

Synthesis of peptides of Carapax Trionycis and their inhibitory effects on TGF- β 1-induced hepatic stellate cells

Chunling Hu, Xiaozhi Peng, Yinpin Tang, Yanwen Liu*

School of Pharmacy, Hubei University of Traditional Chinese Medicine, Hubei, China.

ABSTRACT: We previously identified the anti-fibrotic active ingredients from Carapax Trionycis as two peptides. Here, we synthesized these two peptides (peptide 1 and peptide 2) by a solid phase method and examined their effects on proliferation and activation of cultured hepatic stellate cells (HSC) which are the main ECM (extracellular matrix)-producing cells in fibrosis progression. We demonstrated that peptide 1 and peptide 2 significantly reduced HSC proliferation and activation in a dose dependent manner. Further, peptide 1 and peptide 2 could interfere with TGF-signaling by down-regulating Smad 3 phosphorylation. Thus, these synthetic peptides of Carapax Trionycis could inhibit proliferation and activation of HSC and might be used as a candidate for treatment of liver fibrosis.

Keywords: Synthesis of peptides of Carapax Trionycis, hepatic stellate cells, extracellular matrix (ECM), TGF- β /Smad

1. Introduction

Liver fibrosis is characterized by the accumulation of excess extracellular matrix (ECM), including collagens (1). Hepatic stellate cells (HSCs) play a pivotal role in liver fibrogenesis (2). HSCs survival is a hallmark of liver fibrosis. The major event in hepatic fibrogenesis is the transdifferentiation of quiescent HSCs to a myofibroblastic cell type. This process is driven by a variety of compounds, including growth factors (3). Hepatic fibrosis is a dynamic process caused by chronic liver injury induced by various etiological factors, such as viral, toxic, metabolic and auto-

immune agents, eventually leading to cirrhosis. The HSC-T₆ cell line has been widely used in fibrosis research because it retains all the features of activated stellate cells, including the expression of collagen I, collagen III, alpha-smooth-muscle actin (α -SMA), matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) (4). HSCs are responsible for collagen deposition in liver fibrosis. Therefore, in the present study we used HSC-T₆ cells to test the effect of two synthetic peptides.

Carapax Trionycis, as a traditional Chinese medicine, originated from the shell of *Trionyx sinensis* Wiegmann. Clinical experience indicated that traditional Chinese medicine Compounds containing Carapax Trionycis showed a curative effect when used for the treatment of liver fibrosis (5-7). Previous studies had demonstrated that extracts of Carapax Trionycis were able to protect liver against fibrosis in CCl₄ animal models (8,9), synthesized an extract of Carapax Trionycis and verified that it could inhibit activation and induce early apoptosis of HSC-T₆ (10,11). Our group isolated two active ingredients from Carapax Trionycis and proved their anti-fibrotic activity (12,13). Further we identified the active ingredients are two peptides with sequences of N-D-D-Y (526.2) and N-P-N-P-T (542.16) respectively (14). The Carapax Trionycis natural source is very limited and extractive procedures are very costly, which widely restricts the application of these active peptides. Here, we synthesized peptides 1 and 2 based on the sequences of active ingredients by a solid phase method. However, how the synthetic peptides inhibit activated HSC-T₆ proliferation and its exact mechanism remains unknown.

The aim of this study was to determine whether synthetic peptides 1 and 2 affect the survival of cultured HSC-T₆ cells and collagen I, collagen III, MMP-1, and TIMP-1 content, and to examine whether the protein expression of cultured HSC-T₆ cells changes following administration of the synthetic peptides. Also, for the mechanism study, we identified whether synthetic peptides show anti-fibrotic effects by decreasing p-Smad 3 expression, at least in part, via the TGF- β ₁/Smad pathway as well as by the elimination of the extracellular matrix.

*Address correspondence to:

Dr. Yanwen Liu, School of Pharmacy, Hubei University of Traditional Chinese Medicine, 1 Huangjia River West Road, Wuhan, Hubei 430065, China.
E-mail: huchunling2007@126.com

2. Materials and Methods

2.1. Materials

Fmoc-AA-OH, Wang resin, and TBTU were acquired from Gil (Shanghai, China). MALDI-TOF MS was supplied by Shimadzu Co. (Tokyo, Japan). The HSC-T₆ cell line was purchased from Fuxiang Biological Co., Ltd (Shanghai, China). Fetal calf serum was purchased from Sanli Biological Co., Ltd (Wuhan, China). Trypsin and High-DMEM were purchased from Gibco (Gibco, NY, USA). TGF- β_1 and MTS were acquired from Sigma (Sigma-Aldrich, USA). Collagen I, collagen III, MMP-1, and TIMP-1 ELISA kits were obtained from R&D (Minneapolis, MN, USA). Monoclonal anti-collagen I, collagen III, Smad 3, p-Smad 3, TIMP-1, and MMP-1 antibodies and horseradish-peroxidase (HRP)-conjugated goat anti-rabbit Ig G secondary antibody were purchased from Santa Cruz (Santa Cruz Biotechnology, CA, USA). The SuperSignal Substrate Chemiluminescence Kit was from Pierce (Rockford, USA).

2.2. Solid-phase synthesis of active peptides of *Carapax Trionycis*

The introduction of solid phase peptide synthesis (SPPS) in 1963 by Bruce Merrifield opened the door for researchers to prepare structurally diverse peptides. In this study, SPPS was used with Wang resin as the carrier and *N*-Fmoc protected α -amino acids as the materials based on the sequences of the active ingredients from *Carapax Trionycis*. After condensation with the reagentmix of TBTU/NMM, and deprotection with 20% piperidine, the synthetic peptide crude products were removed from the Wang resin by the cleavage reagents TFA/TIS/H₂O. By analysis and purification with RP-HPLC, we finally obtained two bioactive peptides 1 and 2. Synthetic peptides of *Carapax Trionycis* were determined by MALDI-TOF MS analysis. The sample was obtained in a volume of 0.5 μ L on the target board of MALDI. After natural drying at room temperature, the sample was obtained from a mixed solution of 0.1% TFA and 50% ACN again, and a blank control was set up at the same time.

2.3. Cell culture and MTS assay

HSC-T₆ cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and incubated at 37°C in a 5% CO₂ humidified atmosphere. HSCs were digested with 0.25% trypsin and adjusted to 6 \times 10⁴ cells/mL when the HSCs were in the exponential growth phase. The cells were planted into 96-well plates, 0.2 mL/well, 4-wells/group and incubated overnight.

Several studies have provided ample evidence that TGF- β_1 is a strong stimulant of hepatic stellate cell proliferation (15). Therefore, we used TGF- β_1 to stimulate HSC-T₆ cells. Cells were pre-treated with TGF- β_1 (800

pg/mL) in each well for 2 h and then treated separately with serum containing synthetic peptides 1 and 2.

Control group cells were cultured in only serum medium. The stimulant group cells were cultured in TGF- β_1 medium. The experimental group cells were treated with synthetic peptides 1 and 2 at different concentrations (0.01, 0.05, 0.1, and 0.5 mg/mL) in TGF- β_1 medium. After cells were incubated for 72 h, the culture medium was removed, and 20 μ L MTS/PMS was added in each well for 1 h. OD values were analyzed by one-way ANOVA test. The rate of inhibition was calculated as follows:

$$\text{IR (\%)} = \frac{[(\text{control value} - \text{blank}) - (\text{test value} - \text{blank})]}{(\text{control value} - \text{blank})} \times 100.$$

2.4. Enzyme-linked immunosorbent assay (ELISA)

HSC-T₆ cells were trypsinized and seeded in triplicate at a density of 6 \times 10⁴ cells/mL in six-well plates for 24 h. Before experiments, the medium was changed to serum-free medium and the cells were incubated for 24 h. They were pre-treated with TGF- β_1 (800 pg/mL) for 2 h and then treated with peptides 1 and 2 (0.01, 0.05, 0.10 mg/mL) for 72 h before analysis by ELISA assay. Cell culture supernatants were collected. Collagen I, collagen III, MMP-1, and TIMP-1 were measured using ELISA kits according to the procedure recommended by the manufacturer.

2.5. Western blot analysis

HSC-T₆ cells were treated as described for ELISA. Western blot analysis was then performed with antibodies directed against collagen I, collagen III, Smad 3, p-Smad 3, TIMP-1, and MMP-1 to detect the expression of these proteins. Control, TGF- β_1 -treated and TGF- β_1 + synthetic peptides-treated (0.01, 0.05, 0.10 mg/mL) HSC-T₆ cells were lysed with RIPA buffer (containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 50 μ g/mL aprotinin), to which 200 μ g/mL phenylmethylsulfonyl fluoride was added immediately after addition of the lysis buffer. Protein samples (30 μ g each) were separated electrophoretically on 10% SDS polyacrylamide gels, and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline for 1 h at room temperature and incubated overnight at 4°C with primary anti-rat antibodies. After washing, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary goat anti-rabbit IgG. The bands were visualized using a SuperSignal Substrate Chemiluminescence Kit.

2.6. Statistical analysis

Each experiment was repeated a minimum of three times. Results were expressed as Mean \pm SD. Statistical analysis was performed by one-way ANOVA test. *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Analysis and identification of synthetic peptides of *Carapax Trionycis*

Two peptides of *Carapax Trionycis* 1 and 2 were synthesized using a classical solid phase method from right to left based on the sequence (sequence 1 is NDDY; sequence 2 is NPNPT) of active ingredients from *Carapax Trionycis*. By the analysis and purification from RP-HPLC, the purity of synthetic peptides was over 98%, chromatographic analysis is shown in Figure 1.

Synthetic peptides of *Carapax Trionycis* were determined by MALDI-TOF MS analysis. The molecular weight of synthetic peptide 1 was 525.981 (Figure 2), which was identical with primary active ingredient (NDDY, molecular weight: 526.2) from *Carapax Trionycis*. The molecular weight of synthetic peptide 2 was 542.083 (Figure 2), which was identical with primary active ingredient (NPNPT, molecular weight: 542.16) from *Carapax Trionycis*. The results suggested that synthetic peptides 1 and 2 had the same sequence structure as the corresponding ingredients from *Carapax Trionycis*.

3.2. Effect of synthetic peptides on HSC-T₆ cells proliferation

To test if our synthetic peptides could inhibit proliferation of cultured hepatic stellate cells, we first treated HSC-T₆ cells with synthetic peptides at various concentrations,

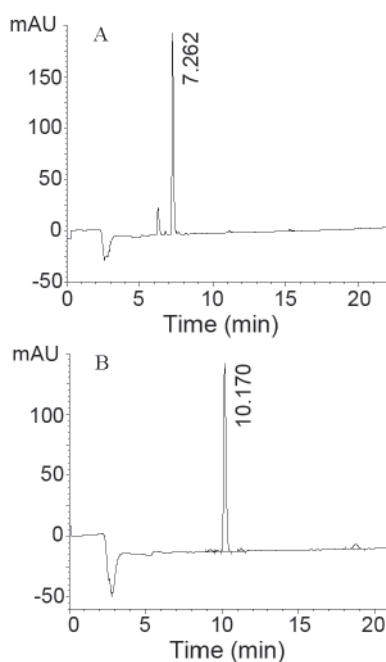


Figure 1. RP-HPLC chromatograms of synthetic peptide 1 (A) and peptide 2 (B). Column: Zorbox Eclipse XDB-C18 (250 × 4.6 mm, 5 μm); mobile phase: acetonitrile (0.05% TFA)-water (0.05% TFA) (acetonitrile: 10-50% in 30 min); flow rate: 1.0 mL/min; detection wavelength: 220 nm.

then we monitored cell proliferation by cell number quantification. As shown in Figure 3, after treatment with synthetic peptides 1 and 2 (0.01 ~ 0.60 mg/mL) for 72 h, HSC-T₆ proliferation was significantly inhibited in a dose dependence manner. The IC₅₀ values of synthetic peptides 1 and 2 for HSC-T₆ cells at 72 h were 0.53 mg/mL and 0.52 mg/mL respectively. Therefore, we chose concentrations of 0.01, 0.05, and 0.10 mg/mL for further analysis.

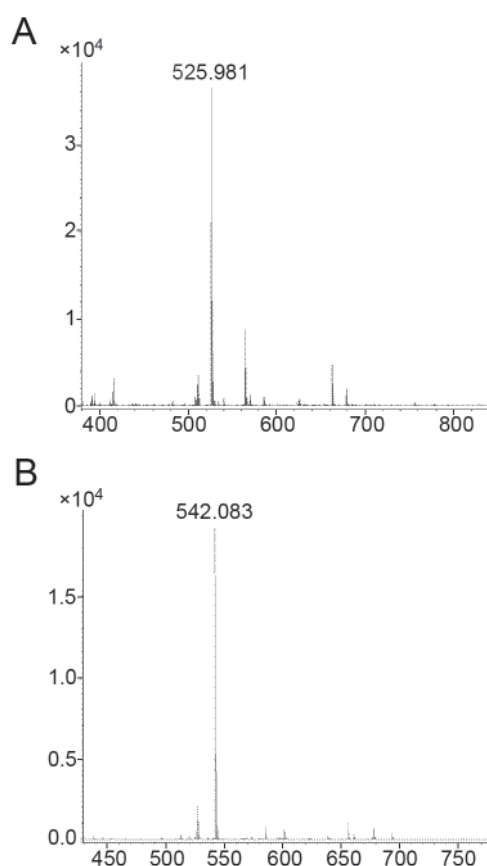


Figure 2. Identification of the synthetic peptides by mass spectrometry analysis. MS/MS spectrum indicated that the major peaks were molecular ion peaks. The m/z 525.981 signal was the molecular ion with loss of a hydrogen ion of synthetic peptide 1 (A) and the m/z 542.083 signal was the molecular ion with loss of a hydrogen ion of synthetic peptide 2 (B). Mass to charge ratio is denoted, m/z.

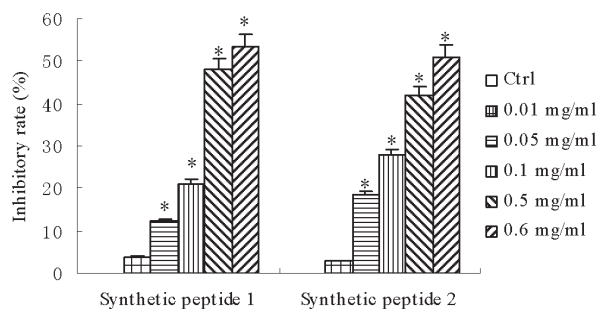


Figure 3. Effect of synthetic peptides on HSC-T₆ proliferation. The inhibitory rate (%) of synthetic peptides 1 and 2 on HSC-T₆ proliferation increased significantly following increase in the synthetic peptides 1 and 2 concentration. * $p < 0.01$ compared with control group ($n = 4$).

3.3. Effect of synthetic peptides on profibrotic mediators and ECM degradation modulators

The extent of liver fibrosis depends on rates of hepatic collagen synthesis and degradation. Collagens are the main components of ECM. Matrix degradation is catalyzed by the activity of MMPs. The activities of MMPs are inhibited by TIMPs (16). So next, we examined the collagen I, collagen III, and TIMP-1 expression levels in the cell culture supernatant after peptide 1 and peptide 2 treatment. As show in Figure 4, their contents were significantly decreased in a dose

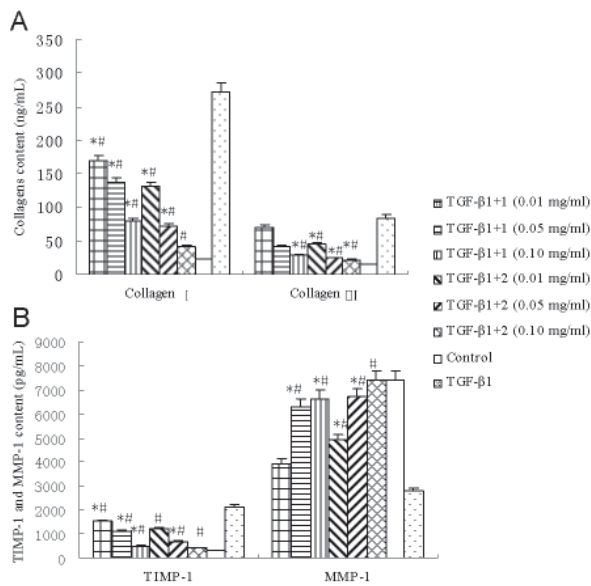


Figure 4. Effects of synthetic peptides 1 and 2 on the content of collagen I, collagen III, TIMP-1, and MMP-1 in HSC-T₆ cells. The cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and were pre-treated with TGF-β1 (800 pg/mL) in each well for 2 h, and then treated separately with serum containing synthetic peptides 1 and 2 for 72 h before analysis with ELISA assay. Cell culture supernatants were collected. * $p < 0.01$ compared with the control group, # $p < 0.01$ compared with the TGF-β1 group ($n = 4$).

dependent manner except for MMP-1. Further analyses indicated that the change of content was greater for 0.05 and 0.10 mg/mL synthetic peptides than for control group and for TGF-β₁ group. Differences among the various concentration groups were notable.

To further test whether the change of collagens was related to the content of MMP-1 and TIMP-1, we determined the protein expression of these fibrogenic genes and found that the levels of collagen I, collagen III, TIMP-1, and α-SMA proteins were lower in the TGF-β₁ + synthetic peptides groups than in the TGF-β₁ groups except for MMP-1 (Figure 5) by Western blot.

3.4. Inhibitory effect of synthetic peptides on TGF-β₁-induced hepatic stellate cells was associated with TGF-β₁/Smad activation

TGF-β₁ is the most potent fibrogenic cytokine described for HSCs and can stimulate HSCs activation and ECM production. We next examined whether the TGF-β₁/Smad signaling pathway is involved in activated HSC-T₆ cells. As demonstrated by Western blot, TGF-β₁ stimulation caused marked increases in expression of p-Smad 3 when compared to the control group, but co-treatment with synthetic peptides 1 and 2 eliminated these changes (Figure 6B). However, the level of Smad 3 protein did not differ significantly in the various groups (Figure 6A).

4. Discussion

During fibrosis progression, the main ECM-producing cells are activated HSCs, which secrete ECM proteins including collagen I and III (17). Cross-remodeling of the ECM in the fibrotic liver is likely to be regulated by the synthesis and enzymatic degradation of the ECM. Expression of MMP-1 can be important in mediating HSC proliferation, potentially by regulating ECM turnover (18) and is one of the major causes of liver fibrosis (19,20). Also, the activities of MMPs are

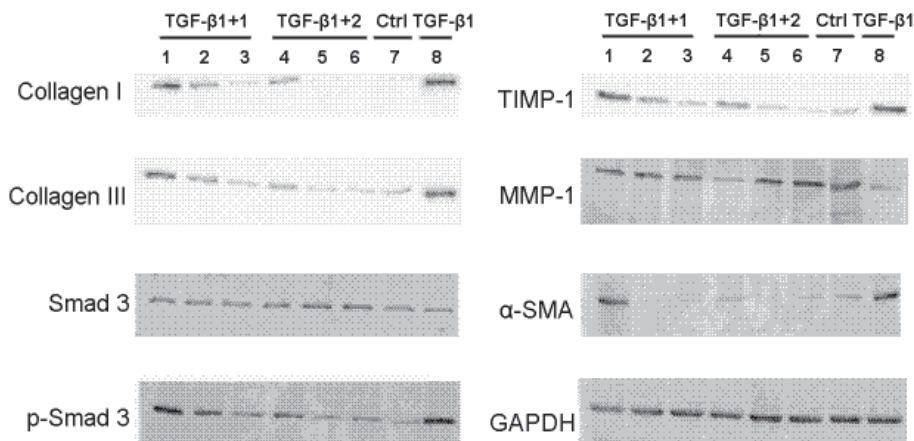


Figure 5. The protein bands for collagen I, collagen III, Smad 3, p-Smad 3, TIMP-1, MMP-1, and α-SMA. The expression levels of above proteins were normalized by an internal control of GAPDH.

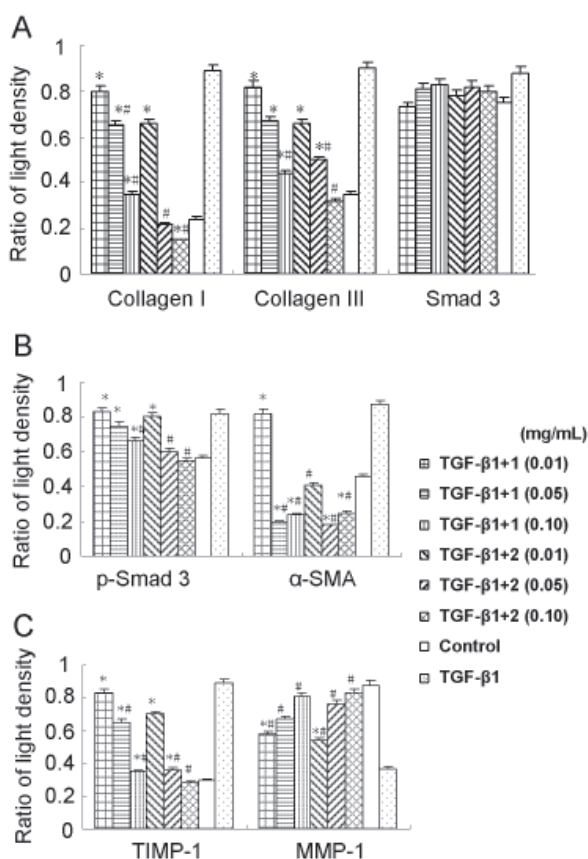


Figure 6. Western blot analysis of collagen I, collagen III, Smad 3, p-Smad 3, TIMP-1, MMP-1, and α -SMA. Panels A-C shows the levels of collagen I, collagen III, p-Smad 3, α -SMA, TIMP-1, and MMP-1 proteins. A drastic difference in protein expression between the TGF- β 1 + synthetic peptides groups and TGF- β 1 groups was observed. * $p < 0.01$ compared with the control group, # $p < 0.01$ compared with the TGF- β 1 group ($n = 4$).

inhibited by TIMPs. Especially, TIMP-1 increases early following liver injury and persists as fibrosis develops (21). Our *in vitro* study showed that synthetic peptides 1 and 2 promoted recovery from liver fibrosis not only by the removal of collagens (Figure 4A), but also by the reduction of TIMP-1 content and the improvement of MMP-1 content (Figure 4B).

A previous study reported that α -SMA occurs in vascular smooth muscle, the luminal portions of bile ducts, and the periphery of hepatocytes, but not in lobules and rarely in HSCs in normal rat liver. However, α -SMA-immunopositive cells are increased in number and reactivity in injured livers. Therefore, α -SMA is accepted as a typical marker of activated HSCs during the fibrotic process and acute liver injury. To evaluate HSCs activation, we performed Western blots for α -SMA. The results indicated that expression of α -SMA protein was reduced in the TGF- β 1 + synthetic peptides groups compared with the TGF- β 1 groups (Figure 6B). Besides, TIMP-1, collagen I and collagen III expression were decreased concurrently with decreased α -SMA expression in cultured HSC-T₆ cells (Figures 6A and 6C).

TGF- β ₁ is the most potent fibrogenic cytokine described for HSCs (22,23), and can stimulate HSCs activation and ECM production. Disruption of TGF- β ₁/Smad signaling markedly reduces fibrosis in experimental models (24,25). In our study, we examined the phosphorylation levels of Smad 3 and found that TGF- β ₁ stimulation led to elevated levels of Smad 3 phosphorylation in cultured HSC-T₆ cells, whereas co-treatment with synthetic peptides 1 and 2 eliminated these changes (Figure 6B). Interestingly, the level of Smad 3 protein wasn't significantly different (Figure 6A). These results suggested TGF- β ₁/Smad signaling may be associated with the therapeutic benefits of the peptides. Therefore, it is likely that inhibition of p-Smad 3 expression plays a major role in the down regulation of collagen production in HSCs (26,27).

In summary, our data indicated that synthetic peptides 1 and 2 efficiently inhibited cultured HSC-T₆ cell activation and proliferation by decreasing p-Smad3 expression, at least in part, via the TGF- β ₁/Smad pathway as well as by the elimination of the extracellular matrix. The present study provides a foundation for the prevention and treatment of liver fibrosis.

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