blood urea nitrogen

EMT - Epithelial-mesenchymal transition

ECM - extracellular matrix

UOO - unilateral ureteral obstruction

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Abstract

Renal fibrosis is distinguished by the abnormal deposition of extracellular matrix (ECM) and progressive loss of nephron function, with a lack of effective treatment options in clinical practice. In this study, we discovered that the Beclin-1-derived peptide MP1 significantly inhibits the abnormal expression of fibrosis and Epithelial-mesenchymal transition (EMT)-related markers, including α-Smooth muscle actin (α-SMA), Fibronectin (FN), Collagen I (Col I), Matrix metallopeptidase 2 (MMP2), Snail1, and Vimentin (Vim) both in vitro and in vivo. Hematoxylin-eosin (H&E) staining was employed to evaluate renal function, while serum creatinine (Scr) and blood urea nitrogen (BUN) were used as main indices to assess pathological changes in the obstructed kidney. The results demonstrated that daily treatment with MP1 during the 14-day experiment significantly alleviated renal dysfunction and changes in Scr and BUN in mice with unilateral ureteral obstruction (UUO).

Mechanistic research revealed that MP1 was found to have a significant inhibitory effect on the expression of crucial components involved in both the Wnt/β-Catenin and TGF-β/Smad pathways, including β-Catenin, C-Myc, Cyclin D1, TGF-β1, and p-Smad/Smad. However, MP1 exhibited no significant impact on either the LC3II/LC3I ratio or P62 levels. These findings indicate that MP1 improves renal physiological function and mitigates the fibrosis progression by inhibiting the Wnt/β-Catenin pathway. Our study suggests that MP1 represents a promising and novel candidate drug precursor for the treatment of renal fibrosis.
Significance statement

Our study indicated that the Beclin-1-derived peptide MP1 effectively mitigated renal fibrosis induced by UUO through inhibiting the Wnt/β-Catenin pathway and TGF-β/Smad pathway, thereby improving renal physiological function. Importantly, unlike other Beclin-1-derived peptides, MP1 exhibited no significant impact on autophagy in normal cells. MP1 represents a promising and novel candidate drug precursor for the treatment of renal fibrosis focusing on Beclin-1 derivatives and Wnt/β-Catenin pathway.
Introduction

Renal fibrosis represents the ultimate consequence and primary pathological alteration in various chronic kidney diseases, characterized by excessive extracellular matrix (ECM) deposition and progressive nephron function decline (Nastase et al. 2018). Excessive deposition of ECM can ultimately induce cellular dysfunction, parenchymal scarring, and renal failure (Humphreys 2018; Nanthakumar et al. 2015). Currently, there is an urgent demand for efficacious pharmaceuticals capable of effectively mitigating or reversing the progression of renal fibrosis in clinic practice.

The epithelial-mesenchymal transition (EMT) drives the progression of renal fibrosis (Lovisa, Zeisberg, and Kalluri 2016). EMT is a transdifferentiation process characterized by the loss of apical-basal polarity and intercellular adhesion properties in epithelial cells, concomitant with the acquisition of mesenchymal characteristics. Transcriptional, translational, and posttranslational processes are tightly regulated by key transcription factors at multiple levels in this intricate biological phenomenon. During EMT, the reprogramming of gene expression is triggered and precisely controlled by extracellular signaling pathways, accompanied by concomitant nontranscriptional modifications. (Lovisa, Zeisberg, and Kalluri 2016; Nieto et al. 2016). Among these pathways, the progression of EMT relies on the active involvement of the Wnt/β-Catenin signaling pathway (Lamouille, Xu, and Derynck 2014).

The Wnt/β-Catenin pathway is involved in important processes during embryonic development and tumorigenesis (Nusse and Clevers 2017). Wnt proteins engage with
a receptor complex of Frizzled and LRP5/6 on the surface of cells and the complex initiates a cascade of downstream events that lead to β-Catenin dephosphorylation. Dephosphorylated β-Catenin exhibits enhanced stability, accumulates within the cytoplasm, and subsequently undergoes nuclear translocation facilitated by Rac1. Within the nucleus, it modulates cellular responses by regulating target gene expression (Hu et al. 2020; Edeling et al. 2016). Numerous researchers have demonstrated that the Wnt/β-Catenin pathway exhibits sustained activation, which has the potential to facilitate EMT and regulate fibrosis in diverse renal disorders (Xiao et al. 2016; Hu et al. 2020; Edeling et al. 2016).

Li Wang et al. demonstrated that depletion of Beclin-1 (encoded by Becn1) enhances the Wnt/β-Catenin pathway through an increase in Lef1 expression, a decrease in the levels of the Wnt inhibitors DKK3 and Sfrp, and facilitated β-Catenin nuclear translocation (Wang et al. 2020). This finding suggests a potential role for increasing Beclin-1 expression in attenuating the activity of the Wnt/β-Catenin pathway, which regulates EMT progression and inhibits fibrosis. Fu et al. demonstrated a significant upregulation of Beclin-1 expression upon treatment with MP1, a peptide derived from the Bcl-2 homology domain 3 (BH3) region of Beclin-1 encompassing amino acids 112-123 (Fu et al. 2020). This finding suggests that MP1 has the potential to inhibit the Wnt/β-Catenin pathway and may contribute to the regulation of fibrosis initiation and progression.

In this study, our findings demonstrated that MP1, a Beclin-1-derived peptide, exhibits antifibrotic activities both in vivo and in vitro. It effectively improves renal
physiological function and ameliorates renal fibrosis. Furthermore, we have verified MP1 that inhibits the Wnt/β-Catenin pathway without activating autophagy. Our study aims to address the urgent demand for drugs in clinical practice and provided a novel and an effective drug precursor candidate molecule for treating renal fibrosis.
Materials and Methods

Peptide synthesis

MP1 (Leu-Ser-Arg-Arg-Met-Lys-Val-Thr-Gly-Asp-Leu-Trp-OH) was synthesized using 2-chlorotrityl resin (Sunresin, Xi'an, China) with Fmoc used to protect the amino acids. The purification of MP1 was achieved through reverse-phase HPLC (Waters, Milford, USA), and was determined using mass spectrometry (Figure S1) (Bruker maXis 4G, Bremen, Germany).

Cell culture and treatments

The mouse embryonic fibroblast cell line NIH-3T3 and the human renal proximal tubular epithelial cell line HK-2 were utilized. NIH-3T3 and HK2 cells were cultured in DMEM and DME/F12 (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, BI, Beit HaEmek, Israel) and 1% penicillin–streptomycin solution (Solarbio, Beijing, China), respectively. Both cell lines were cultured at 37°C and 5% CO₂, with the medium was refreshed every other day. Experiments were conducted using actively proliferating cells from passages two to three. After incubating the cells in 6-well plates using complete medium, they were subsequently shifted to serum-free medium for an additional 12-hour period to maintain a consistent cellular state. Subsequently, the model group and MP1 treatment group cells were induced by 5 ng/ml TGF-β1, while the MP1 treatment group cells were cultured in serum-free medium containing either 50 μM or 100 μM MP1 respectively. The control group cells were incubated with serum-free medium without MP1. When studying phosphorylated proteins, a 1-hour incubation period was
required and non-phosphorylated proteins necessitated a 24-hour incubation period.

**MTT Assay**

NIH-3T3 and HK2 cells were density of 7000 cells per well in 96-well plates, followed by incubation with various concentrations (0-200 μM) of MP1 in serum-free medium for an additional 24 hours. Subsequently, each well was treated with 10 μL of MTT (5 mg/mL, Sigma, St. Louis, USA). After a 4-hour incubation period, aspirated medium and replaced with 150 μL of DMSO to completely dissolved it for a period of 10 minutes. And, microplate reader (Bio-Rad, Philadelphia, USA) was utilized to measure the absorbance at 490 nm of the wells in the 96-well plates.

**Animal Model**

6-8 weeks old male mice of C57BL/6 (20-22g) were purchased from the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Lanzhou, China). After a one-week acclimatization period, the mice were housed in standard isolator cages, which were regularly cleaned and disinfected, with five individuals per cage under controlled environment consisting of a light-dark cycle of 12-hour and a temperature maintained at 22 ± 2°C. They were provided with an adequate supply of nourishment and ensured access to uncontaminated potable water, while bedding material was sterilized, dusted off, and replaced periodically to maintain cleanliness and dryness.

According to Wei et al., the recommended minimum number of small animal experiments in Pharmacological Experiment Methodology is 10 mice in each group (Wei et al., 2010). The mice were allocated into 5 groups (n = 10) using a
randomization procedure: sham group, UUO model group, MP1 treatment groups at
doses of 0.625 mg/kg/d and 1.25 mg/kg/d, and positive control group treated with
captopril at a dose of 20 mg/kg/d.

After administering anesthesia through intraperitoneal injection of pentobarbital
sodium at a dose of 50mg/kg, the lateral skin of the abdomen in mice was incised, and
the ligatures were used to tie off the left ureter, then, cut the left ureter between the
two ligated points to establish UUO models. In the sham group, the lateral skin of the
abdomen was also incised and then sutured after exposing the left ureter. On the day
following surgery, subcutaneous injections of MP1 at doses of 0.625 mg/kg and 1.25
mg/kg were administered to the respective treatment groups, while PBS was injected
into both sham and UUO groups, and captopril was subcutaneously injected into the
positive control group at dose of 20 mg/kg. After continuous administration for a
period of 14 days, euthanasia by cervical dislocation method was performed on all
mice. The left kidney tissues and serum from each mouse were harvested immediately
after euthanasia and subsequently preserved at a temperature of -80°C.

The animal experiments in this study followed the guide for the care and use of
laboratory animals (Institute of Laboratory Animal Resources, 2021), and were
approved by Lanzhou University's Ethics Committee. (license number: SYXK Gan
2018-0002).

Biochemical Analysis

The assay kit (Nanjing Jiancheng, Nanjing, China) was used to quantify the levels of
BUN and Scr. The experimental procedure and subsequent data analysis were
conducted following the manufacturer’s instructions.

**Hematoxylin-eosin (H&E), Masson's trichrome and immunohistochemistry staining**

Hematoxylin-eosin (H&E), Masson trichrome staining, and immunohistochemical staining were performed using 4-μm thick sections. The SP kit (ZSGB-Bio, Beijing, China) was used for immunohistochemical staining. After deparaffinization and rehydration steps, antigen retrieval was carried out. To inhibit endogenous peroxidase activity, a blocking agent was applied for 10 minutes, subsequently, incubation with goat serum for 15 minutes. The sections were then incubated overnight at 4 °C with primary antibodies (Table 1). Secondary antibodies were used for another 15-minute incubation at 37 °C. DAB served as a chromogen. Following staining, the sections underwent dehydration using gradient ethanol treatment before sealing them with neutral gum.

**Western Blot Analysis**

RIPA solution (Beyotime, Shanghai, China) with 1% PMSF (Solarbio, Beijing, China) was utilized to lyse the cells and kidney tissue for total protein extraction. To extract phosphorylated proteins, a supplementary 1% protein phosphatase inhibitor (Solarbio, Beijing, China) was included. The BCA kit (Thermo, Rockford, USA) was utilized to quantify the concentration of total protein in accordance with the manufacturer's instructions. Protein samples underwent electrophoresis and transfer, after that, a blocking step was performed using skim milk powder with a concentration of 5%. Primary antibodies and HRP-labeled secondary antibodies were used for incubation.
The band signals were visualized through the use of developer (NCM Biotech, Suzhou, China). ImageJ 1.53f software (Bethesda, USA) was utilized for conducting quantitative analysis.

**Real-Time qPCR Analysis**

Cells and tissue were subjected to RNA extraction, reverse transcription was performed using the PrimeScript RT Reagent Kit (Yeasen, Shanghai, China) according to manufacturer's instructions. Real-time quantitative PCR was performed using the qPCR SYBR Green Master Mix (Yeasen, Shanghai, China) on the ABI QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). The primer sequences used for both human and mouse genes are listed in Table 2.

**Statistical Analysis**

From a statistical perspective, the biological indicators we detect are not fixed values but are assumed to follow a normal distribution. To enhance confidence in these measurements and the assumed distribution, it is recommended to conduct at least three repetitions, with particular emphasis on mean and SEM. Therefore, all Western Blot and Real-Time qPCR experiments were performed with 3-5 independent replicates, while biochemical indicators were tested with 6-7 independent replicates. All data are presented as the mean ± SEM. GraphPad Prism 8.3.0 (San Diego, USA) was utilized to analyze the intergroup differences using one-way ANOVA according to Tukey’s test. Statistical significance was considered at p < 0.05.
Results

MP1 inhibits fibrosis in vitro

The MTT assay results showed that MP1 (0-200 μM) did not exhibit significant cytotoxicity to NIH-3T3 cells (Figure 1 B, Figure S2 A). To evaluate the potential effectiveness of MP1 in reducing fibrosis, we measured the expression levels of fibrosis-related proteins. Compared with the control group, fibrotic NIH-3T3 cells exhibited a substantial increase in α-SMA, FN, Col I, and MMP2 expression. However, the upregulation was significantly suppressed by both high and low concentrations of MP1 (Figure 1 C-G). Additionally, MP1 effectively suppressed the mRNA levels of Acta2, Fn1, Coll1a1 and Mmp2 (Figure 1 H-K). These results indicated that MP1 had antifibrotic activity in vitro.

MP1 inhibits EMT in vitro

The MTT assay results showed that MP1 (0-100 μM) did not exhibit significant cytotoxicity to HK2 cells, except at a concentration of 200 μM where it displayed notable cell toxicity against HK2 cells (Figure 2 A, Figure S2 B). The findings of this experiment are consistent with the outcomes observed in HK2 cell lines using the 48-hour MTT assay conducted on MP1, as an extended processing time did not result in increased cellular toxicity. Therefore, it can be inferred that MP1 may exhibit acute toxicity towards HK2 cells. Notably, MP1 significantly suppressed the expression of α-SMA, FN, COL, MMP2, Snail1, and Vim, which are closely associated with fibrosis and EMT. These proteins exhibited increased expression in response to exogenous TGF-β1 (Figure 2 B-I). Moreover, the mRNA levels of ACTA2, FN1,
COL1A1, MMP2, SNAI1 and VIM in the transdifferentiated HK2 cell EMT model were effectively suppressed by MP1 (Figure 2 J-O). These results indicated that MP1 suppressed EMT and transdifferentiation of HK2 cells induced by exogenous TGF-β1.

**MP1 inhibits the Wnt/β-Catenin and TGF-β/Smad pathways in vitro**

The activity of the Wnt/β-Catenin pathway is typically limited in adult kidneys, but can be reactivated in chronic kidney disease patients, playing a crucial role in fibrosis and EMT. We performed Western blot analysis to evaluate the expression levels of β-Catenin, C-Myc, and Cyclin D1. (Figure 3 A-D, Figure 4 A-D). The results showed that MP1 effectively suppressed the Wnt/β-catenin pathway in NIH-3T3 cells and HK2 cells, which was consistent with the changes in the transcript levels of Ctnnb1, Myc and Ccnd1 (Figure 3 F-H, Figure 4 F-H).

The TGF-β/Smad pathway represents the primary regulatory mechanism involved in fibrosis and EMT. MP1 treatment significantly attenuated the expression of phosphorylated-Smad (P-Smad) and p-Smad/Smad in NIH-3T3 cells and HK2 cells, as compared to exogenous TGF-β1-induced cells (Figure 3 E, Figure 4 E). These results indicated that MP1 inhibited fibroblast-to-myofibroblast differentiation and EMT by suppressing the Wnt/β-Catenin and TGF-β/Smad pathways.

**MP1 does not induce autophagy in vitro**

MP1 effectively inhibits cancer cell proliferation by inducing the autophagy; however, there is an ongoing debate regarding the involvement of autophagy in fibrosis. Yuta Takagaki et al. demonstrated that autophagy is essential for maintaining endothelial cell integrity, and disruption of endothelial autophagy could result in significant
pathological IL6-dependent EndMT and organ fibrosis. In contrast, Man J Livingston et al. reported that persistent induction of autophagy in kidney tubular cells promotes renal interstitial fibrosis in UUO models. (Livingston et al. 2016). therefore, our objective is to explore the potential involvement of MP1 in the regulation of autophagy.

The LC3 system controls the expansion and completion of autophagosomes, and P62 serves as a versatile scaffolding protein that participates in the modulation of autophagy. The LC3II/LC3I ratio and P62 were utilized for evaluate the autophagy level. According to the results of Western blot, MP1 exhibited no significant impact on either the LC3II/LC3I ratio or P62 levels (Figure 5 A-C). These results indicated that MP1 did not induce autophagy in vitro.

MP1 attenuates UUO-induced renal injury

To explore the potential renoprotective benefits of MP1 in vivo, we established a murine UUO model. The evaluation of renal function in clinical practices primarily relies on assessing Scr and BUN levels. Generally, more than half of kidney function is lost when Scr levels rise, and BUN rises when glomerular filtration function is severely impaired. the 14-day of continuous administration of MP1 improved renal function and significantly reversed the changes of Scr and BUN, which were upregulated after obstruction (Figure 6 A, B).

The pathological changes in obstructed kidney were evaluated through H&E staining. In the UUO model group, detachment of renal tubular epithelial cells into the lumen, loss of structure in the proximal renal tubules, and dilation of the distal renal tubules
as well as glomerular atrophy. However, treatment with MP1 significantly alleviated the pathological changes (Figure 6 C). These results indicated that MP1 mitigated UUO-induced renal injury.

**MP1 inhibits UUO-induced fibrosis and EMT in vivo**

Masson's trichome staining revealed that massive collagen deposition was reversed after 14-day of continuous administration of MP1 (Figure 6 D, Figure S3 A). We assessed the expression of several proteins and mRNAs associated with fibrosis and EMT, which were significantly suppressed in obstructed kidneys upon treatment with MP1 (Figure 7 A-N). These findings were further supported by immunohistochemical staining results (Figure 7 O-Q, Figure S3 B-D). Collectively, our findings indicated the potent inhibitory effects of MP1 on fibrosis and EMT in vivo.

**MP1 inhibits the Wnt/β-Catenin and TGF-β/Smad pathways in vivo**

In contrast to the sham group, a significant upregulation of β-Catenin, C-Myc and Cyclin D1, TGF-β1 and p-Smad/Smad was observed in obstructed kidneys (Figure 8 A-F). However, MP1 effectively reversed this effect by downregulating mRNA levels of *Ctnnb1*, *Myc*, *Ccnd1* and *Tgfb1* in obstructed kidneys (Figure 8 G-J). The immunohistochemical staining results were consistent with the findings from Western blot and qPCR (Figure 8 K, L, Figure S3 E, F). These results indicated that MP1 mitigated renal fibrosis induced by UUO through the suppression of both the Wnt/β-Catenin and TGF-β/Smad pathways.
**Discussion**

Renal fibrosis is the second most common type of organ fibrosis (Zhao et al. 2020). Currently available therapies mainly target the primary disease that causes renal fibrosis, including hypertension and diabetes (Peng et al. 2005; Li et al. 2018), which mitigate or delay progression but have several restrictions during the intermediate and advanced stages of renal fibrosis (Klinkhammer et al. 2017; Nastase et al. 2018).

The findings of this study demonstrate that MP1 does not exhibit significant cytotoxicity and effectively suppresses the expression of fibrosis- and EMT-associated protein *in vitro* and *in vivo*. Renal function was evaluated using H&E staining, while Scr and BUN were used to assess pathological changes in obstructed kidney. The findings demonstrated that MP1 exhibited antifibrotic effects and effectively suppressed EMT, *in vitro* and *in vivo*.

The involvement of the Wnt/β-Catenin pathway is essential in the pathogenesis and recovery of renal injury caused by various insults. Appropriate activation of this pathway in tubules can effectively prevent tubular cell death and acute kidney injury, while persistent activation of this pathway may lead to progressive kidney fibrosis in tubular cells (Feng et al. 2018). Li Wang et al. demonstrated that Beclin-1 (encoded by Becn1) depletion enhanced the canonical Wnt/β-Catenin pathway activation (Wang et al. 2020); in other words, upregulating Beclin-1 expression inhibits fibrosis by attenuating the activity of the Wnt/β-Catenin pathway.

The expression of β-Catenin, C-Myc, and Cyclin D1 is effectively reduced by MP1 both *in vitro* and *in vivo*. These findings demonstrate that MP1 exerts its therapeutic
effects on renal fibrosis through inhibition of the Wnt/β-Catenin pathway. The Myc gene is involved in multiple biological processes, including renal fibrosis genesis and developmental processes (Lemos et al. 2018; Lovisa et al. 2020). And pharmacological blockade of c-Myc effectively mitigated renal fibrosis in mouse models by UUO and folic acid-treatment (Shen et al. 2017), which is consistent with our experimental findings.

Moreover, MP1 significantly ameliorated renal fibrosis through the inhibition of the TGF-β/Smad pathway. However, further investigations are warranted to elucidate whether MP1 exerts its inhibitory effects on the TGF-β/Smad pathway through modulation of the Wnt/β-Catenin pathway or vice versa.

Autophagy plays a crucial role in maintaining tissue homeostasis and overall health (Fernandez et al., 2018). However, there are still unresolved queries regarding the protective or pathological role of autophagy in renal fibrosis, as well as its effects on different types of kidney cells and diseases (Tang et al., 2020). Beclin-1, a pivotal component in autophagy, contains protein-binding domains including the BH3 domain, CCD, and ECD, each playing distinct physiological roles (Seo et al. 2020). Sanae Shoji-Kawata et al. demonstrated that a specific sequence of amino acids (TNVFNATFEIWHDFEGT) located at positions 267-284 within the Beclin-1 ECD could induce autophagy (Shoji-Kawata et al. 2013), significantly reduce cyst formation, and restore kidney function in a pkd1 mutated zebrafish model (Zhu et al. 2017). Leila Peraro et al. identified that the minimal active segment of Beclin-1 is 269VFNATFEIWHD279, with an optimized peptide achieved through substitutions of
Phe2 to Trp and Glu7 to His. The diversity-oriented stapling approach was employed to generate intrinsically cell-penetrant autophagy-inducing peptides (TB11: TAT-VWNATFHIWHD) (Peraro et al. 2017). Mingjun Shi et al. demonstrated that administration of TB11 induced autophagy both prior to and following (2-5 days) ischemia reperfusion injury, thereby conferred renoprotection and suppressed kidney fibrosis (Shi et al. 2022). Conversely, E Wirawan et al. identified TDVD$^{133}$ and DQLD$^{149}$ as two caspase cleavage sites within Beclin-1, cleavage at these sites generates fragments lacking the ability to induce autophagy (Wirawan et al. 2010). MP1, derived from amino acids 112-123 of Beclin-1 BH3, has demonstrated the ability to trigger autophagy in tumor cells (Fu et al. 2020). However, our experimental findings suggest that MP1 does not exert significantly affect on autophagy in normal cells. The findings mentioned above suggest that distinct fragments of Beclin-1 exhibit diverse biological activities, while the identical fragment of Beclin-1 plays diverse roles across distinct cell types. Currently, there is a lack of systematic studies on specific fragments and their corresponding biological activities, and our study provides an experimental foundation for further exploration in this field.

David Lagares et al. reported that the differentiation of fibroblasts into myofibroblasts is driven by increased matrix rigidity, which upregulates BIM, a protein involved in mitochondrial apoptotic priming, and the antiapoptotic protein BCL-XL. These proteins bind to each other to ensure the survival of myofibroblasts. Downregulation of BCL-XL leads to the release of BIM, which directly interacts with and triggers the activation of BAX and BAK, thereby inducing apoptosis in myofibroblasts (Kuehl
and Lagares 2018; Lagares et al. 2017). The “BH3 mimetic” drug ABT-263 (Navitoclax) displaces BIM binding to BCL-XL, enables BIM to activate apoptosis in myofibroblasts, and effectively alleviates bleomycin-induced scleroderma, pulmonary fibrosis, and cardiac fibrosis(Pan et al. 2017; Walaszczyk et al. 2019). Chenggong Fu et al. utilized molecular docking and dynamics simulations to investigate the binding mechanisms of MP1 and demonstrated its ability to competitively bind to BCL-XL through the BH3 domain in vitro(Fu et al. 2020). These findings indicate that MP1 may exhibit similar mechanisms and functions to ABT-263, which blocks BCL-XL binding to BIM and induces apoptosis. To investigate whether MP1 activates apoptosis, we assessed the expression of PARP fragment as an early apoptotic marker in fibrotic NIH-3T3 cells. However, our findings revealed that MP1 exerted a suppressive effect on apoptosis in fibrosis model cells and had no significant impact on myofibroblasts survival rate (Figure S4). These results demonstrate that MP1 differs from ABT-263 in terms of the mechanism and activity of apoptosis. At present, there is no conclusive evidence supports the interaction between MP1 to BCL-XL.

In summary, our study demonstrated that MP1 effectively mitigates renal fibrosis caused by UUO through the inhibition of both the Wnt/β-Catenin pathway and TGF-β/Smad pathway in both in vitro and in vivo, without activating autophagy. Our study aimed to address the urgent need for drugs in clinical practice and provided a novel and a promising candidate drug precursor for the treatment of renal fibrosis.
Data availability statement

The data of this study are available on request from the corresponding author.
Author contributions

Participated in research design: J.F. Zhang, Feng, Yang, B.Z. Zhang

Conducted experiments: J.F. Zhang, Feng, Yang, Bai, Gao

Performed data analysis: J.F. Zhang, Feng, Yang

Wrote or contributed to the writing of the manuscript: J.F. Zhang, Yang, B.Z. Zhang
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Footnotes

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The authors declare that there are no conflicts of interest.
Figure legends

Figure 1. MP1 inhibits fibrosis in TGF-β1-treated NIH-3T3 cells. (A) Chemical structure of MP1. (B) Cytotoxicity of MP1 in NIH-3T3 cells. (C-G) Western blot analysis was used to quantify the protein levels of α-SMA, FN, COL and MMP2 in NIH-3T3 cells. (H-K) Real-time qPCR analysis was used to quantify the mRNA levels of Acta2, Fn1, Colla1 and Mmp2 in NIH-3T3 cells. The data are presented as the mean ± SEM (B, E, F, H-K, n = 3) and (D, n = 4) and (G, n = 5). ⁎P < 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.001, ⁎⁎⁎⁎P < 0.0001 vs. the control group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the TGF-β1 group.

Figure 2. MP1 inhibits EMT in TGF-β1-treated HK2 cells. (A) Cytotoxicity of MP1 in HK2 cells. (B-I) Western blot analysis was used to quantify the protein levels of α-SMA, FN, COL, MMP2, Snail1, Vim in HK2 cells. (J-O) Real-time qPCR analysis of ACTA2, FN1, COL1A1, MMP2, SNAI1 and VIM in HK2 cells. The data are presented as the mean ± SEM (A, D, F-H, J-O, n = 3) and (E, I, n = 4). ⁎P < 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.001, ⁎⁎⁎⁎P < 0.0001 vs. the control group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the TGF-β1 group.

Figure 3. MP1 inhibits the Wnt/β-Catenin and TGF-β/Smad pathway in NIH-3T3 cells. (A-E) Western blot analysis was used to quantify the protein levels of β-Catenin, C-Myc, Cyclin D1 and p-Smad/Smad. (F-H) Real-time qPCR analysis of Ctnnb1, Myc and Ccnd1. The data are presented as the mean ± SEM (B-D, F-H, n = 3) and (E, n = 4). ⁎P < 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.001, ⁎⁎⁎⁎P < 0.0001 vs. the control group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the TGF-β1 group.
Figure 4. MP1 inhibits the Wnt/β-Catenin and TGF-β/Smad pathway in HK2 cells. (A-E) Western blot analysis was used to quantify the protein levels of β-Catenin, C-Myc, Cyclin D1 and p-Smad/Smad. (F-H) Real-time qPCR analysis of CTNNB1, MYC and CCND1. The data are presented as the mean ± SEM (B-D, F-H, n = 3) and (E, n = 4). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. the control group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the TGF-β1 group.

Figure 5. Regulatory role of MP1 in NIH-3T3 cells. (A-C) Western blot analysis was used to quantify the protein levels of LC3II/LC3I and P62. The data are presented as the mean ± SEM (B, n = 4) and (C, n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. the control group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the TGF-β1 group.

Figure 6. MP1 attenuates UUO-induced renal injury. (A-B) Scr and BUN levels in serum. (C) Representative images of H&E staining kidney sections (original magnification, 100×). (D) Representative images of Masson's trichrome staining (original magnification, 100×). The data are presented as the mean ± SEM (A, n = 7) and (B, n = 6). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. the sham group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the UUO group.

Figure 7. MP1 inhibits fibrosis and EMT in UUO mice. (A-F, K-L) Western blot analysis was used to quantify the protein levels of α-SMA, FN, COL, MMP2, Snail1, Vim. (G-J, M-N) Real-time qPCR analysis of Acta2, Fn1, Col1a1, Mmp2 and Snai1, Vim. (O-Q) Representative images of immunohistochemical staining of α-SMA, FN, Vim (original magnification, 100×). The data are presented as the mean ± SEM (D-J,
n = 3) and (C, K, n=4). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. the sham group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the UUO group.

**Figure 8.** MP1 inhibits the Wnt/β-Catenin and TGF-β/Smad pathway in UUO mice. (A-F) Western blot analysis was used to quantify the protein levels of β-Catenin, C-Myc, Cyclin D1, TGF-β1 and p-Smad/Smad. (G-J) Real-time qPCR analysis of Ctnnb1, Myc, Ccnd1 and Tgfb1. (K-L) Representative images of immunohistochemical staining of β-Catenin and TGF-β1 (original magnification, 100×). The data are presented as the mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. the sham group; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs. the UUO group.
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Figure 2

A

Cell Viability

MP1 0 12.5 25 50 100 200 μM

B

TGF-β - + + +
MP1 - - 50 100 μM

α-SMA 42 kDa
Fibronectin 285 kDa
Collagen 220 kDa
MMP2 74 kDa
GAPDH 36 kDa

C

TGF-β - + + +
MP1 - - 50 100 μM

Snail 1 29 kDa
Vimentin 57 kDa
GAPDH 36 kDa
Figure 3

A

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β-Catenin 92 kDa
C-Myc 65 kDa
Cyclin D1 36 kDa
p-Smad 52/60 kDa
Smad 52/60 kDa
GAPDH 36 kDa

B

β-Catenin/GAPDH

C

C-Myc/GAPDH

D

Cyclin D1/GAPDH

E

p-Smad/GAPDH

F

Climb1/GAPDH

G

Myc/GAPDH

H

Gadd153/GAPDH
Figure 4

A

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β-Catenin   | 92 kDa |
C-Myc       | 65 kDa |
Cyclin D1   | 36 kDa |
p-Smad      | 52/60 kDa |
Smad        | 52/60 kDa |
GAPDH       | 36 kDa |

B

C

D

E

F

G

H
Figure 5

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B

![Graph B](image)

C

![Graph C](image)
Figure 6

A

B

C

D

Sham  UUO  0.625 mg/kg  1.25 mg/kg  CAP

H&E  Masson
Figure 8

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B

- β-Catenin/GAPDH
- C-Myc/GAPDH
- Cyclin D1/GAPDH

C

D

E

F

G

H

I

J

K

L

Sham  UUO  0.625 mg/kg  1.25 mg/kg

β-Catenin

TGF-β1