



## Research Article

# Psoralen inhibits hepatitis B viral replication by down-regulating the host transcriptional machinery of viral promoters

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## ABSTRACT

The hepatitis B virus (HBV) is a global public health challenge due to its highly contagious nature. It is estimated that almost 300 million people live with chronic HBV infection annually. Although nucleoside analogs markedly reduce the risk of liver disease progression, the analogs do not fully eradicate the virus. As such, new treatment options and drugs are urgently needed. Psoralen is a nourishing monomer of Chinese herb and is known to inhibit virus replication and inactivate viruses. In this study, we evaluated the potential of psoralen as an anti-HBV agent. Quantitative PCR and Southern blot analysis revealed that psoralen inhibited HBV replication in HepG2.2.15 cells in a concentration-dependent manner. Moreover, psoralen was also active against the 3TC/ETV-dual-resistant HBV mutant. Further investigations revealed that psoralen suppressed both HBV RNA transcription and core protein expression. The transcription factor FOXO1, a known target for PGC1 $\alpha$  co-activation, binds to HBV pre-core/core promoter enhancer II region and activates HBV RNA transcription. Co-immunoprecipitation showed that psoralen suppressed the expression of FOXO1, thereby decreasing the binding of FOXO1 co-activator PGC1 $\alpha$  to the HBV promoter. Overall, our results demonstrate that psoralen suppresses HBV RNA transcription by down-regulating the expression of FOXO1 resulting in a reduction of HBV replication.

## 1. Introduction

Hepatitis B, a potentially life-threatening liver infection caused by the hepatitis B virus (HBV), is a major global health problem. In 2019, the World Health Organisation statistics estimate that 296 million people were living with chronic hepatitis B and almost 1.5 million new infections are detected annually (WHO, 2019). Appropriately one million people die annually from diseases triggered by the HBV infection such as cirrhosis and hepatocellular carcinoma (Pierra Rouviere et al., 2020). The main therapeutic strategies that are currently being used to treat chronic hepatitis B are pegylated-interferon alpha (PEG-IFN $\alpha$ ) and nucleoside analogs, such as: tenofovir alafenamide fumarate (TAF), entecavir (ETV) and tenofovir (TDF) (Mazzaro et al., 2019). PEG-IFN $\alpha$

only provides clearance of infection in less than 7% of patients or 15% of patients when used in combination with ETV or TDF, respectively (Phillips et al., 2017). Long-term consumption of these drugs to control the infection eventually triggers drug resistance. Therefore, there is an urgent need to discover new drugs that combat chronic HBV infections.

The HBV genome is a 3.2 kb partially double-stranded relaxed-circular DNA (rcDNA) (Oropeza et al., 2020). The HBV life cycle begins with virion attachment and entry into hepatocytes. After viral entry, the virion is de-enveloped to release naked nucleocapsid and then transported to the nuclear pore complex where the rcDNA is released into the nucleus. The HBV rcDNA is modified by cellular factors resulting in the conversion of rcDNA into covalently closed circular DNA (cccDNA). The cccDNA serves as a template for transcription of the major HBV mRNA with 0.7,

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2.4/2.1, and 3.5 kb sized transcripts (Shen et al., 2020). Transcription of these RNAs is under the control of four distinct promoters, preS1 (SP I), preS2 (SP II), Enhancer I/X (En I) and Enhancer II/core (En II) promoters, and mediated by host RNA Polymerase II (Chen et al., 2016). The 3.5 kb mRNA includes HBV pregenomic RNA (pgRNA) that encodes the core protein (HBcAg), and the slightly longer precore mRNA that encodes the precore protein (p25), the precursor to the secreted hepatitis B virus e antigen (HBeAg). The 2.4 kb PreS1 and 2.1 kb PreS2/S RNA encode three HBV surface (envelope) antigens (HBsAg) i.e., the large surface protein (LHBs), middle surface protein (MHBs) and small surface protein (SHBs), while the small 0.7 kb mRNA encodes the HBV X protein (HBx) which may exert diverse regulatory functions (Hong et al., 2021).

During the HBV life cycle, RNA transcription is essential for HBV replication and secretion. If the viral life cycle is controlled at the transcriptional level, the effect of simultaneously inhibiting virus replication and antigen secretion can be achieved (Quasdorff and Protzer, 2010). This makes HBV DNA transcription an attractive target for the identification and development of potential therapeutics. HBV transcription is regulated by the four HBV promoters, while transcription factors control transcriptional activity by interacting with the upstream DNA sequence of the HBV genes (Turton et al., 2020). A number of transcription factors are important for the formation of different RNA transcripts resulting from HBV cccDNA transcription.

The forkhead box-O (FOXO) family is a subclass of winged helix/forkhead transcription factors widely expressed in mammals, which are homologues of the *Caenorhabditis elegans* transcription factor Dauer Formation-16 (DAF-16) that programs cells for resistance to oxidative stress, DNA repair and cell cycle control (Hedrick, 2009). The FOXO transcription factors include four members (FOXO1, FOXO3, FOXO4 and FOXO6) which regulate cell survival and apoptosis, proliferation, energy metabolism, and oxidative stress (Burgering, 2008; Chen et al., 2019; Graves and Milovanova, 2019). The transcription factor FOXO1 regulates protein expression, nuclear translocation, DNA binding and interaction with other proteins and is highly expressed in the liver and pancreas (Carbajo-Pescador et al., 2014). FOXO1 is well known as a tumor suppressor in multiple cancers, including hepatocellular carcinoma (HCC) (Yang et al., 2014; Xing et al., 2018; Ioannilli et al., 2020). Recent evidence increasingly suggests that FOXO1 is an activator of HBV transcription through its binding to the HBV core promoter, and expression of FOXO1 is up-regulated to promote HBV replication and expression (Shlomai and Shaul, 2009; Wang and Tian, 2017). This activation of HBV transcription is further enhanced in the presence of peroxisome proliferator-activated receptor  $\gamma$  co-activator 1 $\alpha$  (PGC1 $\alpha$ ), suggesting that FOXO1 interacts with PGC1 $\alpha$  to co-activate hepatitis B virus transcription (Wang and Tian, 2017). PGC-1 $\alpha$  is a metabolic regulator, which co-activates HBV transcription, resulting in fluctuations in HBV gene expression. As a well-known target for PGC1 $\alpha$  co-activation, FOXO1 binds to HBV enhancers II and activates only the pre-core/core promoter in a PGC1 $\alpha$ -dependent manner (Shlomai and Shaul, 2009).

Psoralen, a principal bioactive component in the dried fruits of *Cullen corylifolium* (L.) Medik (*syn. Psoralea corylifolia* L), displays multiple bioactivities which contribute to the treatment of osteoporosis, tumors, viral and bacterial infection, and inflammation. The mechanism of action for psoralen in treating these medical problems has been elegantly reviewed in Ren et al. (2020). Previous studies of the antiviral pharmacology of psoralen had shown that psoralen in combination with UV irradiation could inactivate DNA and RNA viruses (Hanson et al., 1978). Photochemical inactivation of DNA viruses by derivatives of psoralen was first demonstrated in 1965 (Musajo et al., 1965), and then the technique was extended to RNA viruses (Hearst and Thiry, 1977). Combined with ultraviolet light (320–380 nm), several psoralen derivatives could efficiently and totally inactivate viruses, such as dengue virus type 1 (DENV-1) (Schneider et al., 2015), Western equine

encephalitis virus (WEE) (Hanson et al., 1978), herpes simplex virus type 1 (HSV-1) (Hanson et al., 1978), and hepatitis B virus (HBV) (Wollowitz, 2001). The psoralen photoreaction only cross-linked the virus nucleic acid to render the virus non-infectious, but did not destroy the particles and surface antigens (Song and Tapley, 1979). The psoralen-inactivated virus could potentially be used for vaccine development (Schneider et al., 2015). For example, psoralen-inactivated DENV-1 maintained the virus's three-dimensional structure, which allowed the production of antibodies with multiple epitopes for priming the immune system (Maves et al., 2010). Additional studies had shown that a candidate psoralen-inactivated DENV-1 vaccine exhibited protection in non-human primates (Maves et al., 2011). Psoralen could also directly inhibit viral replication, for example, psoralen inhibited murine gamma herpes virus 68 (MHV-68) replication and the lytic cycle of human  $\gamma$  herpesviruses, such as Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Cho et al., 2013). Psoralen inhibited the replication of H1N1 virus and induced macrophages to secrete antiviral cytokines such as interferon-beta (IFN- $\beta$ ) (Choi et al., 2017). Psoralen could also inhibit HBV replication and decrease HBsAg and HBeAg secretion (Parvez et al., 2019).

In this study, we examined the effect of psoralen on the HBV life cycle to explore the possible anti-HBV mechanism of psoralen. First, using the cell-based anti-HBV drug screening system, we confirmed the anti-HBV activity of psoralen. We then demonstrated that psoralen reduced FOXO1 protein expression. As noted above, the FOXO1-PGC1 $\alpha$  co-activator binds to HBV pre-core/core promoter enhancer II region to enhance virus transcription, therefore, a decrease in FOXO1 protein expression negatively affects pgRNA and precore RNA transcription. Thus, psoralen exhibits anti-HBV activity by acting as a transcriptional regulator of FOXO1 protein expression.

## 2. Materials and methods

### 2.1. Reagents

Psoralen (99.34% HPLC purity) was purchased from Topscience, Shanghai, China. Psoralen (40 mmol/L dissolved in DMSO) was stored at  $-30^{\circ}\text{C}$ , and different dilutions were prepared from this stock solution using cell culture medium. Lamivudine, Entecavir and Tenofovir (99.1% HPLC purity) were purchased from Tianfeng Chemical Technology Co, LTD (Suizhou, Hubei, China).

### 2.2. Plasmids

The plasmid *pHBV1.3* contains a 1.3-mer over-length copy of the HBV genotype A2 genome (Yang et al., 2016). The plasmid *pHBV1.3 rtM204V/L180M* contains a dual resistant HBV genome about Lamivudine and entecavir (Yang et al., 2014). These plasmids were donated by Professor Yang of Wuhan Tongji Medical College.

### 2.3. Cell culture, transfection and infection

HepG2.2.15 cells were cultured in Modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, US). HepG2.2.15 cells with two integrated dimers of the HBV genome (GenBank accession number: U95551) were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, US) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, US) and 380  $\mu\text{g}/\text{mL}$  G418 (GibcoBRL) (Xu et al., 2014). Human hepatoma cell line Huh7 was cultured in DMEM supplemented with 10% FBS. Huh7 cells were transfected with plasmids by using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol (Yang et al., 2014). All cells were cultured at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ .

## 2.4. Cytotoxicity assay

The cytotoxic effects of psoralen were assessed by MTT assay. HepG2.2.15 cells and Huh7 cells were cultured in 96-well plates at  $1 \times 10^4$  cells per well. The time when cells were cultured was denoted as day 0. At day 1, cells were treated with psoralen at an initial concentration of 800  $\mu\text{mol/L}$  and two-fold serial dilution. At day 3, the supernatant of Huh7 cells were collected and remaining cells were treated with 1 mg/mL MTT at 90  $\mu\text{L}$  per well for 2 h at 37 °C. Then, MTT lysate was added at 10  $\mu\text{L}$  per well overnight at 37 °C. Next, the absorbance value of lysate was determined by using a SpectraMax® 190 at 570 nm. At the 4th day, culture medium of HepG2.2.15 was replaced by newly diluted compound medium. At the 8th day, the procedures of processing HepG2.2.15 cells were the same as those of Huh7 cells.

## 2.5. Detection of HBsAg and HBeAg

HepG2.2.15 cells and Huh7 cells were treated with different concentrations of psoralen as described above. On the indicated days, the culture supernatants were collected for analysis of HBsAg and HBeAg levels using ELISA kits (Sino-American biotechnology company, Henan, China).

## 2.6. Western blot analysis

Huh7 cells were seeded at a concentration of  $8 \times 10^5$  cell/mL in 6-well culture plates overnight. Cells were then transfected with pHBV1.3 plasmid. Total cell proteins were obtained using Pierce™ RIPA lysis buffer (Thermo Fisher Scientific, MA, US), and the concentrations were determined by the BCA assay (Thermo Fisher). Total proteins were separated by SDS-PAGE and protein bands were transferred to Nitrocellulose membranes (GE). Protein bands were blocked for 1 h at room temperature, and then incubated with the indicated primary antibodies overnight at 4 °C and visualized by chemiluminescence the next day. The primary antibodies were FOXO1 (cst, 2880), HNF4 $\alpha$  (cst, 3113), PGC1 $\alpha$  (santa cruz, sc-13067), RXR $\alpha$  (santa cruz, sc-515929), PPAR $\alpha$  (Abcam, ab19122), HNF6 (Proteintech, 25137-1-AP), GAPDH (Abcam, ab9482), HBcAg (Abnova, PAB14506), goat anti-mouse HRP (Thermo Scientific, 31430), goat anti-rabbit HRP (Bio-Rad, 1705046), and  $\beta$ -actin (Abcam, ab49900).

## 2.7. Southern blot analysis

Huh7 cells were cultured as described above. The cells were collected and lysed with cold lysis buffer (0.5% NP40, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.0). The DNA samples were separated by 1% agarose gel electrophoresis and denatured by alkali solution. Then, DNA bands were transferred to Amersham™ Hybond-N+ membranes (GE), which was hybridized with Dig-labeled full-length HBV genome probe overnight and washed in different concentration of SSC/SDS, washing buffer, detection buffer. Next, the membrane was incubated with anti-DIG antibody, and exposed to X-ray film.

### 2.7.1. Native agarose gels electrophoresis analysis

Cell lysate containing capsids was prepared by lysing cells in cold NP-40 buffer (0.5% NP-40, 50 mmol/L Tris•HCl, 1 mmol/L EDTA•2 Na, pH 7.0) and loaded onto 1.2% agarose gel. For analysis of capsid assembly, the capsids were transferred directly to a Nitrocellulose membrane (GE) using the capillary transfer method with  $10 \times \text{SSC}$  overnight, then immunoblotted with the primary antibody recognizing HBcAg (Abnova, PAB14506), followed by detection of secondary antibody by chemiluminescence. For analysis of capsid-associated DNA, HBV capsids were transferred directly to a nylon membrane under the same conditions, but the encapsidated nucleic acid was released from the capsids by denaturation (Yang et al., 2016). Prehybridization and hybridization were performed identically to that for southern blot analysis.

## 2.8. Northern blot analysis

Huh7 cells were cultured as described above. The extracted RNA was separated by denaturing 1% formaldehyde agarose gel electrophoresis and transferred to a Hybond-N+ membrane (Amersham Biosciences) in  $20 \times \text{SSC}$  (Invitrogen), and the membrane was crosslinked by UV cross-linking for 10 min and incubated with pre-warmed DIG Easy Hyb buffer at 42 °C overnight. The membrane was washed with the DIG Wash and Block Buffer kit (Roche) according to the manufacturer's instructions the next day.

## 2.9. Real time PCR assay

The Qiasymphony Nucleic acid extraction and purification kits (Qiagen, Hilden, Germany) were used for the extraction of HBV DNA from the cells and cell culture supernatants. The sequences of the HBV-specific fluorescent probe (HBVayw/Probe) and primers for HBV DNA (HBVayw/Sp and HBVayw/Asp) were described previously (Yang et al., 2014). The quantitative standard curve was established by setting the concentration of standard sample at  $10^7$ ,  $10^5$ ,  $10^3$  copies/mL respectively. ABI 7900H real-time PCR system (Life Technologies, NY, US) was operated according to the following thermocycling parameters: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s.

Total RNA from cells was extracted using the Trizol reagent (Invitrogen), followed by reverse transcription of RNA to generate cDNA. Then, cDNA was subjected to quantitative real-time PCR assay with an ABI 7900H real-time PCR system (Life Technologies, NY, US) using a QuantiNova™ Internal Control RNA and Assay from QIAGEN (Dusseldorf, Germany). Time PCR System (Thermo Fisher Scientific) was operated according to the following thermocycling parameters: 50 °C for 10 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. Whether the reaction was normal or not was judged according to the dissolution curve, and then the relative mRNA level was calculated by comparing the CT values of the samples. The primer sequences were described in Supplementary Table S1.

### 2.9.1. Luciferase reporter assay

To generate expression plasmids for the luciferase linked the promoters including preS1 (SP I), preS2 (SP II), Enhancer I/X (En I) and Enhancer II/core (En II) promoter respectively, the coding sequence of each promoter was obtained from the published viral genome (GenBank) and optimized for mammalian expression. An optimized coding sequence was chemically synthesized (Inovogen) and cloned into the pGL3 Basic vector, which was verified by sequencing. We seeded Huh7 cells at a concentration of  $3 \times 10^5$  cell/mL in 12-well culture plates overnight, and then transfected with the plasmids mentioned above. After 72 h of culture, we drained the supernatant culture, and added  $1 \times \text{Glo}$  Lysis Buffer (Promega). The luciferase activity was assessed by a bioluminometer using the Bright-Glo luciferase assay system (Promega) according to the manufacturer's instructions (Yang et al., 2021).

## 2.10. Immunoprecipitation

Huh7 cells were lysed following the instructions of the kit (Thermo Fisher scientific, MA, US) to obtain the adjusted concentration of the cell lysate. The cell lysate was incubated with primary antibody (FOXO1, CST2880, 1:100) in a shaker overnight at 4 °C, and then incubated with a spin column containing protein A/G enhanced agarose the next day for 2 h. The final eluent was applied on SDS-PAGE, and the subsequent steps were the same as those of Western blot.

## 2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism8.0 and statistics were analyzed by one-way analysis of variance (ANOVA) in appropriate circumstances. A *P* value < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Psoralen inhibited HBV RNA and core protein expression as well as DNA replication

The chemical structure of psoralen is shown in Fig. 1A. To determine the cytotoxicity and antiviral activity of psoralen, HepG2.2.15 cells were treated with increasing concentrations of psoralen for 8 days. The 50% cytotoxic concentration (CC<sub>50</sub>) of psoralen was determined to be 413.5 ± 12.98 μmol/L by the MTT assays (Fig. 1B). The amount of HBV DNA was quantified using qPCR and the 50% inhibitory concentration (IC<sub>50</sub>) of psoralen on supernatant HBV DNA was 126.4 ± 13.13 μmol/L (Fig. 1C) while the IC<sub>50</sub> for intracellular capsid-associated HBV DNA was 63.7 ± 14.53 μmol/L (Fig. 1D). The SI values (selectivity index, SI = CC<sub>50</sub>/IC<sub>50</sub>) were 3.26 and 6.49, respectively. Psoralen also inhibited the secretion of HBsAg and HBeAg in a dose-dependent manner. As a polymerase inhibitor, nucleoside analog TAF had no inhibitory effect on HBsAg and HBeAg levels (Fig. 1E and F).

We further detected the inhibitory effect of psoralen on HBV DNA, RNA, and core protein levels. Southern blot results showed that psoralen inhibited the various forms of HBV intracellular DNA replication intermediates, including relaxed circular (RC) and single-stranded (SS) HBV DNA, in a dose-dependent manner in HepG2.2.15 cells. As a positive control, the nucleoside analog TAF significantly inhibited the synthesis of HBV DNA (Fig. 1G). Northern blot results showed that HBV RNA synthesis was also influenced by psoralen, especially the 3.5 kb RNA, whereas the 2.4/2.1 kb RNAs had no obvious change compared to the control group. As expected, after TAF treatment, HBV RNA levels were unchanged (Fig. 1G). HBV core protein expression was also suppressed by psoralen, which led to a subsequent reduction in capsid formation. β-Actin was used as a loading control (Fig. 1H).

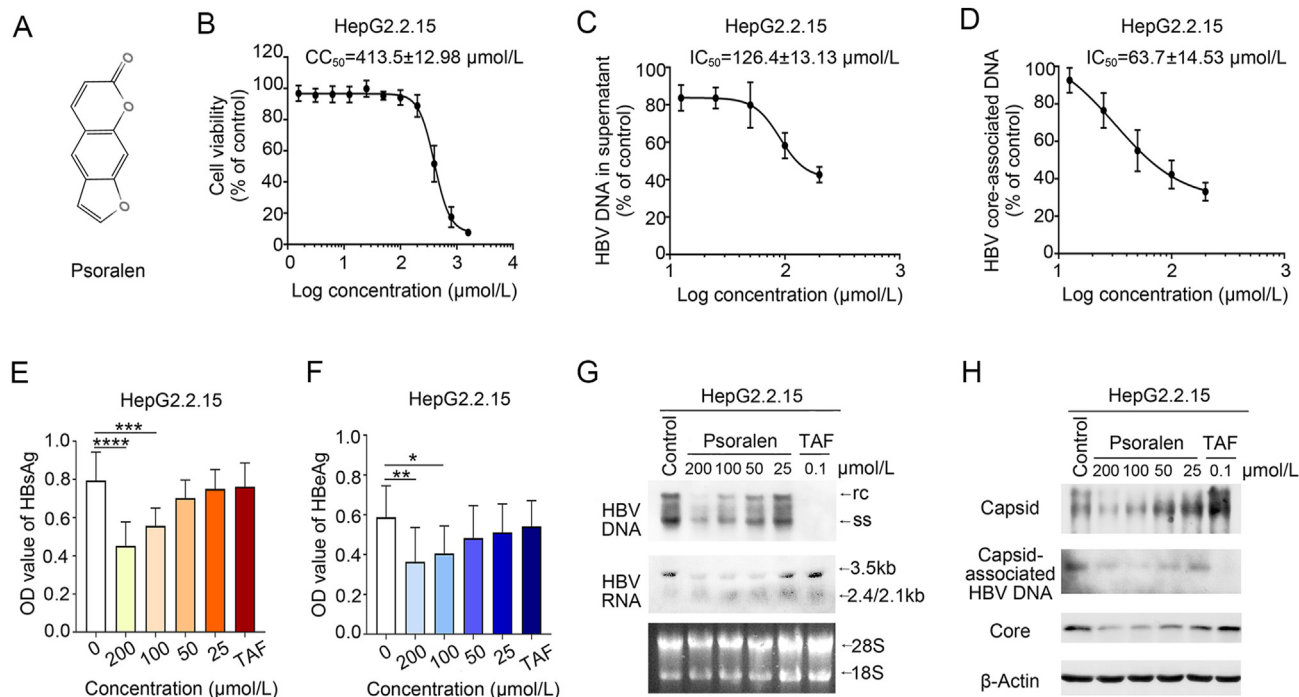
The anti-HBV effects of psoralen were confirmed in Huh7 cells transfected with wild type pHBV1.3. As shown in Fig. 2, the CC<sub>50</sub> was 398.2 ± 25.49 μmol/L (Fig. 2A), and the IC<sub>50</sub> of intracellular HBV DNA was 81.2 ± 10.27 μmol/L with an SI value of 4.90 (Fig. 2B). In addition, the secretion of HBsAg and HBeAg decreased in a dose-dependent manner (Fig. 2C–D). We also detected the effects of psoralen on HBV RNA, HBcAg and nucleocapsid. The data showed that psoralen inhibited the secretion of HBV DNA, RNA (Fig. 2E), nucleocapsid and core proteins (Fig. 2F) in Huh7 cells, similar to that observed in HepG2.2.15 cells.

#### 3.2. Psoralen inhibited the replication of 3TC/ETV-dual-resistant HBV variant

Patients undergoing nucleos(t)ide therapy may become resistant leading to treatment failure. We tested the activity of psoralen against the HBV polymerase rtM204V/L180M variant, which is a common clinical 3TC/ETV dual resistant mutation. As shown in Fig. 3A, psoralen maintained activity against wild-type and rtM204V/L180M variant, while the rtM204V/L180M variant was resistant to 3TC and ETV. The HBV rtM204V/L180M mutant remained sensitive to inhibition by TAF, but the inhibition effect was reduced compared to wild type HBV suggesting that while psoralen could inhibit the replication of the HBV variant rtM204V/L180M, inhibition was less efficient compared to wild type HBV (Fig. 3B).

#### 3.3. Time-course study of psoralen action

To explore the mechanism of the psoralen anti-HBV activity, a time-course study on the inhibition of HBV RNA transcription, DNA replication, core protein expression and HBeAg/HBsAg resection was performed on pHBV1.3 transfected Huh7 cells. Huh7 cells were transfected with pHBV1.3 and treated by psoralen for the indicated time periods, after



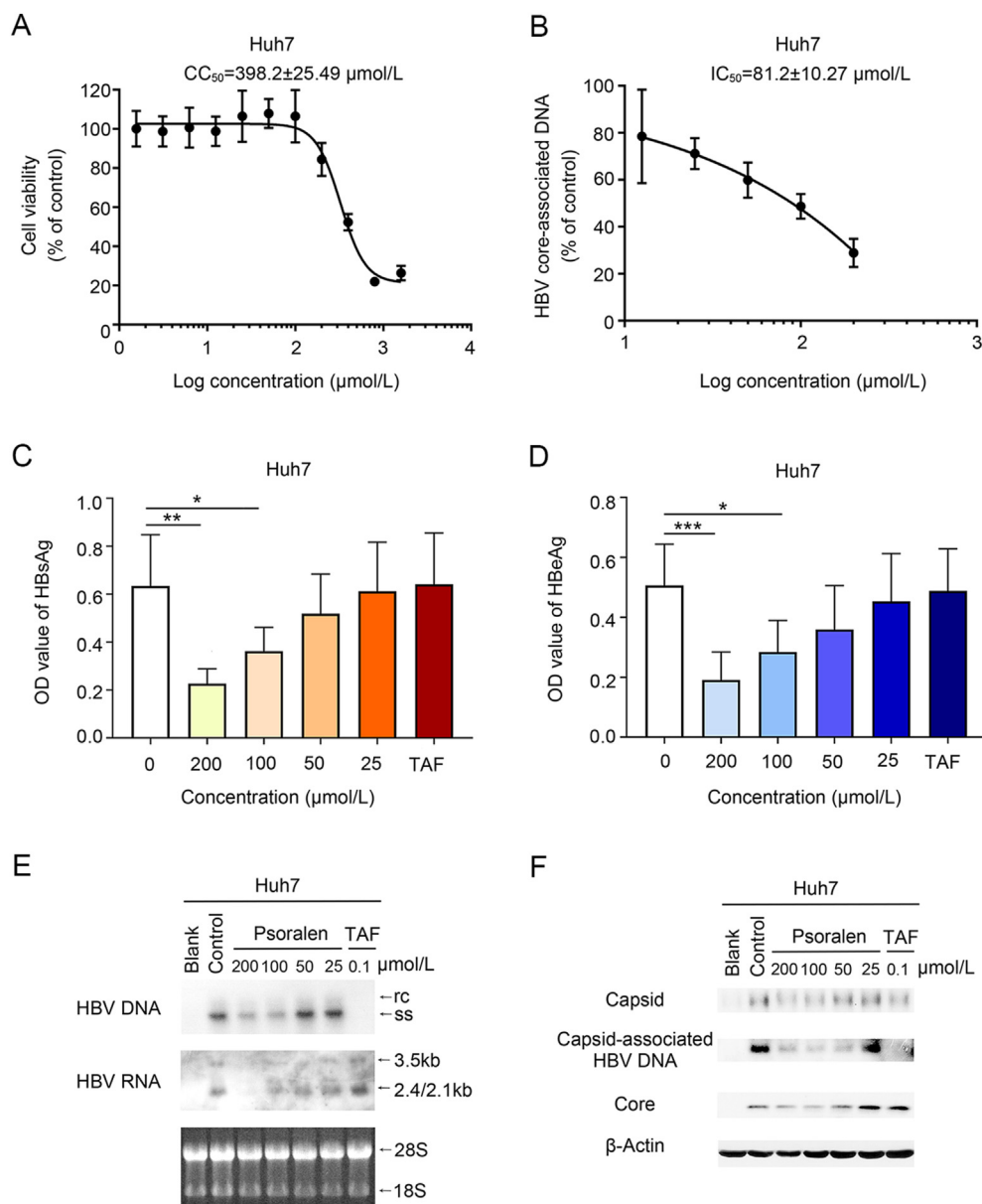
**Fig. 1.** Toxicity and antiviral activity of psoralen on HepG2.2.15 cells. HepG2.2.15 cells were seeded in 96-well plates, treated with psoralen for 8 days, and the cytotoxicity was detected by the MTT assay. HepG2.2.15 cells treated with psoralen were harvested and the virus particles secreted into the supernatant were collected after 8 days. **A** Chemical structure of psoralen. **B** Cytotoxicity was detected by the MTT assays. **C–D** The intracellular and supernatant HBV DNA were extracted and quantified by qPCR. **E–F** The supernatant was used for analyzing the levels of secreted HBsAg and HBeAg by ELISA. The supernatant of HepG2.2.15 cells without psoralen was used as the control. Values are means ± SD of 3 independent experiments. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. **G** HBV intracellular core-associated DNA was analyzed using Southern blot. rcDNA, relaxed circular DNA; ssDNA, single-stranded DNA. Total RNA was detected by northern blot. 28S and 18S RNA serve as loading control. **H** The intracellular capsid and capsid associated-DNA in core particles were detected by native agarose gel electrophoresis. Core protein was detected by Western blot. β-actin was used as the loading control.

which cells were harvested within 72 h for analysis (Fig. 4A). The HBV RNA, DNA and viral protein were detected by Northern blot, Southern blot, Western blot and ELISA, respectively. When psoralen was added to cultures within 12 h after transfection, the amounts of HBV RNA (Fig. 4B), DNA (Fig. 4C) and viral proteins (Fig. 4D and E) decreased in a dose-dependent manner. On the contrary, when cells were treated with psoralen after 24 h post-transfection, there was no effect on transcription, replication, expression and secretion (Fig. 4B–D). What’s more, these viral markers after a 48-h psoralen treatment were also studied. The results of 48-h administration of psoralen were the similar to 72-h administration, that is, HBV RNA, DNA and viral proteins were suppressed (Supplementary Fig. S1). Therefore, we speculate that psoralen is effective at the early stage of the HBV life cycle in the infected cells.

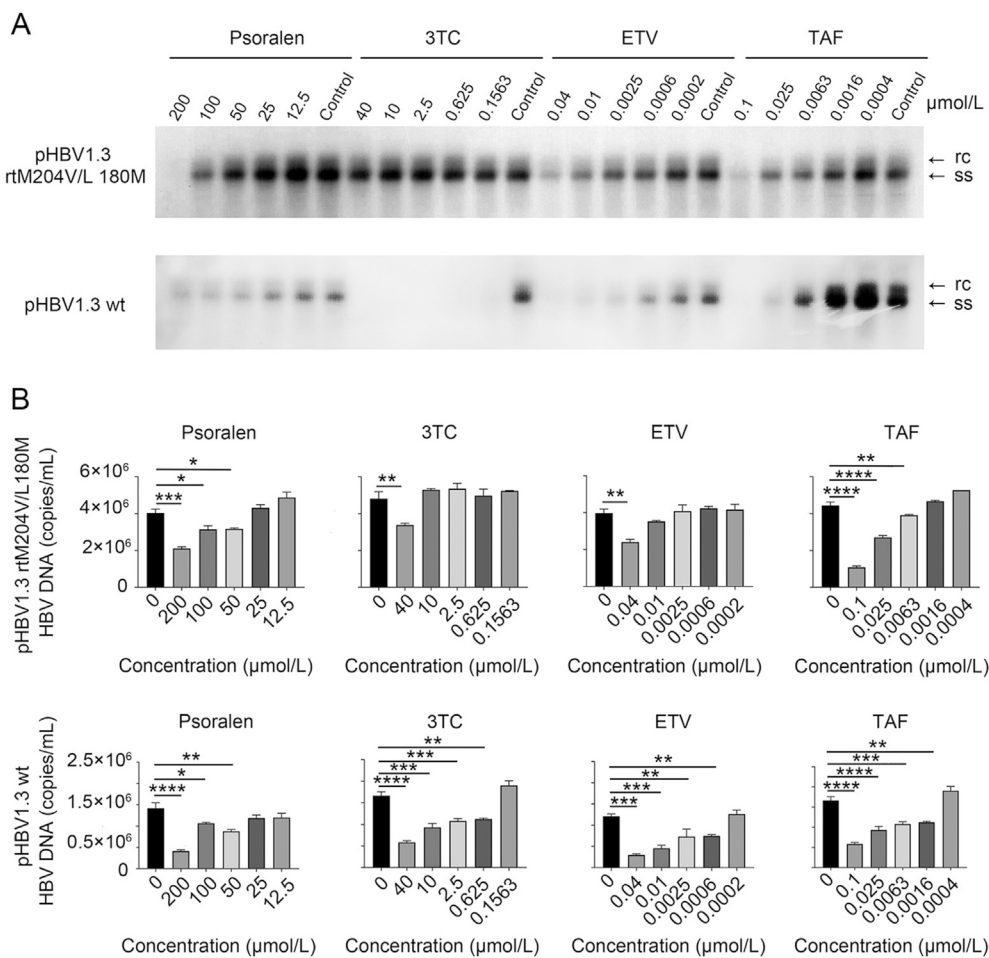
3.4. Psoralen inhibits HBV-related mRNA transcription

The nuclear cccDNA serves as template for the synthesis of pgRNA (3.5 kb), precore RNA (3.5 kb) and three additional subgenomic RNAs,

PreS1, PreS2/S and HBx RNA (Kim et al., 2016). To fully understand the inhibitory effect of psoralen on HBV, we examined gene expression level of the major HBV RNAs by qRT-PCR. When compared to untreated cells, the levels of pgRNA and precore RNA in infected cells treated with psoralen were inhibited in a dose-dependent manner, but PreS1, PreS2/S and X mRNA levels were unchanged (Fig. 5A). To ascertain if psoralen reduced promoter transcriptional initiation activity, we performed the luciferase reporter assay to detect the effect of psoralen on Enhancer II/core, preS1, preS2 and Enhancer I/X promoters. We transfected Huh7 cells with plasmids carrying individual constructs of the luciferase gene fused downstream of the promoters for preS1 (SP I), preS2 (SP II), Enhancer I/X (En I) and Enhancer II/core (En II) respectively, and treated the transformed cells with different concentrations of psoralen for 72 h following which the cells were lysed to detect luciferase fluorescence. The results showed that psoralen inhibited transcription from the Enhancer II/core promoter but not the PreS1, PreS2 or Enhancer I/X promoters (Fig. 5B).



**Fig. 2.** Psoralen inhibited the HBV RNA and core protein in pHBV.1.3 transfected Huh7 cells. **A** Cytotoxicity was detected by the MTT assays. **B** HBV DNA was extracted from cells, and the DNA content was detected by qPCR. **C–D** The supernatant was used for analyzing the levels of secreted HBsAg and HBeAg by ELISA. The supernatant of huh7 cells without psoralen treatment was used as the control sample. Values are means ± SD of three independent experiments. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. **E** HBV DNA was detected by Southern blot and HBV RNA was detected by Northern blot. 28S and 18S RNA serve as loading control. **F** The native agarose gels electrophoresis was used to detect the intracellular capsid and capsid associated-DNA.



**Fig. 3.** Psoralen inhibited drug-resistant mutant and wild-type HBV. Huh7 cells were transfected with wild-type HBV or 3TC/ETV-dual-resistant HBV mutant (polymerase rtM204V/L180M variant), cells were treated with indicated concentrations of psoralen, 3TC, ETV, TAF and harvested 3 days later. **A** HBV replication intermediates were analyzed by southern blot hybridization. **B** HBV core-associated DNA in cells were quantified using qPCR. Values are means  $\pm$  SD of 3 independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001.

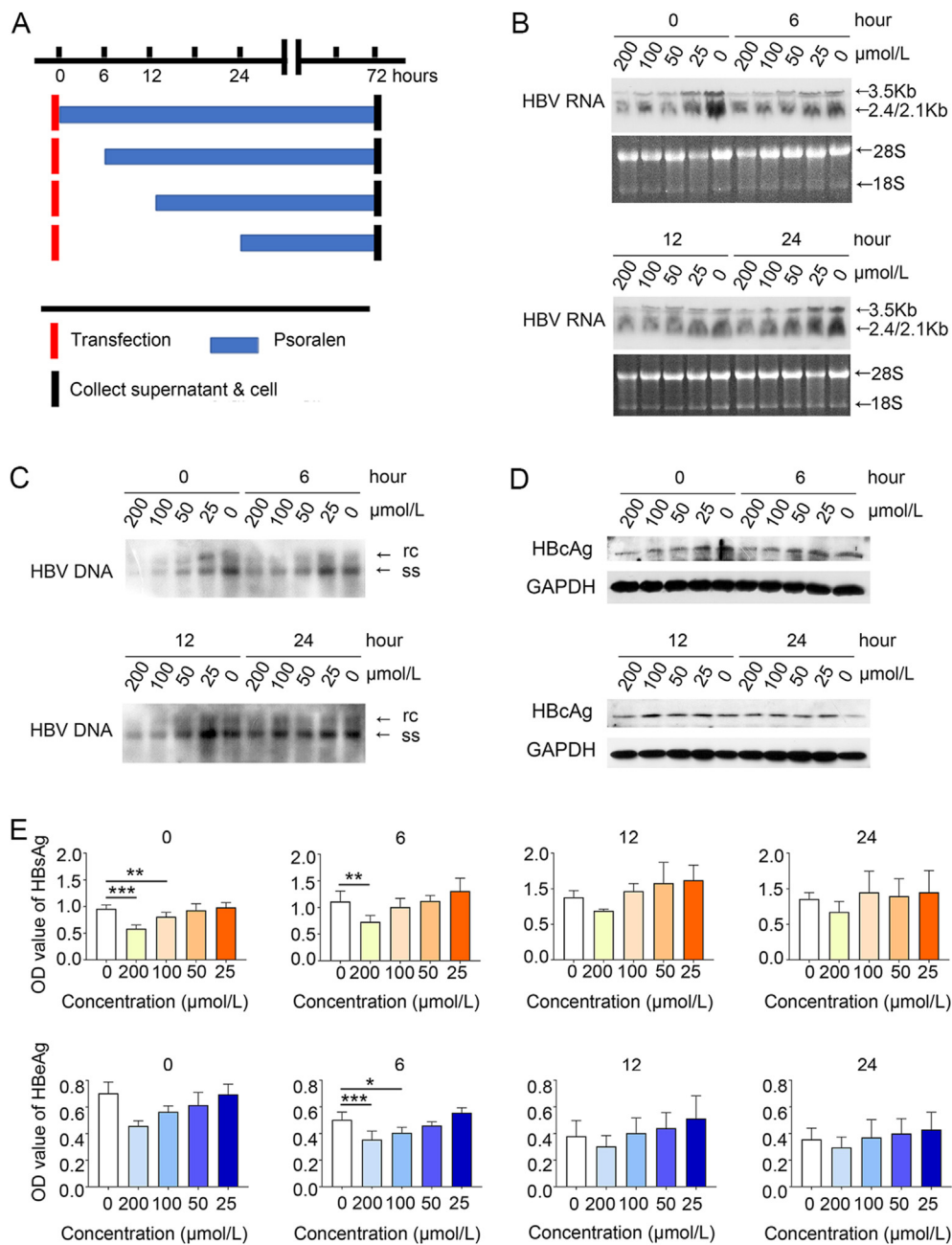
### 3.5. Psoralen suppressed HBV RNA synthesis by regulating FOXO1-*PGC1 $\alpha$* expression

Previous studies have demonstrated that transcription of HBV genes was regulated by the precise and orderly recruitment of chromatin modifiers and various host factors, including transcription factors (Turton et al., 2020). Therefore, we tested if psoralen could inhibit expression of these transcription factors. We observed that expression of FOXO1, a known target for PGC1 $\alpha$  co-activation, was significantly inhibited in the presence of psoralen. Furthermore, psoralen inhibited FOXO1 protein expression in a concentration-dependent manner (Fig. 6A). Psoralen did not reduce the levels of other classic transcription factors, such as HNF4 $\alpha$  (Supplementary Fig. S2). In addition, psoralen had no significant effect on PGC1 $\alpha$ , the co-activator of FOXO1 (Fig. 6B). It is known that FOXO1 is dependent on PGC1 $\alpha$  to activate transcription (Wang and Tian, 2017). We tested if psoralen interfered with FOXO1-*PGC1 $\alpha$*  interaction using co-immunoprecipitation. The results showed that PGC1 $\alpha$  efficiently co-precipitated with FOXO1 but this decreased when levels of FOXO1 were reduced while the amount of total PGC1 $\alpha$  did not change (Fig. 6C).

## 4. Discussion

Nucleos(t)ide analogs can significantly inhibit the replication of HBV DNA but the development of drug resistance will prevent some patients from achieving ideal therapeutic effect. New knowledge on HBV replication and virus-host interaction should assist the development of new anti-HBV treatment strategies. The occurrence of liver cancer is reported

to be associated with the secretion of HBV viral proteins. In this paper, we demonstrated that psoralen could inhibit HBV RNA transcription, DNA replication, and viral protein expression in a dose-dependent manner (Figs. 1 and 2). In addition, this inhibitory effect was also noted for the 3TC/ETV-dual-resistant HBV variant (Fig. 3) proposing that the inhibitory effect of psoralen was superior to that of nucleos(t)ide analogs. From a time-course assessment, we noted that psoralen was effective in the early stages of the HBV life cycle i.e., the RNA transcription stage (Fig. 4). To study the role of psoralen on the transcriptional stage of HBV, the levels of HBV mRNA and the activity of HBV promoters were detected by qRT-PCR and luciferase reporter assay, respectively. The results showed that psoralen could significantly inhibit the activity of Enhancer II/core promoter resulting in decreased pgRNA and precore RNA transcription (Fig. 5). Both pgRNA and precore RNA transcription are known to be initiated from the Enhancer II/core promoter (Hong et al., 2021). Enhancer II is a 105-bp region located upstream of the basal part of the core promoter. As many transcriptional regulators are known to bind with promoter/enhancer elements to regulate HBV transcription, we tested mRNA levels of different transcription factors and noted that the transcription of the forkhead transcription factor FOXO1 decreased following psoralen treatment. FOXO1 binds to the HBV Enhancer II/core promoter region to activate transcription (Shlomai and Shaul, 2009). FOXO1 activity is enhanced by the co-activator PGC-1 $\alpha$  (Yamagata et al., 2007; Zhang et al., 2021). We examined the RNA and protein level of PGC-1 $\alpha$  and observed that psoralen had no significant effect on PGC1 $\alpha$ . An immunoprecipitation assay confirmed the interaction between PGC1 $\alpha$  and FOXO1, however, the amount of FOXO1-*PGC1 $\alpha$*  complex decreased



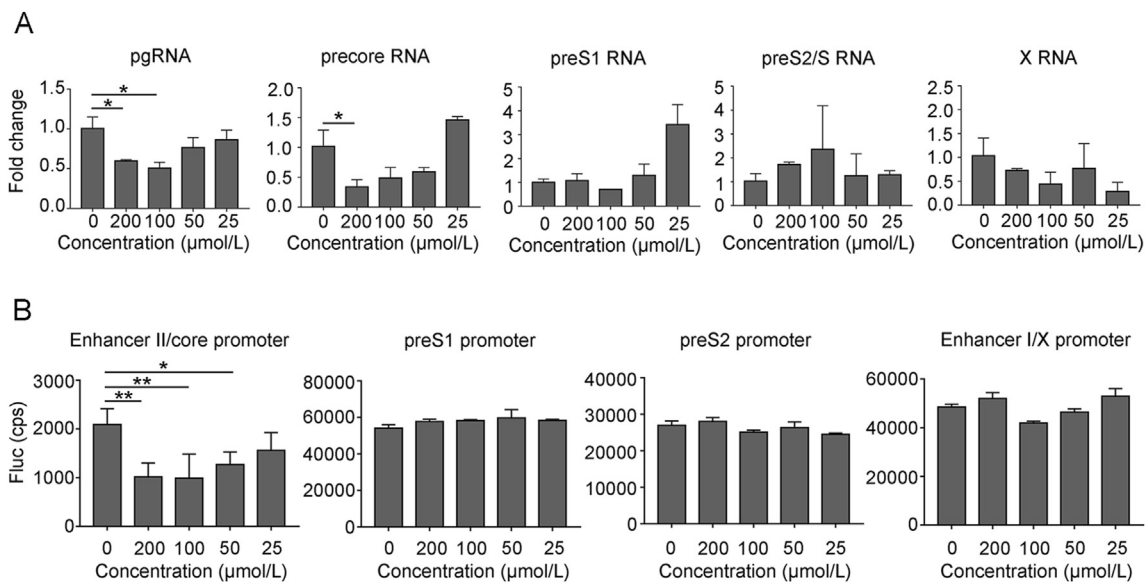
**Fig. 4.** Time-course of HBV RNA, DNA, protein expression and HBeAg/HBsAg resection after Psoralen treatment. Similarly, pHBV1.3 transfected Huh7 cells were treated with psoralen for the indicated time periods, harvested after 72 h and analyzed. **A** Schematic diagram of the administration of psoralen. **B** HBV RNAs were detected using northern blot with 18s rRNA or 28s rRNA as reference. **C** HBV replication intermediates at different time points were analyzed by Southern blot hybridization. **D** Core proteins were detected by Western blot. **E** Supernatant was used to analyze the levels of secreted HBsAg and HBeAg by ELISA. Values are means ± SD of 3 independent experiments. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

with the reduction in FOXO1 implying that psoralen did not directly affect PGC1 $\alpha$  but acted on FOXO1 to affect the FOXO1-PGC1 $\alpha$  complex (Fig. 6). Thus, our results propose that the antiviral effect of psoralen might be achieved through a down-regulation of FOXO1 which then down-regulate transcription from the Enhancer II/core promoter. This reiterates that FOXO1 is an important regulator of HBV transcription (Fig. 7). We also observed that psoralen did not significantly affect the expression levels of the PreS1 and S gene regions which encode the envelope protein. Intact hepatitis B virus particles are composed of an outer membrane and a nucleocapsid, and the viral outer membrane or envelope is composed of HBsAg. Previous experimental results have shown that psoralen can inhibit the secretion of HBsAg. While psoralen itself did not affect the transcription and protein translation of PreS1 and PreS2/S, it could inhibit the secretion of intact viral particles by suppressing the

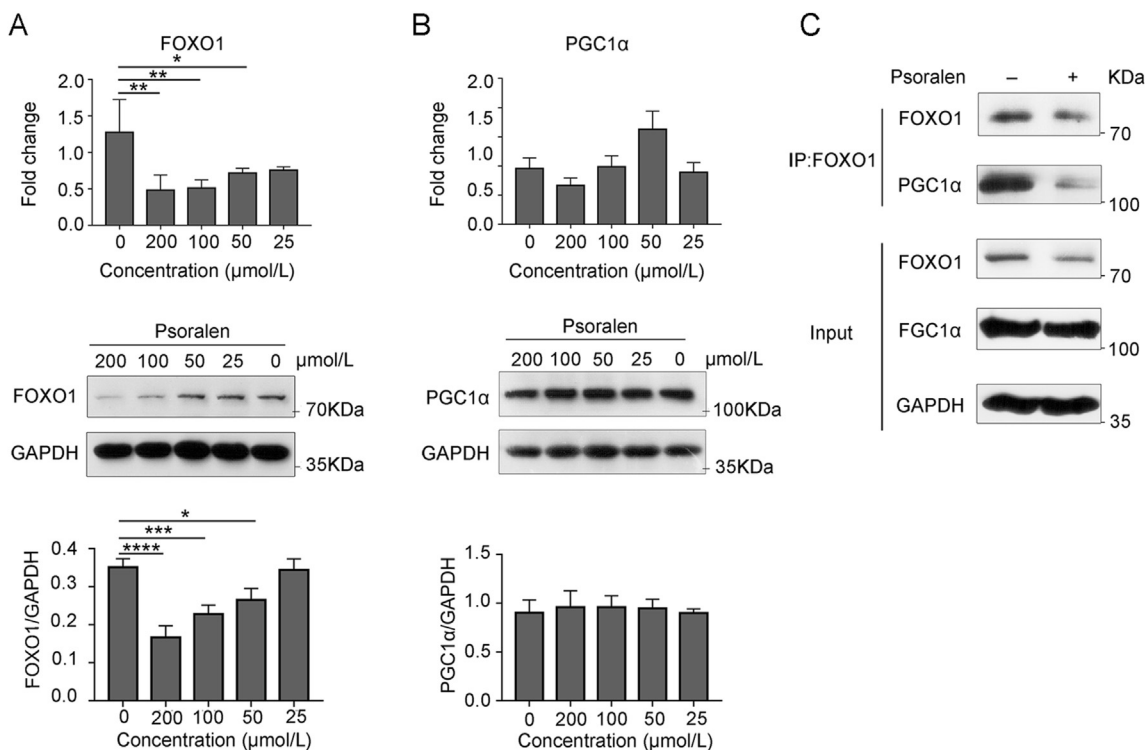
transcription of pgRNA and precore RNA, thereby reducing the amount of S protein in the culture supernatant.

### 5. Conclusions

In summary, psoralen inhibited HBV replication by negatively regulating the HBV RNA transcription regulated by FOXO1-PGC1 $\alpha$ . The unique anti-HBV mechanism suggests that psoralen may be a hit compound as an HBV inhibitor. Thus, if psoralen and psoralen derivatives could be further optimized or modified by subsequent SAR study and drug-like optimization, a more powerful anti-HBV candidate may be discovered. That may provide a new option for further development of HBV therapeutics for the treatment of chronic hepatitis B.



**Fig. 5.** Psoralen inhibited the transcription of pgRNA and precore RNA by affecting the activity of Enhance II/core promoter. **A** Huh7 cells were transfected with pHBV1.3 plasmid and treated with psoralen for 72 h. Levels of pgRNA, precore RNA, PreS1 RNA, PreS2/S RNA and X RNA were detected by qRT-PCR. GAPDH was used as a control. **B** The luciferase reporter assay was used to detect promoter activity. Values are mean  $\pm$  SD of 3 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Fig. 6.** Psoralen inhibited HBV transcription by down-regulating FOXO1 levels. The pHBV1.3 transfected Huh7 cells were treated with psoralen for 3 days. **A** The mRNA and protein levels of FOXO1 were detected by qRT-PCR and Western blot. **B** The mRNA and protein level of PGC1 $\alpha$  using qRT-PCR and Western blot. Image J software was used to quantitatively analyze the protein results. GAPDH was used as a control. Values are means  $\pm$  SD of 3 independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . **(C)** The cells were immunoprecipitated with anti-FOXO1 antibody and immunoblotted to determine the interaction between FOXO1 and PGC1 $\alpha$ .



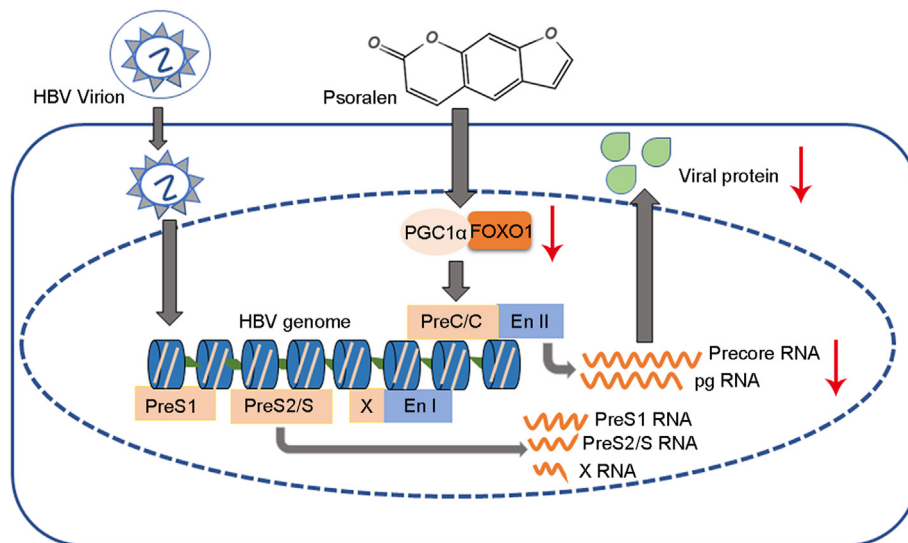


Fig. 7. A schematic presentation of the mechanism for Psoralen's anti-HBV activity. Psoralen exerted the antiviral effect against HBV replication through a down-regulation of FOXO1-PCG1 $\alpha$  complex, and then inhibited the activity of Enhance II/core promoter.

### Data availability

Data supporting the findings of this study are reported in the main text and figures and in Supplementary Fig. S1, Fig. S2 and Table S1. All reagents and materials generated in this study are available from the corresponding authors.

### Ethics statement

This article does not contain any studies with human or animal subjects performed by any of the authors.

### Author contributions

Jianping Zuo: conceptualization, supervision. Li Yang: conceptualization, supervision. Xinna Ma: methodology, formal analysis, resources, Writing – original draft. Heng Li: methodology, formal analysis, resources, Writing – original draft. Ying Gong: validation. Feifei Liu: validation. Xiankun Tong: Writing – review & editing. Fenghua Zhu: project administration. Xiaoqian Yang: project administration.

### Conflicts of interest

The authors declare that they have no conflict of interest.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.01.027>.

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