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Antiviral activity of chemical compound isolated from *Artemisia morrisonensis* against hepatitis B virus *in vitro*

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ABSTRACT

The compound *p*-hydroxyacetophenone (PHAP) isolated from *Artemisia morrisonensis* was found to have potential anti-HBV effects in HepG2 2.2.15 cells. We clarified its antiviral mode further and HBV-transfected Huh7 cells were used as the platform. During viral gene expression, treatment with PHAP had no apparent effects on the viral precore/pregenomic RNA. However, the 2.4-kb preS RNA of viral surface gene increased significantly relative to the 2.1-kb S RNA with PHAP. Promoter activity analysis demonstrated that PHAP had a potent effect on augmenting the viral preS promoter activity. The subsequent increase in the large surface protein and induce endoplasmic reticular (ER) stress has been reported previously. Interestingly, PHAP specifically reduced ER stress related GRP78 RNA/protein levels, but not those of GRP94, in treated Huh7 cells while PHAP also led to the significant intracellular accumulation of virus. Moreover, treatment with the ER chaperone inducer thapsigargin relieved the inhibitory effect of PHAP based on the supernatant HBV DNA levels of HBV-expressed cells. In conclusion, this study suggests that the mechanism of HBV inhibition by PHAP might involve the regulation of viral surface gene expression and block virion secretion by interference with the ER stress signaling pathway.

1. Introduction

Hepatitis B virus (HBV) infections are a major global health problem despite the availability of an effective vaccine. Over two billion people are infected at present and about 400 million are chronically infected carriers of this virus (McMahon, 2005; Popper et al., 1987). These carriers are at risk of severe liver diseases including acute hepatitis, chronic active hepatitis, cirrhosis, and eventually hepatocellular carcinoma (Arbuthnot and Kew, 2001; Feitelson, 1999; Park et al., 2006). Although interferons and nucleotide analogs have been used widely to treat chronically infected patients, the rapid development of resistance and their undesirable side-effects mean that the development of an antiviral drug is urgently needed to treat HBV.

Previous studies have shown that several naturally occurring constituents of medicinal plants have anti-HBV activities and it was suggested that their antiviral activities were mediated via the regulation of endoplasmic reticulum (ER) homeostasis (Huang et al., 2006, 2009). The ER is a cytoplasmic compartment where proteins and lipids are synthesized. Chaperones have been proposed to facilitate correct protein folding in the ER and they also prevent protein unfolding, which causes the aggregation of proteins in the ER and induces ER stress (Lambert and Prange, 2003). Two well-characterized ER chaperone proteins are the 78-kDa glucose-regulated protein (GRP78 or BiP) and GRP94, which are known to be induced by ER stress (Boelens et al., 2013; Dey et al., 2006; Eletto et al., 2010; Kaufman, 1999; Lee, 2005; Patil and Walter, 2001) and they are involved with the ER stress signaling pathway. GRP78 was also demonstrated to function during HBV viral morphogenesis where it interacts with the large surface protein of HBV and regulates its posttranscriptional topological reorientation (Cho et al., 2003; Lambert and Prange, 2003). However, the mechanisms by which natural products affect the ER signaling pathway and inhibit HBV propagation remain to be elucidated. Therefore, it is important to clarify the interaction between HBV gene expression and ER stress to facilitate anti-HBV drug development.

Artemisia morrisonensis is an endemic alpine species in Taiwan. Traditionally, *A. morrisonensis* was used to treat rheumatoid arthritis, allergic rhinitis, headache, and edema by the aboriginal people. Relatives of this species have long been used as traditional medicines with antiviral and antibacterial activities, while they have also been used to treat related liver disease (Tan et al., 1998).

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However, the active constituents of this species and their therapeutic effects in liver disease remain unknown. Recently, we have made substantial progress in elucidating the chemical and pharmacological properties of this species (Chou et al., 2012). Solvent extracts of the leaves of *A. morrisonensis* have yielded a major component with potential antiviral activity against HBV. Advanced purification of this extract identified the compound *p*-hydroxyacetophenone (PHAP). We found that PHAP inhibited HBV by regulating the viral surface gene and interfering with the ER stress signaling pathway. In the present study, we showed that the antiviral mechanism of PHAP is mediated by changes in the expression ratio of the HBV large surface protein relative to the small and middle proteins. Thus, inhibition of the chaperone protein by PHAP is through affecting the gene regulation level.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies against the GRP78, GRP94, HBV-preS2 surface antigen and albumin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The DIG high prime DNA labeling and detection starter kit was obtained from Roche (Mannheim, Germany). Power SYBR Green PCR master mix was purchased from Applied Biosystem (Foster City, CA, USA). A dual-Luciferase reporter assay kit was purchased from Promega (Madison, WI, USA). The Trizol® total RNA isolation solution and Lipofectamine 2000 transfection reagent were from Invitrogen (Carlsbad, CA, USA). Thapsigargin (TG), lamivudine (2′,3′-dideoxy-3′-thiacytidine, commonly known as 3TC), DNase I and anti-α-tubulin antibody were purchased from Sigma (St Louis, MO, USA).

2.2. Preparation of PHAP

p-Hydroxyacetophenone (PHAP) (Fig. 1A) used in this study was purified from the leaves of *A. morrisonensis* as major component, i.e., 130 mg/g in an ethanol extract, by gel filtration chromatography using Sephadex LH-20, as described previously (Chou et al., 2012). The purity of PHAP was determined as more than 99.5% by HPLC–MS analysis and ^{1}H NMR spectroscopy. PHAP was dissolved in dimethyl sulfoxide (DMSO) before treatment. The final concentration of DMSO did not exceed 0.5% and it caused no detectable effects during treatment.

2.3. Cell culture

HepG2 2.2.15 cells derived from HepG2 human hepatocellular carcinoma cells and stably transfected with a head-to-tail HBV DNA dimer (Sells et al., 1987) were maintained in MEM with heat-inactivated 10% fetal bovine serum (FBS) (GIBCO/BRL, Invitrogen, Carlsbad, CA, USA) and 1% antibiotics. In parallel experiments, human hepatoma Huh7 cells were maintained in DMEM supplemented with heat-inactivated 10% FBS and 1% antibiotics. HepG2 2.2.15 and Huh7 cells were both grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.4. Cell cytotoxicity assay

The viabilities of HepG2 2.2.15 and Huh7 cells were determined using a CellTiter 96® AQ_{ueous} one solution cell proliferation assay kit (MTS) (Promega, Madison, WI, USA). Briefly, HepG2 2.2.15 or HBV-transfected Huh7 cells were plated into 96-well plates at a density of 4×10^4 cells/ml for 24 h. Next, the cells were treated with serial dilutions (36.8–2352.9 μM) of PHAP every 3 days for up to 9 days. The toxicity was measured in cells according to the manufacturer's protocol. All measurements included four replicates and the results were expressed as percentages relative to the control group. Noncytotoxic drug concentrations were used in the antiviral activity assays.

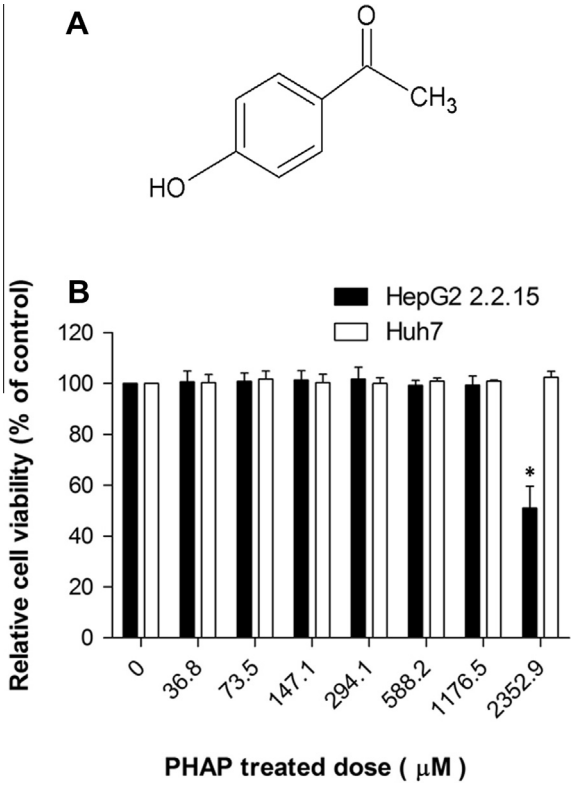

Fig. 1. (A) Schematic illustration of PHAP structure. (B) Cytotoxic effects of PHAP on HepG2 2.2.15 and Huh7 cells. To determine the cytotoxicity, cells were plated in 96-well plates for 24 h and treated with serial dilutions (36.8–2352.9 μM) of PHAP every 3 days for up to 9 days. After treatment, the cells were subjected to a cytotoxicity assay. The data are expressed as mean and the standard deviation of the mean. ($n = 4$) (*$P < 0.01$ vs untreated cells).

2.5. Determination of HBsAg and HBeAg

After treating the HepG2 2.2.15 cells or HBV-transfected Huh7 cells, the levels of the viral surface antigen (HBsAg)-and e antigen (HBeAg) were measured in the culture media using an enzyme immunoassay (EIA) kit (Johnson and Johnson, Skillman, NJ, USA), according to the manufacturer's instructions.

2.6. Immunoprecipitation of extracellular virions for viral DNA extraction

The immunoprecipitation of virions from cultural media was described previously (Shih et al., 2008). In briefly, the cultural media were centrifuged at 3000 rpm to remove cell debris. Then the viral particles from 4 ml clear cultural media were pelleted by centrifugation at 40,000 rpm at 4° C for 5 h in a SW41 rotor (Beckman-Coulter Inc., Brea, CA, USA) through a 20% sucrose cushion and resuspended in 0.5 ml 0.1 × TE buffer (0.1 mM EDTA, 1 mM Tris–HCl, pH8.0). The samples were immunoprecipitated with 1 μg anti-HBV preS2 surface antibody (S26 clone) (Santa Cruz, CA, USA) for overnight at 4° C. The enveloped virions were precipitated using protein A-Sepharose beads (GE Healthcare, Buckinghamshire, UK) at 4° C for 4 h and virion particle bound beads was washed with wash buffer (0.5% Tween-20, 0.5% SDS in PBS). The precipitated samples were digested with 20 U/ml DNase I at

37° C for 30 min and then inactivated by adding EDTA (pH 8.0) to final concentration of 2.5 mM. The samples were pelleted by centrifugation at 12,000 rpm for 10 min and resuspended in TEN buffer (10 mM Tris–HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.1 mg/ml proteinase K, pH 8.0) at 50° C for 2 h. After incubation, the viral DNA samples were extracted by phenol/chloroform and precipitated with ethanol. The pellets were dissolved in 0.1 × TE buffer and subjected to Southern blot analysis (see Section 2.7).

2.7. Southern blot and Real-time PCR analysis of intracellular HBV-DNA synthesis

Encapsidated HBV DNA in cells was extracted from core particles and fractionated on 1.0% agarose gels and transferred onto Hybond N^+ membrane (GE Healthcare, Buckinghamshire, UK) as described by Pugh et al. (1988). HBV DNA was detected by Southern blot analysis using the DIG-labeled full-length HBV probe. In addition, HBV DNA in the virion from conditioned media that were untreated or treated with PHAP or Lamivudine (3TC) was isolated as described in Section 2.6 and subjected to Southern blot analysis as described above. For Real-time PCR analysis of HBV DNA, the forward primer was 5′-AGGAGGCTGTAGGCATAAATTGG-3′ and the reverse primer was 5′-CAGCTTGGAGGCTTGAACAGT-3′ (Feng et al., 2009). The PCR reactions were performed using SYBR Green PCR master mix and the primer pair with the following program: initial denaturation at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15 s and annealing/extending at 58 °C for 1 min.

2.8. Western blot analysis

The whole cell proteins of Huh7 were prepared by suspending in RIPA buffer with protease inhibitors. The suspensions were subjected to shear stress several times using a syringe with a 25-gauge needle and centrifuged at 14,000 rpm for 20 min at 4° C. The cellular protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) and 25 μg of protein from each sample was used for sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gel electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham, GE Healthcare, Buckinghamshire, UK) by electroblotting and then blocked for 1 h with PBST (PBS buffer with 0.05% Tween-20) containing 4% nonfat milk. The immunoblots were incubated with primary antibody followed by HRP-conjugated secondary antibody. The antibody-bound proteins were detected using ECL reagent (Amersham, GE Healthcare, Buckinghamshire, UK). The same membranes were then stripped and reprobed using anti-α-tubulin antibody (Sigma, St Louis, MO, USA) as the loading control.

2.9. Transfection

Huh7 cells were seeded in 60-mm dishes at the same density (as described in the cytotoxicity assay) for 24 h and then transfected with the pHBV1.2 plasmid (a plasmid containing the 1.2-fold of HBV adw2 serotype genome (nt 2186–1986), which was cloned into the *Dra*I and *Bgl*II sites of pGEM-7Zf(+) vector; Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 10% FBS containing DMEM for 24 h, according to the manufacturer's protocol. The cells were then treated with PHAP for a further 48 h.

2.10. Northern blot and RT-PCR analysis

Huh7 cells were plated in 60-mm culture dishes, transfected with the pHBV1.2 plasmid for 24 h, and treated with PHAP in DMEM with 2% fetal bovine serum for 48 h. The total RNA was extracted from the treated cells using Trizol® isolation buffer (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. To control the variations in transfection efficiency, cells were co-transfected with pCMV-β (Clontech, Palo Alto, CA, USA), which contains the CMV immediate-early promoter driving the β -galactosidase (β-*gal*) gene. Five micrograms of total cellular RNA was subjected to Northern blot analysis using a DIG-labeled (Roche Mannheim, Germany) full-length HBV genome probe. The β-*gal* gene probe derived from the restricted digestion of pCMV-β plasmid with *Not*I enzyme was used to normalized transfection efficiency among treatments. In addition, the GAPDH probe derived from pGAPDH plasmid was used as loading control. Before the RT-PCR analysis, RNA samples were prepared from HBV-transfected and PHAP-treated Huh7 cells, and the RNA was reverse-transcribed at 42 °C for 90 min using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR was performed using specific primers for the GRP78 gene, i.e., (5′-GATAATCAACCAACTG TTAC-3′ and 5′-GTATCCTCTTCACCAGTTGG-3′), GRP94 gene, i.e., 5′-CAGTTTTGGATCTTGCTGTGG-3′ and 5′-CAGCTGTAGATTCCTTT GC-3′, and the β-actin gene, i.e., 5′-TCGTCACCAACTGGGACGACA TGG-3′ and 5′-GATCTTGATCTTCATTGTGCTGGG-3′ (Wang et al., 2002; Wang et al., 2008). The PCR products were electrophoresed on a 2% agarose gel and detected by ethidium bromide staining.

2.11. Plasmid construction and the HBV promoter luciferase reporter assay

HBV surface gene promoter-reporter plasmids were constructed using standard recombinant DNA techniques (Sambrook et al., 1989). Briefly, HBV genomic fragments that corresponded to the Core (nt 1636–1851), S (nt 3114–220), PreS (nt 2438–2855) and X (nt 1071–1357) gene promoter regions were amplified by PCR using the pHBV1.2 plasmid as the template and subsequently inserted into the *Sac*I/*Xho*I sites of the pGL4.17 luciferase-reporter expression vector (Promega, Madison, WI, USA). All of the DNA sequences were verified by appropriate restriction enzyme digestion and direct sequencing. For transfection and the luciferase assay, the cells were plated into 24-well culture plate and transfected with promoter-reporter constructs (1 μg/well) and pRL-SV40 in serum-free DMEM for 24 h, washed with 1× PBS, incubated in DMEM supplemented with 2% FBS, and treated with or without PHAP (294.1 μM) for 2 days. The luciferase assay was performed according to manufacturer's instructions (Promega, Madison, WI, USA). For promoter-reporter construct transfection, the pRL-SV40 Renilla luciferase expression plasmid (0.02 μg/well) (Promega, Madison, WI, USA) was co-transfected and used to normalize the basal level luciferase activity.

2.12. Analysis of mRNA stability after the inhibition of transcription by actinomycin D

For detection of mRNA stability of GRP78 during treatment, the polypeptide-containing transcription inhibitor, actinomycin D, was used as specific inhibitor of formation of new RNA. In this experiment, Huh7 cells were transfected with pHBV1.2 for 24 h and then treated with actinomycin D (10 μg/ml) and DMSO or actinomycin D concomitant with PHAP (294.1 μM) at different time periods (2–24 h). After treatment, the cells were subjected to total RNA extraction and RT-PCR analysis of GRP78 mRNA as described in the Section 2.10.

2.13. Statistical analysis and quantification of data

The data were expressed as the mean and the standard deviation (SD) of the mean from three independent experiments using

GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Variance analysis and the Student's *t*-test were used for data analysis. Differences were considered significant when $P < 0.01$. Quantitative data from Northern, Western blot and RT-PCR analysis were obtained using computing densitometer and TotalLab Quant software (Nonlinear Dynamics Ltd.).

3. Results

3.1. Cellular toxicity of PHAP

To exclude the possibility of HBV production being suppressed by PHAP due to its cytotoxicity, the cell viability of PHAP on HepG2 2.2.15 cells was tested using the MTS assay. In the 9-days PHAP treatment period, the cytotoxic effects of PHAP on HepG2 2.2.15 cells were only observed when the treatment dose was >1.176 mM (Fig. 1B). The toxicity of HBV-transfected Huh7 cells was also examined during an additional analysis of the mechanism. In the same treatment period, PHAP had no significant toxic effects on Huh7 cells at a dose of up to 2.353 mM (Fig. 1B). In the following study, noncytotoxic doses were used for antiviral treatment.

3.2. Effects of PHAP on HBV virions and antigen secretion in HepG2 2.2.15 cells

To investigate the effect of PHAP on the expression of viral antigens, the secretion rates of HBsAg and HBeAg by HepG2 2.2.15 cells were measured using EIA. Cells were treated with various concentrations (73.5, 147.1, and 294.1 μM) of PHAP and the secretions of HBsAg and HBeAg were suppressed significantly in a dose-dependent manner compared with the vehicle control. The inhibitory effects of PHAP on HBsAg secretion were higher than those on HBeAg (Fig. 2A). To further examine the function of PHAP during virus secretion, the virion DNA was isolated from immunoprecipitated virion particles in the conditioned medium and Southern blot was performed. The results showed that treatment with PHAP significantly reduced the secretion of virion in a dose-dependent manner. The maximum dose of PHAP-caused inhibition of virion secretion was compared with 3TC (Lamivudine, a commonly used antiviral drug) at the same treatment level at a dose of 43.6 μM (Fig. 2B).

3.3. The effect of PHAP on HBV gene expression

To further examine the molecular regulatory effects of PHAP on HBV gene expression, Huh7 cells were transfected with the pHBV1.2 HBV genome-containing plasmid (Blum et al., 1991) and treated with two doses (73.5 and 294.1 μM) of PHAP for 2 days to analyze the HBV viral RNA expression levels. Treatment with PHAP appeared to have no effect on the level of precore/pregenomic RNA. However, the expression of the major S 2.1-kb mRNA was suppressed concomitantly with the increased dose of PHAP. By contrast, the level of the 2.4-kb preS mRNA was enhanced by PHAP treatment (Fig. 3A).

3.4. Effect of PHAP on the activity of the HBV surface gene promoter

The regulatory effects of PHAP on HBV gene expression were highest in the surface gene. The four promoters that corresponded to the viral gene were isolated and cloned into the pGL4.17 luciferase reporter vector and we tested the effect of PHAP on the promoter activity level. In this study, pCore-Luc, pS-Luc (S promoter), pPreS-Luc (preS promoter) or pX-Luc constructs were transfected into Huh7 cells for 24 h and treated with the maximum

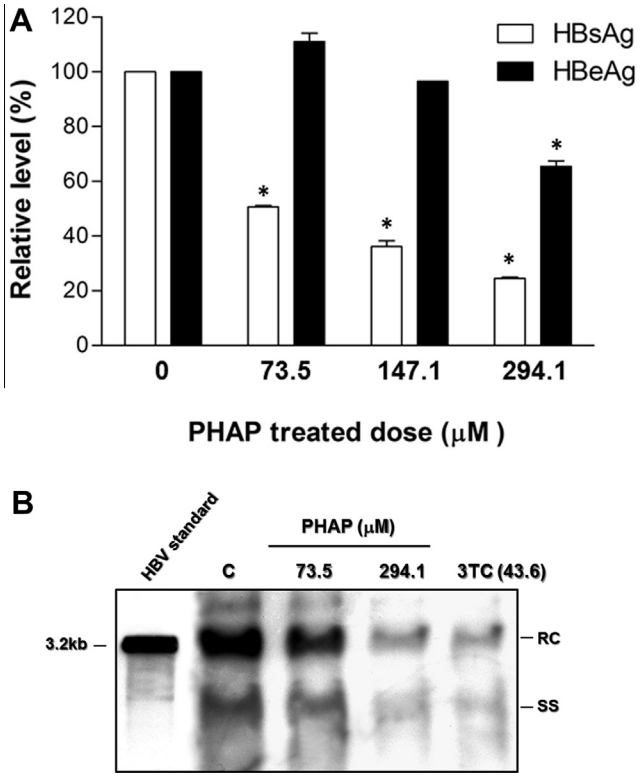

Fig. 2. The effects of PHAP on the secretion of (A) HBV viral antigen, and (B) viral particles in HepG2 2.2.15 cells. Cells were treated with two concentrations (73.5 and 294.1 μM) of PHAP for 3 days and the conditioned media were collected for EIA analysis of HBsAg and HBeAg. In addition, the HBV DNA was isolated from immunoprecipitated virion particles in conditioned media and subjected to Southern blot analysis. HBV standard, 2 ng of 3.2 kb linear HBV genome marker; RC, relaxed circular; SS, single stranded. The data are expressed as the mean and the standard deviation of the mean. ($n = 3$) (*$P < 0.01$ vs untreated cells).

inhibitory dose of PHAP before performing a luciferase assay. The results showed that PHAP treatment did not affect the Core, S and X promoter activity whereas it significantly increased the preS promoter activity (Fig. 4). Furthermore, the promoter activity assay was also performed in HepG2 2.2.15 cells to clarify if PHAP affect viral promoter activity through feedback regulation of viral proteins. The results showed that promoter activity conducted in HepG2 2.2.15 is similar that in Huh7 cells except for the S promoter. In HepG2 2.2.15 cells, PHAP significantly reduced the S promoter activity. This result indicated that PHAP could regulate the HBV surface gene via a different pathway and feedback regulation of viral protein is involved in surface gene expression.

3.5. ER chaperones are essential for the inhibitory effects of PHAP on HBV

The blocking effects of PHAP on HBV viral particle secretion and the changes in the surface gene were examined further to determine whether the effect of PHAP was caused by ER stress induction. Previous studies have shown that two ER chaperone proteins (GRP78 and GRP94) may be indicators of ER stress while GRP78 can interact directly with the large surface protein of HBV, which regulates viral morphogenesis and viral particle secretion (Huang et al., 2009). Surprisingly, PHAP treatment significantly suppressed the GRP78 mRNA and protein levels but not those of GRP94 in RT-PCR (Fig. 5A) and Western blot (Fig. 5B) analyses. In addition, the supernatant HBV DNA level was reduced significantly by PHAP treatment. However, the inhibitory effect on the supernatant HBV DNA level caused by PHAP was reversed

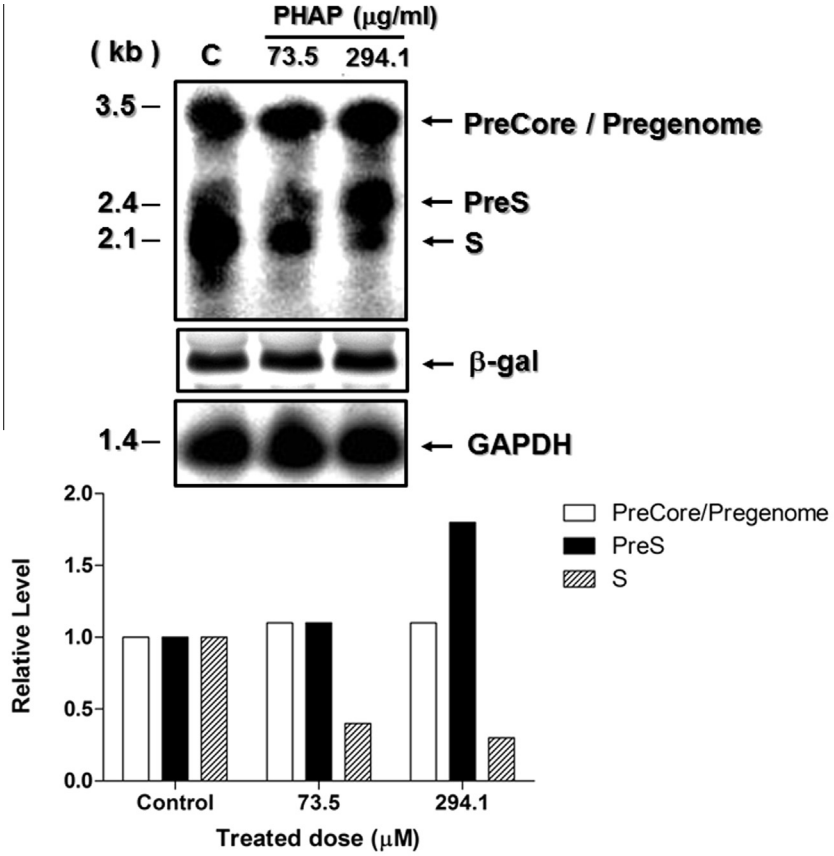

Fig. 3. The effects of PHAP on HBV gene expression in Huh7 cells. Huh7 cells were transfected with the pHBV1.2 plasmid for 24 h and treated with two concentrations (73.5 and 294.1 μM) of PHAP for 2 days. Treated cells were harvested and subjected to total RNA isolation. The total RNA from transfected and treated Huh7 cells was subjected to a Northern blot analysis using HBV whole genome DNA as the probe, as described in the Material and methods. GAPDH was used as an RNA loading control and β -gal was used to detect the efficiency of each transfection. The intensity of each RNA band was quantitated with densitometer and the relative amount was normalized with loading control. The data shown are representative of three replicate experiments.

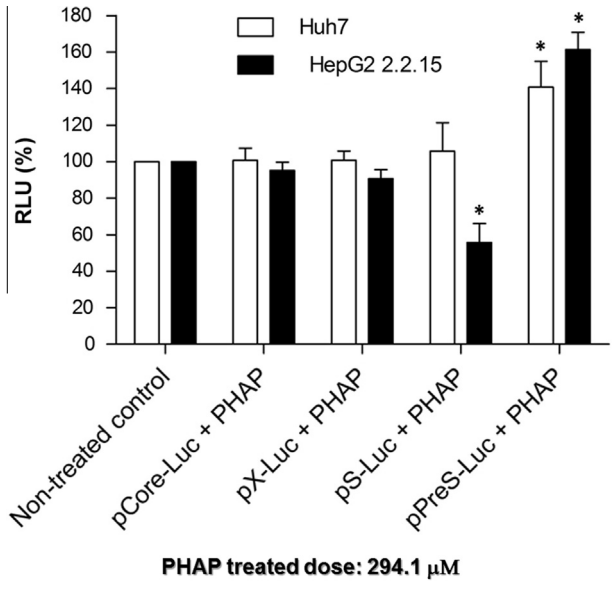

Fig. 4. The effect of PHAP on HBV gene promoter activity. Huh7 cells were seeded in 24-well plates for 24 h and transfected with either pCore-Luc, pS-Luc, pPreS-Luc or pX-Luc viral gene promoter-luciferase reporter constructs, together with pRL-SV40, for 24 h and then treated with PHAP (294.1 μM) for a further 24 h. The cellular lysates were prepared for luciferase assay (see the Section 2). The data are expressed as the mean and the standard deviation of the mean. ($n = 3$) (*$P < 0.01$ vs untreated cells).

when thapsigargin (TG) was cotreated with PHAP (Fig. 5C). Consistently, the amount of GRP78 protein was also observed that decreased by PHAP and potent increased when the treatment of PHAP was combined with TG in immunoblot. Moreover, treatment with TG alone can increase GRP78 protein but not the supernatant HBV DNA level. However, PHAP or TG treatment did not affect the expressed level of albumin; a secretory protein that is exclusively synthesized in hepatocytes and its secretion is occurred independently of GRP78. These results suggest that PHAP may specifically regulate the ER signaling pathway by inhibiting *GRP78* gene expression and by interrupting the secretion of HBV virions in HBV-transfected Huh7 cells (Fig. 5C). In addition, to test the possibility of effect of PHAP on GRP78 mRNA stability, the assay was conducted using a polypeptide-containing antibiotic, actinomycin D, as transcription blocker in determining whether PHAP could change the stably status of GRP78 RNA during treatment. After treatment of actinomycin D at different time period, PHAP did not alter GRP78 mRNA stability as comparison to vehicle (DMSO) control (Fig. 5D).

3.6. PHAP-induced accumulation of virus in HBV-transfected Huh7 cells

As a consequence of the ER regulation dysfunction and the discrepancy in the expressed levels of intra- and extracellular HBV viral surface proteins by PHAP, it was expected that PHAP treatment might lead to the retention of viral surface proteins in cells. To test this possibility, Huh7 cells were transfected with

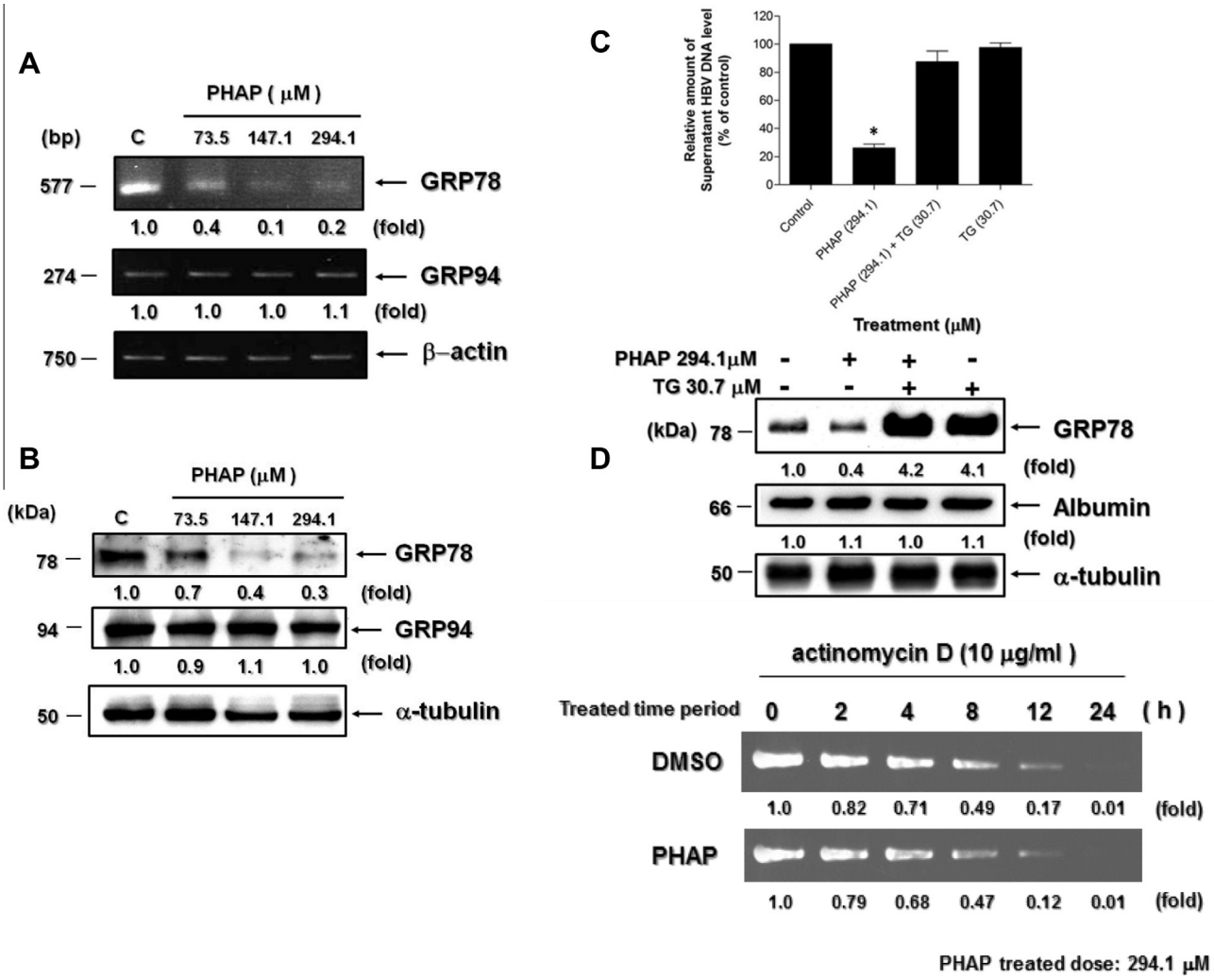

Fig. 5. Effect of chaperone proteins on the inhibitory effects of PHAP on HBV viral capsid secretion. Huh7 cells were transfected with pHBV1.2 for 24 h, treated with TG (30.7 μM) or various concentrations (73.5, 147.1, and 294.1 μM) of PHAP, or cotreated with TG, for 2 days. (A) The total RNA was extracted from cells and subjected to RT-PCR analysis to determine *GRP78* and *GRP94* chaperone gene expression. (B) The total cellular protein was extracted and immunoblotting was used to detect the GRP78/94 chaperone proteins for comparison. (C) The supernatant HBV DNA level was quantified by Real-time PCR. In the same reaction, total cellular protein was used to detect GRP78, α-tubulin and albumin, a secretory protein in hepatocyte in immunoblotting for comparison. (D) GRP78 mRNA stability was conducted using actinomycin D as blocker in newly synthesized transcripts. At different time period, cells that treated with DMSO or PHAP were collected for total RNA extraction and subjected to RT-PCR analysis. The intensity of each RNA, cDNA and protein band was quantitated with densitometer and the relative amount was normalized with internal control. The data for Real-time PCR are expressed as the mean and the standard deviation of the mean. ($n = 3$) ($^*P < 0.01$ vs untreated cells).

the HBV genome and treated with increasing doses of PHAP. The conditioned medium and total cellular proteins were collected and subjected to EIA analysis to determine the intra- and extracellular levels of HBsAg. The EIA results indicated that PHAP treatment dramatically enhanced the accumulation of intracellular HBsAg (Fig. 6A). To test whether ER chaperones are essential for the effect of PHAP on viral surface protein accumulation, the chaperone inducer TG was applied and co-treated with PHAP at a dose of 30.7 μM. The results showed that TG treatment significantly reduced the intracellular accumulation of HBsAg caused by PHAP. In addition, the viral DNA from HBV-expressed cells that treated with PHAP or cotreated with TG was isolated and performed Southern blot analysis. As showed in Fig. 6B, PHAP potently augments the intracellular HBV DNA level in a dose dependent manner and this effect can be relieved by TG cotreatment. However, TG alone did not affect HBV DNA level. These results suggest that PHAP could block HBV viral particle secretion through ER chaperone gene regulation. In addition, the abundant immature viral replicative intermediates (RI) were also been observed at lower area of blot filter. This phenomenon has been described previously in cells that transduced with HBV genome (Ren and Nassal, 2001).

4. Discussion

In this study, we demonstrated that PHAP from *A. morrisonensis* inhibited HBV HBsAg and HBeAg secretion, while it also reduced the supernatant HBV virion DNA level in HepG2 2.2.15 cells. To clarify the inhibitory effects of PHAP on HBV, we used HBV-transfected Huh7 cells as a platform to determine how this compound regulates viral gene expression and viral propagation, which was facilitated by the low sensitivity of determination for viral gene products in HepG2 2.2.15 cells. PHAP significantly downregulated S RNA but increased preS RNA expression but without affecting the precore/pregenomic RNA levels that transcribed from transfected epigenetic HBV genome. This suggests that PHAP specifically regulates the surface gene at the transcription level in transfection condition.

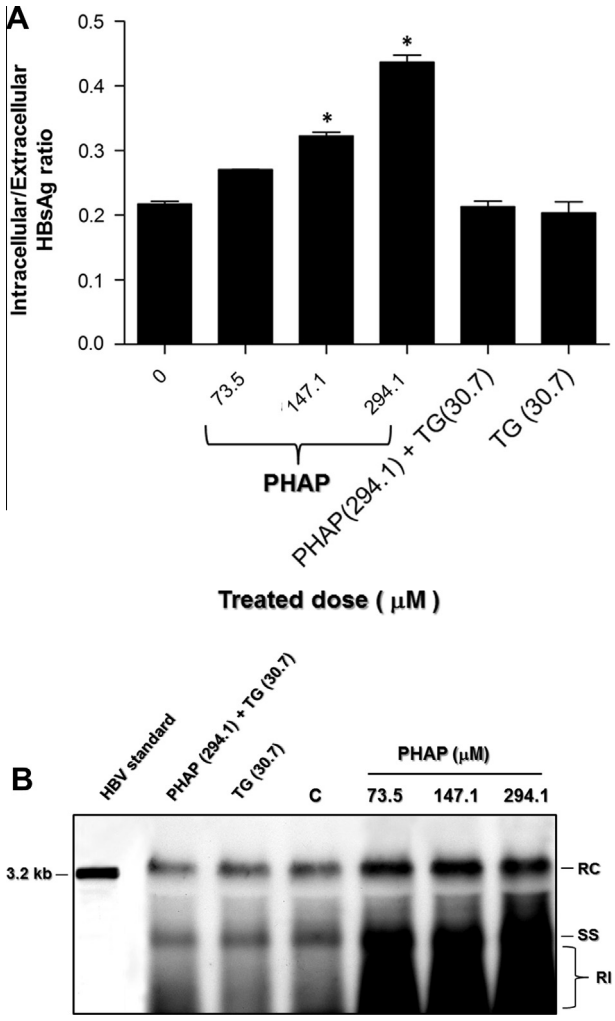

Fig. 6. Effect of PHAP on the (A) HBsAg and (B) HBV viral particle intracellular accumulation. Huh7 cells were transfected with the HBV genome, treated with various concentrations (73.5, 147.1, and 294.1 μM) of PHAP or PHAP (294.1 μM) concomitant with the ER inducer thapsigargin (TG) (30.7 μM) for 2 days, and the conditioned media and extracted cellular proteins were subjected to HBsAg analysis using EIA. In the same reaction, intracellular viral particle was isolated and HBV DNA was extracted for Southern blot analysis. RC, relaxed circular; SS, single stranded; RI, replicative intermediates. The data are expressed as the mean and the standard deviation of the mean. ($n = 3$) (*$P < 0.01$ vs untreated cells).

We also examined the viral promoter activity in response to PHAP treatment and four major promoters of the surface gene were isolated so a reporter analysis could be conducted. The results indicated that the preS promoter, but not the Core, X, and S promoter, was increased by PHAP treatment. Surprisingly, in our study indicated that the RNA level of S was significantly decreased by PHAP when the entire HBV genome was present in cells. By contrast, the S promoter activity was not affected by PHAP treatment of cells and it was not expressed by HBV. These results suggest that the molecular mechanism by which PHAP affects the surface gene expression might be coordinated via the feedback regulatory effects of viral proteins on gene expression, at least for the surface gene. To test this possibility, HepG2 2.2.15 cells were transfected with viral promoter-reporter gene constructs, respectively, which were treated with PHAP, followed by a luciferase analysis. The activity of the S promoter was decreased significantly with PHAP treatment when HBV was present in the cells. In the other set of experiments, Huh7 cells were co-transfected of pCMV-LHBs (plasmid that expressed HBV large surface protein) or pCMV-MSHBs (plasmid that expressed middle/small surface proteins) with promoter-reporter constructs, followed by PHAP treatment and performed the luciferase assay. However, the promoter activities were similar with condition that Huh7 cells were transfected with promoter-reporter alone without pCMV-LHBs or pCMV-MSHBs co-expression (data not shown). These results suggest the possible feedback regulatory effects of viral proteins other than surface antigens on surface gene expression.

Previously, the ratio of large surface protein relative to that of the middle and small surface proteins synthesized by HBV were shown to be crucial for viral replication (Persing et al., 1986). Xu et al. demonstrated that the accumulation of large surface protein and its intracellular retention could induce ER stress and trigger the expression of viral middle and small surface protein via a regulatory feedback mechanism (Xu et al., 1997). Our study suggests that the regulatory effects of PHAP on the surface gene might lead to intracellular blockage of the HBsAg secretory pathway in ER. In addition, PHAP regulates surface gene expression is not only affecting the feedback regulation of viral proteins on S promoter but also augmenting the preS promoter directly.

ER is an important compartment for the modification and folding of membrane or secretary proteins while it is also the site where the synthesis of cellular lipids and sterols occurs (Kaufman, 1999). It is known that viral infection and the overexpression of viral proteins may lead to an unfolded protein response (UPR) (DuRose et al., 2006; He, 2006) and subsequent ER stress. The ER luminal chaperones are induced to catalyze the correct folding of proteins within the ER. The best-characterized chaperones in the ER are GRP78 and GRP94. These two chaperone proteins are induced to maintain ER homeostasis (Kaufman, 1999). In addition, many molecular signaling mechanisms coordinate the complex ER stress response at the translational and transcriptional level (Chapman et al., 1998). However, the mechanism that coordinates HBV infection via the ER signaling pathways in hepatocytes to establish chronic infections in HBV carriers remains unknown. Thus, the elucidation of the mechanisms that regulate HBV viral gene expression and ER homeostasis are important for the development of new anti-HBV therapies. We found that the viral surface gene was regulated by PHAP while the ER signaling pathway was also involved with HBV maturation and morphogenesis. Surprisingly, the expression level of the *GRP78* gene chaperone was specifically downregulated by PHAP treatment. However, the stability of GRP78 mRNA did not change during treatment indicating that the action of PHAP on GRP78 expression is through gene regulation. Furthermore, the inhibitory effect of PHAP on the supernatant HBV DNA level was reversed and the secretion of viral particles occurred when the GRP78 inducer, thapsigargin, was introduced concomitantly with PHAP into HBV-transfected Huh7 cells. Recently, Huang et al. used an immunoprecipitation analysis to show that GRP78 can interact with the HBV core and surface proteins (Huang et al., 2009), which indicates that GRP78 may be involved with the process of nucleocapsid envelopment. It is expected that PHAP-induced suppression of GRP78 protein expression will lead to the blockage of viral particle assembling and secretion, which suggests that the GRP78 protein has an important role in virion maturation and secretion. However, the interaction between GRP78 and viral proteins during viral gene regulation is coordinated via ER homeostasis remains to be elucidated. Furthermore, differential regulation of ER stress may dictate viral replication and pathogenesis (He, 2006).

For medicinal applications, whether the virus can be controlled well enough to ameliorate disease is the most crucial issue that affects antiviral therapeutic strategies, as well as preventing the viral resistance commonly caused by viral reverse transcriptase inhibitors. In our study, we showed that PHAP can regulate HBV surface gene expression while it also controls the *GRP78* chaperone gene

during ER stress, instead of inhibiting the viral reverse transcriptase activity. In conclusion, the anti-HBV effect of PHAP is coordinated via pathways that link viral surface gene regulation and ER stress homeostasis. In addition, GRP78 is a key regulator during virus replication and morphogenesis, as well as maintaining ER homeostasis. Overall, we have elucidated the mechanism underlying the anti-HBV effects of PHAP and we identified a novel therapeutic target for HBV drug development.

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