Original Article

Induction of apoptosis of HepG2 cells by sophoridine via miR-144/cyclin B1 signaling

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Abstract: Objective: The aim of this study was to clarify the effect and possible mechanism of sophoridine on the induction of apoptosis in HepG2 cells. Methods: After treating HepG2 cells with sophoridine, cell viability was measured via MTS assay and apoptosis was investigated via annexin V-propidium iodide staining. After collecting cell samples, the expression of miR-144 and cyclin B1 (CCNB1) was detected via quantitative polymerase chain reaction and western blotting. Subsequently, differentially expressed miR-144 and CCNB1 were transfected into HepG2 cells to detect changes in cell proliferation and apoptosis. Results: Sophoridine reduced the proliferation and increased the apoptosis, migration, and invasion of HepG2 cells in a concentration-dependent manner. Sophoridine also increased miR-144 levels and reduced CCNB1 levels in a concentration-dependent manner. CCNB1 interference or miR-144 mimic overexpression reduced the proliferation of HepG2 cells, while CCNB1 interference or miR-144 mimic overexpression increased apoptosis of HepG2 cells. The effects of Sophoridine on cell proliferation and apoptosis was reversed by CCNB1 interference or miR-144 overexpression. Conclusion: Sophoridine induced apoptosis in HepG2 cells by targeting miR-144/CCNB1 signaling.

Keywords: Apoptosis, HepG2, sophoridine, signaling

Introduction

Currently, cancer mortality rate is second only to that of cardiovascular disease. Hepatocellular carcinoma (HCC) is one of the most serious malignancies currently threatening human health [1, 2]. According to reports, the mortality rate of liver cancer in Chinese men and women in 2011 was ranked 2nd and 5th, respectively, across all fatal diseases. Surgical treatment is the preferred treatment method for liver cancer, but the high recurrence rate and low surgical resection rate remain an outstanding problem. Certain patients do not receive prompt treatment and are only diagnosed at an advanced stage, thus losing the opportunity of undergoing surgical treatment. In recent years, targeted drug therapy and chemotherapy have also become important treatment strategies for patients with liver cancer. However, the toxic side effects associated with long-term treatment are important. Moreover, some patients develop drug resistance, which limits clinical application. It is therefore important to carry out research and develop new anti-HCC drugs.

Natural medicines are important for the prevention and treatment of human diseases. The development of new drugs with active ingredients is a shortcut for the study of anti-tumor drugs. Sophoridine is a monomeric alkaloid extracted from Sophora alopecuroides and is considered to be a potential natural anti-tumor product with high application value [3]. It has inhibitory effects on various tumor cells such as lung cancer, sarcoma, and epidermis. However, the effect of sophoridine on HCC and its related mechanisms remain unclear.

A variety of signaling pathways are involved in the process of staging, recurrence, and metastasis of liver cancer. Among them, the miR-144/cyclin B1 (CCNB1) regulatory axis plays an important role in the pathogenesis of liver cancer [4]. MiR-144 is a tumor suppressor gene whose expression is reduced in HCC. CCNB1 is a product of a proto-oncogene. MiR-144 can negatively regulate the expression of CCNB1, thereby inhibiting the activity of mitogen-promoting factors and blocking cell division and proliferation. Therefore, the miR-144/CCNB1
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signaling pathway may be considered as a potential new therapeutic target for the treatment of liver cancer. Based on this, we investigated the effect and possible mechanism of sophoridine on the apoptosis of HepG2 cells.

Material and methods

Materials and reagents

Human HCC HepG2 cells were purchased from the Shanghai Cell Storage Center, Chinese Academy of Sciences, Shanghai, China. A cell cycle and apoptosis assay kit was purchased from the Biyuntian Biotechnology Research Institute, Nantong, China. The electrochemiluminescence (ECL) Immunoblot Substrate Kit was purchased from Millipore (Burlington, MA, USA). CCNB1 antibodies were purchased from Abcam, Cambridge, UK. The MTS cell proliferation kit was purchased from Sigma (St. Louis, MO, USA). Sophoridine was purchased from Ningxia Bauhinia Flower Co., Ltd., Ningxia, China.

Preparation of sophoridine solution [5]

An amount of 32 mg sophoridine was accurately weighed, dissolved in 100 mL phosphate buffered saline (PBS), filter-sterilized, and stored at 4°C. Before use, this solution was diluted with RPMI-1640 to prepare solutions at concentrations of 5, 10, and 20 μg/mL.

Cell culture and treatments

Human hepatoma HepG2 cells were cultured in a petri dish and kept in an incubator at 37°C, in an atmosphere with 5% CO₂ and saturated humidity. The medium used was 90% Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were sub-cultured using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and logarithmic growth-phase cells were used in all experiments. HepG2 cells were divided into five groups: the control group (untreated cells), 5 μg/mL sophoridine treatment group, 10 μg/mL sophoridine treatment group, 20 μg/mL sophoridine treatment group, and positive control group (cells treated with 100 μg/mL 5-fluorouracil). This concentration was set to 100 μg/mL after preliminary experiments.

Lentivirus-infected cells

Recombinant lentiviral particles expressing CCNB1 small interfering RNA (siRNA) or control short siRNA, as well as miR-144 mimic and miR-control were purchased from Shanghai Jima Biotech Co., Ltd. HepG2 cells were cultured to varying degrees of confluency and infected with lentiviral particles in complete medium for 48 h as recommended by the manufacturer’s instructions. To increase infection efficiency, cells were co-treated with the cationic polymer polybrene (8 μg/mL aqueous solution). Neither siRNA nor polybrene affected cell viability. siRNA showed no off-target effects and no effect on HepG2 adhesion, shape, or viability at the indicated multiplicity of infection (MOI) and for the duration of treatment.

Cell proliferation

Logarithmic growth-phase cells (100 μL) were added to a 96-well plate at a density of 3×10⁴ cells/mL. Cells were treated according to each treatment group. After 72 h, the medium was aspirated, MTS reagent was added according to the kit instructions, and the absorbance was finally measured at 490 nm using a microplate reader to calculate the proliferation rate. Fluorescence microscopy was then performed to detect changes in cell morphology. Logarithmic growth-phase cells (100 μL) were added to 96-well plates at a density of 3×10⁴ cells/mL. Cells were treated according to each treatment group. After 24 h, the medium was aspirated, the cells were centrifuged, washed four times using PBS solution, and stained with 20 μL of 10 mg/L Hoechst 33342 at 37°C. After incubating for 15 min, the cells were centrifuged at 1000×g for 10 min, the supernatant was discarded, and the cells were washed with PBS solution. Three slides were made from each group for observation, and the effect of sophoridine on the morphology of HepG2 cells was examined via fluorescence microscopy.

Cell migration assay

Logarithmic growth-phase cells (100 μL) were seeded in a 6-well plate at a density of 2.5×10⁵ cells/mL and incubated at 37°C overnight. When the cells had grown to cover the bottom of the well plate, a scratch wound was made. A 10-μL tip was held perpendicular to the well bottom, and a cross-shaped scratch was made without tilting the plate. The cells were then washed twice with PBS solution to remove any cells under the scratches, and fresh serum-free medium was added along with the corresponding drugs. The scratch position was marked
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and a photo was taken. Cells were then incubated at 37°C for 24 h in an atmosphere with 5% CO₂ and saturated humidity. Samples were collected at the marked position and photographed to calculate the scratch-healing rate.

Cell invasion assay

A single-cell suspension of HepG2 cells in the logarithmic growth phase was made with a density of 1.0×10⁵ cells/mL. Cells were added to a 6-well plate at a volume of 2 mL per well and incubated at 37°C. Cells were treated with drug for 48 h. Matrigel (100 μL) was diluted with serum-free medium and added to each transwell upper chamber and placed in a 37°C incubator for approximately 1 h to render the mixture gelatinous. FBS (10%) was added to the lower chamber, the cells were washed twice with PBS, and the cell concentration was adjusted to 5.0×10⁵ cells/mL with serum-free medium. Single-cell suspension (100 μL) was added to the upper chamber and cells were cultured at 37°C for 24 h. Subsequently, the filter was removed, and the gel on the membrane and upper-chamber cells was gently wiped off using a cotton swab. Cells were fixed with 4% paraformaldehyde for 15 min, stained with 0.1% crystal violet for 10 min, and washed twice with PBS. The cell membrane was observed under a microscope and photographed. Three fields of view were randomly selected from each well to count the number of invading cells and photographed.

Flow cytometry to detect changes in cell cycle and apoptosis

Cells (100 μL) were added to a 6-well plate at a density of 5×10⁶ cells/mL and incubated at 37°C overnight. Cells were then treated with the appropriate drugs, incubated at 37°C for 24 h, and collected after digestion. After staining with propidium iodide (PI)/annexin V-fluorescein isothiocyanate (FITC), the cell cycle was investigated via flow cytometry after incubation for 30 min in the dark.

Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted from the cells using TRIzol reagent. Reverse transcription was performed using the PrimeScript RT kit (Promega, Madison, WI, USA). The PCR reaction conditions were as follows: 94°C for 4 min, 94°C for 40 s, 52°C for 40 s, and 72°C for 40 s, for a total of 40 cycles. qPCR was performed on an ABI 7500 instrument using a SYBR Premix ExTaq (Takara). Analysis of the PCR results was performed using the 2-ΔΔCt method. U6 was used as the internal standard. The PCR primers were designed and synthesized by Shanghai Biotech Co., Ltd. The primer sequences were as follows: miR-144, upstream 5’-GG-GAGATCAGAGGTGATT-3’, downstream 5’-GTG-CAGGGTC-CGAGGT-3’; upstream of U6 5’-CTCGCTTTCGG-CAGCACA-3’, downstream 5’-AACGCTTACGAA-TTGGCT-3’.

Western blotting

After protein separation, the expression of CCNB1 was detected via western blotting. The western blot method is briefly described as follows: cellular proteins were analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk, the anti-CCNB1 monoclonal antibody (1:100) was used for incubation, and the GAPDH (1:200) monoclonal antibody was used as the internal standard. The protein expression was examined via ECL. The protein bands obtained were quantified using Image J software and protein levels were analyzed.

Statistical analysis

The difference in the expression of cellular miRNA and protein between two groups was determined using an unpaired t test (two-tailed) and the data are expressed as means ± standard deviation (SD). Cell growth and apoptosis were compared using one-way analysis of variance (ANOVA) with post hoc Bonferroni test. p < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 6.0 and SPSS 18.0 statistical software.

Results

Effect of sophoridine on the proliferation and apoptosis of HCC HepG2 cells

To clarify the effect of sophoridine on liver cancer cells, we first examined the effect of sophoridine on the proliferation and apoptosis of hepatoma HepG2 cells. Figure 1A shows that
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Figure 1. Effect of sophoridine on the proliferation and apoptosis of hepatocellular carcinoma (HCC) HepG2 cells. A. Sophoridine reduced the proliferation of liver cancer cells in a concentration-dependent manner; B. Sophoridine increased the apoptosis of liver cancer cells in a concentration-dependent manner. 5-Fu, 5-fluorouracil; C. FCM figures for cell apoptosis of liver cancer cells. * indicates p < 0.05 compared to the untreated control; ** indicates p < 0.01 compared to the untreated control; # indicates p < 0.05 compared with treated group.

Figure 2. Effect of sophoridine on apoptosis morphology in HepG2 cells. A. Untreated cells; B. Cells treated with 5 μg/mL sophoridine; C. Cells treated with 15 μg/mL sophoridine; D. Cells treated with 20 μg/mL sophoridine; E. Cells treated with 100 μg/mL 5-Fu. 5-Fu, 5-fluorouracil.

the effects of sophoridine are concentration-dependent. Sophoridine treatment reduced the proliferation of liver cancer cells, and cell proliferation rates were 100.0% ± 5.8%, 93.2% ± 5.7%, 74.5% ± 4.9%, and 60.3% ± 6.7% at concentrations of 0, 5, 10, and 20 μg/mL sophoridine, respectively. The positive control group showed a cell proliferation rate of 59.6% ± 5.7% at a concentration of 100 μg/mL 5-Fu, which was similar to that after treatment with 20 μg/mL sophoridine (Figure 1B). Sophoridine increased the apoptosis of liver cancer cells in a concentration-dependent manner. At concentrations of 0, 5, 10, and 20 μg/mL sophoridine, the percentage cell apoptosis was 5.0% ± 3.8%, 14.8% ± 3.0%, 42.4% ± 6.7%, and 59.6% ± 4.7% respectively. In the positive control group, cells treated with 100 μg/mL 5-Fu showed a percentage apoptosis of 64.8% ± 7.1%, which was similar to that in cells treated with 20 μg/mL sophoridine. This result suggests that sophoridine reduced the proliferation and increased the apoptosis of liver cancer cells in a concentration-dependent manner.

Changes in cell morphology

To clarify the effect of sophoridine on the apoptosis of HCC HepG2 cells, we performed fluorescence microscopy (Figure 2). The liver cancer cells displayed normal morphology, with round nuclei, and evenly distributed blue flou-
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In order to clarify the inhibitory effect of alkaloids on the migration of hepatoma HepG2 cells, we used a cell scratch test to obtain the results. As shown in Figure 3, sophoridine inhibited the migration of liver cancer cell lines in a concentration-dependent manner. The cell migration rates at 5, 10, and 20 μg/mL were (61.9 ± 5.7)%, (46.5 ± 6.7)%,(36.1 ± 5.9)%, and (29.6 ± 4.4)%, respectively. The cell mobility at 100 μg/mL was (32.7 ± 6.4)%, similar to the effect of sophoridine. This result suggests that sophoridine can inhibit the migration of liver cancer cell lines in a concentration-dependent manner.

**Inhibitory effect of sophoridine on HepG2 cell invasion**

To clarify the inhibitory effect of alkaloids on HCC cell invasion, we performed a cell invasion assay. As shown in Figure 4, sophoridine inhibited the invasion of liver cancer cells in a concentration-dependent manner. The number of invasive cells at sophoridine treatment concentrations of 0, 5, 10, and 20 μg/mL was 167.7 ± 15.9, 147.03 ± 12.5, 131.7 ± 10.6, and 109.5 ± 10.7, respectively. The number of invasive cells in the positive control group treated at a concentration of 100 μg/mL 5-Fu was 112.7 ± 11.3, which was similar to that in the sophoridine-treated groups. This result suggests that sophoridine inhibited the invasion of liver cancer cells in a concentration-dependent manner.

**Cell cycle changes induced by sophoridine**

To clarify the effect of sophoridine on cell cycle changes in liver cancer cells, we used flow cytometry (Figure 5). After 24 h, the proportion of cells in G1 phase increased in a sophoridine concentration-dependent manner. This suggests that sophoridine arrests HepG2 cells in the G0/G1 phase in a concentration-dependent manner (Table 1, Figure 5).

**Effect of sophoridine on the miR-144/CCNB1 signaling pathway**

The miR-144/CCNB1 signaling pathway was previously reported to participate in the biological behavioral changes of hepatoma HepG2 cells, and therefore, we examined this pathway. Our results show that sophoridine increased the level of miR-144 expression in a concentration-dependent manner, with 20 μg/mL sophoridine treatment producing effects similar to those of treatment with 10 μg/mL 5-Fu (Figure 6A). Furthermore, our results showed...
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Table 1. Effect of sophoridine on cell cycle of HepG2 cell line

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<tr>
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<th>G0/G1 phase cell ratio (%)</th>
<th>S phase cell ratio (%)</th>
<th>G2/M phase cell ratio (%)</th>
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<tr>
<td>Sophoridine 0 µg/mL group</td>
<td>63.04 ± 3.50</td>
<td>26.39 ± 1.32</td>
<td>10.57 ± 2.88</td>
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<td>Sophoridine 5 µg/mL group</td>
<td>69.21 ± 4.65</td>
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<tr>
<td>Sophoridine 10 µg/mL group</td>
<td>76.22 ± 3.03</td>
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<td>5.74 ± 1.88</td>
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<tr>
<td>Sophoridine 20 µg/mL group</td>
<td>89.65 ± 3.31</td>
<td>6.72 ± 1.65</td>
<td>3.63 ± 1.94</td>
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Effect of CCNB1 interference on the proliferation and apoptosis of HCC HepG2 cells

As CCNB1 was reported to mediate some of the effects of sophoridine on hepatoma cell lines [5], we further investigated changes in cell proliferation and apoptosis after RNA interference (RNAi) with CCNB1. Our results showed that CCNB1 interference redtosis of HepG2 cells (Figure 7). This result further confirmed that CCNB1 is involved in the regulation of the biological behavior of liver cancer cells.

Effect of miR-144 overexpression on proliferation and apoptosis of HCC HepG2 cells

As miR-144 was reported to mediate some of the effects of sophoridine on hepatoma cell lines, we further investigated changes in cell proliferation and apoptosis after miR-144 mimic overexpression. Our results showed that miR-144 mimic overexpression reduced the proliferation and increased the apoptosis of HepG2 cells (Figure 8). This result further confirmed that miR-144 is involved in the regulation of the biological behavior of liver cancer cells.

Regulation of HepG2 cell proliferation and apoptosis by CCNB1/MiR-144 pathway

In order to further observe whether sophoridine inhibits the proliferation effect of hepatoma cell line by regulating miR144/CCNB1 signaling pathway, we further observed the proliferation and apoptosis of hepatocellular carcinoma cell line HepG2 by sophoridine. Our results show that sophoridine can reduce the proliferation of HepG2 cells and increase cell apoptosis, and its effect is similar to the effect of CCNB1 interference or miR-144 overexpression on HepG2.
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Figure 6. Effect of sophoridine on miR-144/CCNB1 signaling. A. Sophoridine can reduce the level of miR-144 in a concentration-dependent manner; B. Sapoin can reduce the level of CCNB1 in a concentration-dependent manner. * indicates p < 0.05 compared to the untreated control; ** indicates p < 0.01 compared to the untreated control; # indicates p < 0.05 compared with treated group.

Figure 7. Effect of CCNB1 interference on the proliferation and apoptosis of HCC HepG2 cells. A. CCNB1 interference reduced the proliferation of HepG2 cells; B and C. CCNB1 interference increased the apoptosis of HepG2 cells. * indicates p < 0.05 compared to blank group siRNA. * indicates p < 0.05 compared to control siRNA.
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HCC is a common malignant tumor with a high incidence and poor prognosis [6, 7]. Surgery has been considered the first line of treatment for HCC for the past few decades [8]. However, most patients with HCC are diagnosed only when the disease is already advanced and therefore have lost the opportunity of undergoing surgery [9, 10]. So far, improving the survival of patients with HCC has remained a challenge [11]. It is therefore necessary to identify new molecular targets to promote early diagnosis and improve targeted therapy of HCC to achieve better survival of patients with HCC.

Sophoridine is an alkaloid with a tetracyclic quinolizidine skeleton structure that is extracted from bitter bean and is used in northwest Chinese herbal medicine. Many studies have shown its anti-tumor [12], anti-arrhythmia [13], and anti-inflammatory properties, among other pharmacological effects. Experimental studies [14, 15] have revealed that sophoridine has significant inhibitory effects on a variety of animal transplanted tumors such as Lewis lung cancer cells, and that its inhibition rate could reach...
Sophoridine inhibits apoptosis of HepG2
Sophoridine inhibits apoptosis of HepG2

The results of the current study showed that sophoridine reduced the proliferation, invasion, and migration of HCC cells in a concentration-dependent manner. Furthermore, the effect of sophoridine on the apoptosis of HCC HepG2 cells was confirmed via fluorescence microscopy. Untreated liver cancer cells displayed normal morphology, with round nuclei, and evenly distributed blue fluorescent staining. However, the number of apoptotic cells increased along with the concentration of sophoridine used for treatment; where the nuclei became concentrated, and the blue-stained chromatin became irregularly aggregated. This suggests that sophoridine inhibits the proliferation, migration, and invasion of HepG2 cells.

miRNAs are a family of non-coding RNA that is involved in cell apoptosis and proliferation. It was recently shown that miRNAs play indispensable roles in the pathogenesis of cancer by regulating oncogenes and anti-oncogenes. Three published studies investigated miR-144 levels in HCC tissue samples and revealed the down-regulation of miR-144 expression in HCC. However, the limited number of samples in these three studies (23 pairs, 33 pairs, and 100 pairs, respectively) may lead to false positive results and weak conclusions. Therefore, further attempts to validate altered miR-144 expression in HCC are required.

To date, several studies have investigated the clinical impact of miR-144 in several cancers and have demonstrated the important role of miR-144 in carcinogenesis. In lung cancer, miR-144 inhibits cell proliferation and induces apoptosis and autophagy. Guo et al. [17] demonstrated that miR-144 regulates the Wnt signaling pathway and promotes cancer cell proliferation in bladder cancer. Gu et al. [18] also demonstrated that miR-144 was associated with radiosensitivity in prostate cancer. However, there are few studies on the role of miR-144 in HCC. Therefore, in this study, we focused on the role of miR-144 in HCC. Our results showed that sophoridine increased miR-144 levels in a concentration-dependent manner.

miR-144 mimic overexpression reduced the proliferation and increased the apoptosis of HepG2 cells.

It is well known that CCNB1 plays a role in the G2/M phase transition of the cell cycle [19, 20]. It forms a complex (maturation-promoting factor) with cyclin-dependent kinase 1 (Cdk1) that induces early events of mitosis by controlling chromosome coagulation, nuclear membrane rupture, and mandrel assembly. This may result in enhanced cell proliferation and up-regulation of CCNB1. Further research is needed to elucidate the mechanisms and roles of this regulation in vivo. Our results showed that sophoridine reduced CCNB1 levels in a concentration-dependent manner. CCNB1 interference reduced the proliferation and increased the apoptosis of HepG2 cells [21]. At the same time, our study also found that Sophoridine can reduce the proliferation of HepG2 cell line and the increase of cell apoptosis, and its effect is indeed similar to the effect of CCNB1 interference or miR-144 overexpression on HepG2 cell proliferation and apoptosis. The effect of fixed alkali on liver cancer cells may be achieved by regulating the miR-144/CCNB1 signaling pathway. The ability to activate gene expression does not rule out the possibility that miRNA/double-stranded RNA (dsRNA) may act through a regulatory mechanism of gene silencing, and that CCNB1 induction is the result of down-regulation of other regulatory genes [22]. If we consider that each functional miRNA and dsRNA has different “seed” sequences and target non-overlapping regions, then by inhibiting common or different upstream regulatory genes, we cannot identify miR-144 and CCNB1 via bioinformatic analysis. There is, however, a direct interaction between them. Therefore, we recommend that these two molecules be included in the relevant regulatory network.

In summary, our results confirm that sophoridine induces apoptosis in HCC HepG2 cells by targeting miR-144/CCNB1 signaling. However, since this study is a preliminary study. We did not delve into the role of miR-144 and CCNB1 in...
the regulation network and will conduct in-depth research in the next step.

Disclosure of conflict of interest

None.

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