Original Paper

A Novel Inhibitor of Homodimerization Targeting MyD88 Ameliorates Renal Interstitial Fibrosis by Counteracting TGF- β 1-Induced EMT *in Vivo* and *in Vitro*

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Key Words

MyD88 • Epithelial-mesenchymal transition • Renal interstitial fibrosis • NF- κ B • Transforming growth factor beta 1

Abstract

Background/Aims: The TLR/MyD88/NF- κ B signaling pathway has been successfully used to treat renal interstitial fibrosis (RIF). However, the exact therapeutic mechanism is still unknown. Here, we assessed the therapeutic efficacy of TJ-M2010-2, a small molecular compound that inhibits MyD88 homodimerization, in RIF induced by ischemia reperfusion injury (IRI). **Methods:** *In vivo*, RIF was induced in mice by IRI, and the mice were prophylactically treated with TJ-M2010-2. *In vitro*, HK-2 cells were incubated with TGF- β 1 to induce EMT, and the cells were pretreated with TJ-M2010-2. **Results:** We found that, compared with the IRI group, the TJ-M2010-2 group showed marked attenuation of RIF and renal function injury; decreased expression of TGF- β 1, α -SMA, vimentin, MMP2 and MMP9; and increased E-cadherin expression. Furthermore, TGF- β 1-induced EMT was blocked by TJ-M2010-2 in HK-2 cells, as evidenced by blocked morphologic transformation, restored E-cadherin expression and inhibited α -SMA expression. In addition, compared to the TGF- β 1 group, the TJ-M2010-2 group showed profound inhibition of the expression of TRAF6, p65 and Snail and upregulation of the expression of I κ B α . **Conclusion:** This MyD88 inhibitor may be a potential therapeutic agent to ameliorate RIF.

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Introduction

Renal fibrosis, an unavoidable consequence of chronic kidney disease (CKD), may progress to end-stage renal disease (ESRD), which requires dialysis therapy or kidney transplantation to circumvent death [1, 2]. The excessive deposition of extracellular matrix (ECM) is the most striking and name-lending feature of tubulointerstitial fibrosis [3], and the extent of tubulointerstitial fibrosis corresponds with the rate of decline in kidney function [4]. Activated tubulointerstitial myofibroblasts are the major cell type that leads to the production of excessive ECM [5]. Emerging evidence suggests that myofibroblasts can be derived from tubular epithelial cells through the process of epithelial-mesenchymal transition (EMT) [2, 6]. Transforming growth factor-beta 1 (TGF- β 1) has been proposed to be the major regulator of the induction of EMT and renal fibrosis [7]. The activation of MMP2 and MMP9 by TGF- β 1 is associated with the degradation of basement membrane stimulation of EMT [8], which could be inhibited by plasminogen activator inhibitor-1 (PAI-1) [9].

The TLR/MyD88/NF- κ B signaling pathway is closely related to renal fibrosis. Skuginna V et al. confirmed that the knockout of TLR4 or MyD88 can reduce the expression of α -smooth muscle actin (α -SMA) and attenuate RIF in unilateral ureteral obstruction (UUO) [10, 11]. The activation of NF- κ B plays a role in various chronic kidney diseases associated with inflammation and fibrosis [12]. Zhou BP et al. found that the transcriptional activity of NF- κ B was required for TNF α -mediated Snail stabilization, which plays a fundamental role in the EMT process, and could produce many cytokines, including TGF- β 1, TNF- α , IL-1 β , IL-6, and MCP1 [13]. Due to the important role of TLR/MyD88/NF- κ B signaling in RIF progression, MyD88 may be a potential target for RIF prevention and treatment.

Previous studies mainly applied various TLR monoclonal antibodies, gene knockouts or gene knockouts downstream of MyD88 to suppress the TLR/MyD88 signaling pathways. These methods can only be used in preclinical study; they have little clinical value. We synthesized a novel MyD88 inhibitor (TJ-M2010-2), which is designed to bind to the TIR domain of MyD88 and interfere with the dimerization of MyD88. Our previous study indicated that TJ-M2010-2 attenuated renal interstitial fibrosis and inhibited TGF- β 1-induced EMT in HK-2 cells [14]. This study aims to explore the mechanism of TJ-M2010-2 in RIF.

Materials and Methods

Animals and ethics

Male Balb/c mice (6–8 weeks old) were obtained from the Huabukang Company (Beijing, China). They were housed in specific pathogen-free facilities and maintained under controlled conditions (22°C, 55% humidity and a 12 h day/night cycle) at Huazhong University of Science and Technology, Wuhan, China. Experimental protocols were approved by the Animal Care and Research Committee of Huazhong University of Science and Technology.

Renal IRI model

The renal IRI model was used as previously reported with some modifications [14]. Briefly, the left kidney in the mice underwent 60 min of ischemia without right nephrectomy followed by long-term observation (28 days). On day 29, the right nephrectomy was performed, and then serum creatinine (Cr) and blood urea nitrogen (BUN) levels were measured to assess the function of the left kidney. The mice were divided into three groups as follows: (1) sham group: Balb/c mice underwent a sham operation; (2) IRI group: BALB/c mice underwent IR; (3) TJ-M2010-2 group: BALB/c mice were pretreated with TJ-M2010-2 and then underwent IR. In the TJ-M2010-2 group, mice were treated with TJ-M2010-2 (100 mg/kg, i.p.) on Days -3, -2, -1, 0, 1, 3, 5, 7, and 14. The same volume of vehicle was also administered to the sham and IRI groups. The IR operation was performed on day 0.

Drugs and administration

TJ-M2010-2 was synthesized at the Academy of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (WIPO Patent, application number: PCT/CN2012/070811). The details are shown in our previous article [14].

Measurement of blood and renal parameters

At twenty-eight days post-I/R (60 min ischemia of the left kidney), right nephrectomy was performed. Blood samples were collected from the inferior vena cava and centrifuged (7500 g, 10 min) for serum collection. Serum Cr and BUN concentrations were measured by the clinical laboratory of Tongji Hospital (Wuhan, China). The kidney weight was measured on day 28.

Histology, Masson's trichrome staining and immunohistochemistry

Left kidneys were dissected from mice 28 days after I/R and fixed in 10% formalin. Formalin-fixed kidneys were embedded in paraffin, and 4 µm sections were stained with hematoxylin and eosin (H&E). E-cadherin, α-SMA, vimentin, MMP2, MMP9 and PAI-1 (Abcam, Cambridge, UK) were evaluated by immunohistochemistry as previously described [15]. Masson's trichrome staining was used to evaluate renal fibrosis. The positive tissue staining area was quantified using Image Pro-plus v 6.0 software to analyze the mean optical density. All morphological analyses were conducted in a blinded fashion.

Cell culture and treatment

HK-2 cells, obtained from the Institute of China Center for Type Culture Collection, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco) in a 37°C humidified 5% CO₂ atmosphere. These cells were seeded in a 6-well plate at 1.0 × 10⁵ cells/ml for 24 h and grown to approximately 60%-70% confluence. The medium was then replaced with serum-free medium. TJ-M2010-2 was added (0 µM or 20 µM) to the culture for 30 min, and then TGF-β1 (4 ng/ml) was added for 72 h. Morphological changes were assessed by phase contrast microscopy [14].

Immunofluorescent staining and fluorescence microscopy

Cells were grown in 6-well glass-bottomed dishes and washed twice with phosphate-buffered saline (PBS) before being fixed in 4% paraformaldehyde for 30 min and then permeabilized with 0.2% Triton X-100 in PBS. Nonspecific binding sites were blocked with normal goat serum (Sigma-Aldrich, USA) diluted in 0.1% Triton X-100 in PBS for 2 h. Then, the cells were incubated overnight at 4°C with anti-E-cadherin (Transduction Laboratories, Lexington, KY, USA) or anti-α-SMA (ab5694) antibody at a 1:200 dilution in blocking buffer. The next day, after 3 washes with PBS, the cells were stained with a FITC-conjugated secondary antibody (1:200) for 1 h at room temperature. DAPI was used to stain the nuclei before images were acquired. The images were acquired using a fluorescence microscope (Olympus, Tokyo, Japan); the green or red fluorescence indicated positive antibody staining, and the blue fluorescence indicated nuclear DAPI labeling.

Real-time PCR

Total RNA was extracted from renal tissues using Trizol reagent (Invitrogen). Reverse transcription was performed using a Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific). Real-time PCR was performed using a SYBR® Green Real-time PCR Master Mix (TOYOBO, Japan) with an ABI PRISM® 7700 instrument. The primer sequences were as follows: TGF-β1 (forward) 5'-ATACGCCTGAGTGGCTGTCT-3' and (reverse) 5'-TGGGACTGATCCCATTGATT-3', CTGF (forward) 5'-TTCCCGAGAAGGGTCAAGCT-3' and (reverse) 5'-TCCTTGGGCTCGTCACACA-3', and GAPDH (forward) 5'-ACAAGATGGTGAAGGTCGGTG-3' and (reverse) 5'-AGAAGGCAGCCCTGGTAACC-3'. The results were expressed using the comparative CT method, with gene expression calculated relative to the expression of the housekeeping gene *Gapdh*.

Western blot analysis

On day 28, total protein was extracted from the renal tissue homogenate. HK-2 cells were lysed with IP lysis buffer containing the protease inhibitor PMSF (both from Beyotime Institute of Biotechnology). Western blot analysis was performed as previously described with some modifications [16]. The membrane

was immunoblotted with antibodies against IKB α , p65 and Snail (Cell Signaling Technology, Danvers, MA, USA), E-cadherin (Transduction Laboratories, Lexington, KY, USA), TRAF6 (ab33915), vimentin (ab16700), α -SMA (ab5694), GAPDH, or β -actin (Beyotime Institute of Biotechnology) and detected with horseradish peroxidase-conjugated secondary antibodies and ECL A/B reagents (Beyotime Institute of Biotechnology). The intensity of each band was quantitatively determined using Gel-Pro Analyzer software (Media Cybernetics Inc., Rockville, MD, USA). The density ratio represents the relative intensity of each band against those of the controls in the experiment.

Statistical Analysis

Data are expressed as the mean $\pm$ SD as indicated. Different groups were compared with a Student's t test or one-way ANOVA, as appropriate. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA). P values < 0.05 were considered statistically significant.

Results

TJ-M2010-2 increased the ratio of LKW to RKW 28 days after IRI

To evaluate the degree of renal atrophy 28 days after IRI, we observed renal size by macrography and measured the ratio of left kidney weight (LKW) to right kidney weight (RKW) among the different groups. As shown in Fig. 1, the size of the left renal tissue was larger in the TJ-M2010-2 group than in the IRI group (A). Furthermore, the ratio of LKW to RKW was significantly greater in the TJ-M2010-2 group than in the IRI group (B).

TJ-M2010-2 reduced pathologic damage and attenuated renal fibrosis after IRI

To evaluate the protective effect of TJ-M2010-2 on histopathological changes and renal fibrosis 28 days after IR surgery, we analyzed slides treated with hematoxylin and eosin (H&E) staining, Masson's trichrome staining and immunohistochemical staining for E-cadherin, vimentin and α -SMA (myofibroblast activation marker). As shown in Fig. 2, severe glomerular collapse, tubular dilation, and tubulointerstitial infiltration as well as a large amount of ECM deposition were observed in the kidneys of the IRI group. In addition, expression levels of α -SMA and vimentin determined by immunohistochemical analysis demonstrated robust increases, but E-cadherin expression decreased in the IRI group. However, all of the results above were quite the contrary to the results in the TJ-M2010-2 treatment group. *P < 0.001 versus sham, *P < 0.01 and #P < 0.001 versus IRI. (n = 6 per group). Original magnification 200 $\times$ with five fields assessed. Bar = 400 μ m in all panels.

Fig. 1. The right (R) and left (L) kidney tissues were collected 28 days after IRI and observed by macrography (A) (n = 6 per group). The ratio of the left kidney weight to right kidney weight was measured (B). (**P < 0.001 versus sham group, #P < 0.05 versus IRI group). Data are presented as the mean $\pm$ SD.

Fig. 2. TJ-M2010-2 reduced pathologic damage and attenuated renal fibrosis after IRI. Left renal tissue was collected 28 days after IRI and stained with hematoxylin & eosin or Masson's trichrome (n = 6 per group). Severe glomerular collapse, tubular dilation and tubulointerstitial infiltration as well as a large amount of extracellular matrix deposition were observed in the kidneys of the IRI group; Original magnification 400× with five fields assessed. Bar = 200 μm in all panels. In addition, α-SMA and vimentin expression determined by immunohistochemical staining demonstrated robust increases, but E-cadherin expression decreased in the IRI group compared to the sham group. However, all of the results listed above were contrary to the results observed in the TJ-M2010-2 treatment group. *P<0.001 versus sham, *P<0.01 and #P<0.001 versus IRI. (n = 6 per group). Original magnification 200× with five fields assessed. Bar = 400 μm in all panels.

TJ-M2010-2 prevents renal dysfunction after IRI

At 28 days post-I/R (60 min ischemia of the left kidney), right nephrectomy was performed, and then serum creatinine (Cr) and blood urea nitrogen (BUN) levels were measured. As shown in Fig. 3, mice that experienced IR exhibited dramatically elevated levels of Cr and BUN compared with the levels of the sham-treated group (**P < 0.001). These increased levels were significantly reduced by TJ-M2010-2 administration (###P < 0.001). These findings suggest that TJ-M2010-2 improves renal function in mice after IR injury.

TJ-M2010-2 decreased the expression of TGF-β1 and CTGF in the renal tissue after IRI

TGF-β1 has received much attention as a major inducer of EMT, and CTGF makes a significant contribution to RIF. Real-time PCR was performed to measure the RNA levels of TGF-β1 and CTGF. As shown in Fig. 4, significantly increased TGF-β1 and CTGF expression was observed in the IRI group compared to the sham group. In contrast, TJ-M2010-2 treatment inhibited the expression of TGF-β1 and CTGF. The protein expression levels of TGF-β1 and CTGF determined by Western blot analysis (B, C) were in accord with the RNA test results (A). The results are expressed as the mean ± SD. **P < 0.01; ***P < 0.001.

Fig. 3. TJ-M2010-2 prevents renal dysfunction after IR surgery. At twenty-eight days post-I/R (60 min ischemia of the left kidney), right nephrectomy was performed, and then serum creatinine (Cr) and blood urea nitrogen (BUN) concentrations were measured. (n = 6 per group). (***P < 0.001 versus sham, ###P < 0.001 versus IRI). Data are presented as the mean $\pm$ SD.

Fig. 4. TJ-M2010-2 decreased the expression of TGF- β 1 and CTGF in renal tissue after IR surgery. Real-time PCR (A) and Western blot (B) analyses for TGF- β 1 and CTGF expression in renal tissue. The Western blot results were examined by densitometry (C). The values represent the density of the α -SMA or CTGF band versus the β -actin band (%). **P < 0.01; ***P < 0.001.

TJ-M2010-2 decreased the expression of MMP2 and MMP9 but increased PAI-1 expression in the renal tissue after IRI

The activation of MMP2 and MMP9 by TGF- β 1 correlates with the degradation of the basement membrane that stimulates EMT. Immunohistochemical staining was performed to detect the expression of MMP2, MMP9 and PAI-1 28 days after IR. As shown in Fig. 5, the TJ-M2010-2 treatment group showed decreased expression of MMP2 and MMP9 but increased PAI-1 expression after IR surgery in the kidneys compared with that in the IRI group.

TJ-M2010-2 maintained the epithelial morphology of HK-2 cells treated with TGF- β 1

HK-2 cells showed a typical epithelial cuboidal shape with a cobblestone morphology. Treatment with TGF- β 1 caused distinct morphological changes with evidence of the gross elongation associated with filopodia formation. HK-2 cells were pretreated with TJ-M2010-2 for 30 min before the addition of TGF- β 1 (4 ng/ml) for 72 h, and this treatment largely maintained the epithelial morphology of the HK-2 cells (Fig. 6).

TJ-M2010-2 blocked the expression of α -SMA and the depression of E-cadherin induced by TGF- β 1 in HK-2 cells

To determine whether tubular epithelial cells can undergo conversion into myofibroblasts *in vitro*, we used TGF- β 1, a well-characterized profibrogenic cytokine, to induce EMT in HK-2 cells. E-cadherin is a tubular epithelial cell-cell adhesion receptor that is essential for the

Fig. 5. TJ-M2010-2 decreased the expression of MMP2 and MMP9 but increased PAI-1 expression in renal tissue after IR surgery. Immunochemical staining with antibodies against MMP2, MMP9 or PAI-1 (A). The expression of MMP2, MMP9 and PAI-1 was quantitatively measured (B). ** $P < 0.01$ and *** $P < 0.001$ versus sham, ## $P < 0.01$ and ### $P < 0.001$ versus IRI. (n = 6 per group). Original magnification 200 $\times$ with five fields assessed. Bar = 400 μ m in all panels.

Fig. 6. TJ-M2010-2 maintained the epithelial morphology of HK-2 cells treated with TGF- β 1. Normal HK-2 cells (A) were treated with TGF- β 1 (4 ng/ml) for 72 h

(B); HK-2 cells were pretreated with TJ-M2010-2 for 30 min before the addition of TGF- β 1 (4 ng/ml) for 72 h (C). HK-2 cells changed from a cuboidal to a spindle shape in response to TGF- β 1, whereas treatment with 20 μ M TJ-M2010-2 blocked this morphologic transformation. Cells were viewed using phase contrast microscopy. The pictures are representative of at least 3 independent experiments performed in duplicate. Original magnification 100 $\times$.

formation and maintenance of the homeostasis and architecture of the renal epithelia; α -SMA is the phenotypic marker of myofibroblasts. Both immunofluorescent staining (Fig. 7A) and Western blot analysis (Fig. 7B and C) indicated that TJ-M2010-2 dramatically abrogated the TGF- β 1-induced expression of α -SMA and simultaneously restored E-cadherin expression in HK-2 cells.

TJ-M2010-2 regulated EMT via the MyD88/NF- κ B/Snail pathway

As expected, the expression of Snail is a strong indicator of EMT. The activation of NF- κ B, which is tightly regulated by MyD88, was critical for the stabilization of Snail. To test the suppression of TGF- β 1-induced EMT by TJ-M2010-2 in HK-2 cells, TRAF6, I κ B α , p65 and Snail were detected by Western blotting. As shown in Fig. 8, markedly increased TRAF6, p65 and Snail expression and decreased I κ B α expression were observed in the TGF- β 1 group. However, TJ-M2010-2 inhibited the expression of TRAF6, p65 and Snail and upregulated I κ B α . The values are represented as the density of the TRAF6, I κ B α , p65 or Snail band versus

Fig. 7. TJ-M2010-2 blocked the expression of α -SMA and the depression of E-cadherin induced by TGF- β 1 in HK-2 cells. Fluorescence micrographs with staining for E-cadherin (green), α -SMA (red) and the nucleus (blue) in HK-2 cells at 400 $\times$. Scale bars= 200 μ m (A). Western blot analyses for α -SMA and E-cadherin expression in HK-2 cells (B). Densitometric analysis of the Western blot results (C). The values are represented as the density of the α -SMA or E-cadherin band versus that of the GAPDH band (%). ***P<0.001 versus control; ##P<0.01 versus TGF- β 1.

that of the GAPDH band (%). *P < 0.05, **P < 0.01, and ***P < 0.001 versus control; ##P < 0.01 and ###P < 0.001 versus TGF- β 1.

Fig. 8. TJ-M2010-2 regulates EMT via the MyD88/NF-κB/Snail pathway. Western blot analyses for TRAF6, IκBα, p65 and Snail expression in HK-2 cells (A). Densitometric analysis of the Western blot results (B). The values are represented as the density of the TRAF6, IκBα, p65 or Snail band versus that of the GAPDH band (%). *P<0.05, **P<0.01, ***P<0.001 versus control; ##P<0.01, ###P<0.001 versus TGF-β1.

Discussion

CKD is a prototypical example of progressive fibrosis leading to organ failure. It includes glomerulosclerosis and tubulointerstitial fibrosis; the latter is the better histological predictor of CKD progression [17] and contributes to the rate of kidney function decline. Therefore, preventing RIF could slow the pace of renal function deterioration [18].

EMT was defined as the stepwise loss of epithelial markers, such as E-cadherin, and the acquisition of mesenchymal markers, such as vimentin and α-SMA [7, 19]. Tubular epithelial cells (TECs) are postulated to contribute to the increase in the ECM through the process of EMT [20]. Importantly, the cells that underwent EMT could secrete some cytokines and recruit bone marrow-derived mesenchymal cells for myofibroblast differentiation, promoting fibrogenesis and persistent inflammation [21].

EMT can be induced or regulated by various factors. The changes in gene expression that contribute to the repression of the epithelial phenotype and activation of the mesenchymal phenotype involve master regulators, including SNAIL, TWIST and the zinc-finger E-box-binding (ZEB) transcription factors. TGF-β1 has received substantial attention as a major inducer of EMT during fibrosis [22]. TGF-β1-induced activation of the receptor complex leads to the activation of Smad2 and Smad3, which increase Snail expression and enhance the progression of EMT [23]. Deletion of Snail in renal epithelial cells significantly attenuates interstitial fibrosis in mouse models of TIF [24]. Moreover, epithelial cells engaged by TGF-β1 express essential MMPs, such as MMP2 and MMP9, for both basement membrane degradation and interstitial invasion, two steps that are ultimately required for successful completion of EMT or detachment and loss into the tubular lumen [3]. CTGF has recently been shown to be a mediator of the fibrogenic effects of TGF-β1, including proliferation, EMT promotion and ECM production [25]. For instance, it is well known that E-cadherin suppression during carcinoma cell EMT is primarily mediated by the Snail transcription factor [26], whereas its suppression in renal tubular epithelial cells during TGF-β1-induced EMT is clearly Snail-independent [27].

The TLR/MyD88/NF-κB pathway is closely related to EMT and renal fibrosis. In a previous study in the unilateral ureteral ligation model (UUO), TLR4^{-/-} and MyD88^{-/-} mice

showed reduced expression of α -SMA and attenuated renal interstitial fibrosis [10, 11]. The activation of NF- κ B plays a role in various chronic kidney diseases associated with inflammation and fibrosis [12]. The transcriptional activity of NF- κ B is required for TNF α -mediated Snail stabilization, which plays a fundamental role in the EMT process and can produce many cytokines, including TGF- β 1, TNF- α , IL-1 β , IL-6, and MCP1 [13]. Enhanced expression of MyD88 promoted EMT properties and tumor-initiating capabilities in HCC cells. MyD88 was found to be able to interact with p85, a regulatory subunit of phosphoinositide 3-kinase (PI3-K), independent of the TLR/IL-1R-mediated response and caused PI3-K/Akt murine thymoma viral oncogene homolog (Akt) activation, which resulted in the subsequent phosphorylation of glycogen synthase kinase-3 β and stabilization of Snail [28].

In an *in vivo* study, we found that TJ-M2010-2 attenuated renal fibrosis and prevented renal dysfunction after IR surgery, and this conclusion was supported by less positive staining with Masson's trichrome and α -SMA and vimentin, more expression of E-cadherin, a higher ratio of left kidney weight to right kidney weight, and lower levels of Cr and BUN in the TJ-M2010-2 group than in the IRI group. TJ-M2010-2 suppressed TGF- β 1, CTGF, MMP2 and MMP9 expression and increased PAI-1 expression in fibrotic kidneys. *In vitro*, TJ-M2010-2 reversed the process of TGF- β 1-induced EMT. Compared to the TGF- β 1 group, the TJ-M2010-2 group showed restored epithelial morphology in HK-2 cells, reduced expression of α -SMA and less depression of E-cadherin expression after induction by TGF- β 1. TJ-M2010-2 treatment inhibited the expression of TRAF6, p65 and Snail and upregulated I κ B α .

Conclusion

Our results demonstrated that MyD88 inhibitors may be a potential therapeutic agent to ameliorate RIF and treat patients with chronic kidney disease (CKD); the inhibitors can suppress TGF- β 1, CTGF, MMP2 and MMP9 expression in fibrotic kidneys and reverse the process of EMT induced by TGF- β 1.

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Disclosure Statement

The authors declare that they have no competing interests.

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