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Antiviral Research 77 (2008) 206–214
Antiviral Research
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The role of helioxanthin in inhibiting human hepatitis B viral replication and gene expression by interfering with the host transcriptional machinery of viral promoters

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Received 24 August 2007; received in revised form 22 November 2007; accepted 21 December 2007

Abstract

A non-nucleosidic compound, Helioxanthin (HE-145), was found to suppress HBV gene expression and replication in HCC cells. To understand the molecular mode of action of HE-145 on HBV gene expression, the effects of HE-145 on four viral promoter activities using luciferase as a reporter were examined. It was found that HE-145 selectively suppresses surface antigen promoter II (SPII) and core promoter (CP) but has no effect on surface antigen promoter I (SPI) or promoter for X gene (Xp). The suppressive effects of HE-145 on either SPII or CP activity is liver-specific, since no suppressive activity of HE-145 was observed when CP or SPII promoter activity was assayed in non-liver cells such as HeLa or 293T. To examine the mode of action of HE-145, EMSA analysis revealed that HE-145 decreased the DNA-binding activity of nuclear extract of HepA2 cells to specific *cis* element of HBV promoter for core antigen, including peroxisome proliferator-activated receptors (PPARs), PPARs binding site (PPRE), α-fetoprotein transcription factor (FTF), and Sp1. Ectopic expression of PPARγ or HNF4α partially reversed the HE-145-mediated suppression of HBV RNA. Therefore, HE-145 may represent a novel class of anti-HBV agents which selectively modulate transcriptional machinery of human liver cells to suppress HBV gene expression and replication.

Keywords: Helioxanthin; Hepatitis B virus; Viral promoters; Hepatic nuclear factors; Human hepatocellular carcinoma cells

1. Introduction

Hepatitis B virus (HBV) infection causes acute and chronic hepatitis, and the affected patients have an increased risk of developing liver cancer (Chang et al., 1997). HBV carries a partially double-stranded DNA genome and replicates through an RNA intermediate. After infecting host liver cells, there are four HBV transcripts transcribed from four different viral promoters (Core, SPI, SPII and X promoter) with 3.5-, 2.4-, 2.1- and 0.7-kb length, respectively. The 3.5-kb transcript is translated to produce the polymerase, core and precore proteins and serves as the pregenomic RNA template. The 2.4- and 2.1-kb transcripts produce the large, middle and small envelope proteins. The 0.7-kb transcript is translated to produce the X protein, which can transactivate both viral and host gene promoters (Chisari, 2000).

Although a preventive vaccine for HBV is available, the therapeutic options for chronically infected patients are still limited (Zuckerman, 2006). Interferon-alpha (IFN-α), lamivu-

Abbreviations: HE-145, helioxanthin; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HCC, hepatocellular carcinoma; CP, core promoter; SPI, surface promoter I; SPII, surface promoter II; XP, X promoter; 3TC, Lamivudine, (−)β-L-2′,3′-dideoxy-3′-thiacytidine; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay.

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doi:10.1016/j.antiviral.2007.12.011

dine [3TC,(−)β-L-2′,3′-dideoxy-3′-thiacytidine], adefovir [9-(2-phosphonylmethoxyethyl)adenine], entecavir (BMS-200475) and telbivudine (L-thymidine) have been approved as the antiviral drugs (Franco and Saeian, 2002; Mohanty et al., 2006; Yuan and Lee, 2007). However, IFN-α is costly and has a narrow range of efficacy, safety, and tolerability (Alberti et al., 1988). 3TC is a nucleoside analogue that inhibits HBV–DNA reverse transcriptase and decreases HBV replication in patients. A major drawback of 3TC treatment is the development of HBV variants with resistance mutations that reduce susceptibility to 3TC and entecavir but remain sensitive to adefovir after a short period of treatment (Delaney et al., 2001). Other non-nucleoside anti-HBV chemicals have been developed. For example, ellagic acid was reported to block hepatitis B virus e antigen (HBeAg) secretion (Shin et al., 2005) and the heteroarylhydropyrimidine Bay41-4109 was shown to inhibit HBV replication by depletion of nucleocapsids (Deres et al., 2003).

Arylnaphthalene lignan lactone, Helioxanthin (HE-145) and its analogues were previously reported to inhibit HBV replication in cultured HepG2.2.15 cells (Li et al., 2005; Yeo et al., 2005). In this study, we examined molecular mechanism of anti-HBV activity of HE-145 and revealed that HE-145 could selectively modulate the host transcriptional machinery in human liver cells to suppress HBV gene expression and replication.

2. Materials and methods

2.1. Materials

The enzyme immunoassay (EIA) kits for hepatitis B virus surface antigen (HBsAg) and e antigen (HBeAg) were purchased from Bio-Rad (Hercules, CA). Fetal calf serum was obtained from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco/BRL (Gaithersbung, MD). [α-^{32}P] dCTP was obtained from PerkinElmer Life Sciences (MA). Sea Kem LE-agarose was purchased from FMC Bioproducts (Rockland, MA). The tetrazolium salt WST-1 for the quantification of cell viability was purchased from Roche (Mannheim, Germany). Other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

The human hepatocellular carcinoma (HCC) HepA2 cell line was derived from HepG2 cells by transfecting a tandem repeated full-length HBV DNA and continually secretes HBsAg and HBeAg into the culture medium (Chang et al., 1987; Yeh et al., 1993). The Hep3B cell line contains one to two copies of integrated HBV DNA and continuously secretes HBsAg into the culture medium (Aden et al., 1979). The 1.3ES2 cell line is a clone derivative of HepG2 cells in which the 1.3 copies of the entire HBV genome was stably integrated in the genome (Chou et al., 2005). 1.3ES2 cells therefore continuously produce HBV viral particles into the culture medium. Stock cultures of HCC cell lines, human cervix epithelial cells line HeLa (Scherer et al., 1953) or human kidney epithelial cell line 293T (Graham et al., 1977) were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics (100 IU/ml each of penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. The cultures were passaged by trypsinization every 4 days. For the bioassays, cells were plated either in 24-well plates at a density of 8×10^4 cells/well or in 100-mm culture dishes at a density of 1.5×10^6 cells/dish in DMEM medium containing 10% fetal calf serum.

2.3. Preparation of HE-145

The heartwood of *Taiwania cryptomerioides* Hayata was extracted with methanol and subsequently partitioned in *n*-hexane and water (1:1, v/v). The *n*-hexane soluble material was fractionated by sequential column chromatography using silica–gel. The active component, HE-145 was eluted using *n*-hexane/EtOAc (95:5, v/v) and was further purified by reverse phase high performance liquid chromatography to homogeneity. HE-145-9, a derivative of HE-145, was organically synthesized following standard methods. The structures of HE-145 and HE-145-9 were determined by H-nuclear magnetic resonance, infrared (IR), and mass spectroscopy. For the bioassays, the compound was dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.25 μm fluoropore filter (Millipore, MA).

2.4. Quantification of HBsAg and HBeAg

Cells were seeded in 24-well plates at a density of 8×10^4 cells/well in DMEM containing 10% fetal calf serum. After 24 h of incubation, the cells were washed twice with PBS, pH 7.0, and treated with various concentrations of drugs in serum-free DMEM for the time indicated. The HBsAg and HBeAg in the culture medium were measured by an enzyme immunoassay (EIA) kit (Bio-Rad, CA). The viability of the cells was determined by a WST-1 cell proliferation assay. For the WST-1 assay, WST-1 (Roche Diagnostics, Mannheim, Germany) was added to each well and incubated for 0.5 h. The amount of formazan dye formed can be correlated to the number of metabolically active cells, which is quantitatively determined using a scanning multi-well spectrophotometer (ELISA reader) at the absorbance of 450 nm.

2.5. RNA isolation and Northern blot analysis

Total cellular RNA was extracted using the phenol and guanidinium isothiocyanate method (Gruffat et al., 1996). The RNA (20 μg) was denatured by 2.2 M formaldehyde, separated on denaturing formaldehyde 1.2% agarose gel, and transferred to a nylon membrane (Hybond-XL, Amersham, Piscataway, NJ). The membrane was hybridized with a ^{32}P-radiolabelled full-length HBV probe. The amount of the total RNA applied was normalized by hybridization of the probe for glyceraldehyde-3-phosphate dehydrogenase.

2.6. Endogenous HBV DNA polymerase activity assay

The culture media of 1.3ES2 cells were clarified by centrifugation at 250,000 × *g* for 1.5 h. The pellets were dissolved in NET buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% of Nonidet P-40 at 4 °C) and precipitated with human antiserum against HBcAg. The endogenous polymerase activity was measured as described previously (Chang et al., 1987; Junker et al., 1987). Briefly, the immunoprecipitates were suspended in HBV polymerase buffer (50 mM Tris-HCl, pH 7.4, 40 mM NH_4Cl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 0.2% 2-mercaptoethanol, 25 μM each of dATP, dGTP, dTTP, 100 μCi [^{32}P]-dCTP) and incubated for 2 h at 37 °C. Then unlabelled dCTP was added at a final concentration of 25 μM, and incubation was continued for 2 h. After washing with NET buffer to remove the free [^{32}P]-dCTP, proteinase K and sodium dodecyl sulfate (SDS) were added to a final concentration of 200 μg/mL and 0.5%, respectively. The samples were incubated for 2 h at 37 °C, followed by phenol/chloroform–isoamyl alcohol extraction. Then HBV DNA was precipitated with two volumes of 100% ethanol, and the DNA pellet was dissolved in 0.1-fold TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), which contained 50 ng/ml RNAase A.

2.7. Quantitative detection of HBV DNA by real-time light cycler PCR

1.3ES2 cells were seeded in 100 mm well plates at a density of 3×10^6 cells/well in DMEM containing 10% fetal calf serum. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.0) and treated with various concentrations of drug in serum-free DMEM for 72 h. For quantification of HBV DNA, viral DNA was extracted from culture media using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the Manufacturer's instructions. A series dilution of known amounts of HBV–DNA was used as a control. The standard curve showed a good linear range when 10^2–10^7 copies of plasmid DNA were used as templates. The PCR primers used were purchased from Tib-Molbiol (Berlin, Germany). The oligonucleotide sequences of primers were: HBV Forward: 5′-CAGGTCTGTGCCAAG-3′ (the accession number of GenBank: AY128092, nt 1168–1182) HBV Reverse: 5′-TGCGGGATAGGACAA-3′ (nt 1359–1345). The PCR cycling program consisted of an initial denaturing step at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 12 s, 54 °C for 20 s.

2.8. Plasmids construction

All plasmids were constructed by standard DNA cloning procedures (Proudfoot and Baralle, 1979) and polymerase chain reaction (PCR) methods (Mullis et al., 1986). To generate pSPI-Luc, pSPII-Luc, pCP-Luc, and pXP-Luc, the *Xbal-HindIII* fragments containing the surface promoter I (SPI), surface promoter II (SPII), or core promoter (CP) from pA3SPICAT, pA3SPIICAT (Kuo and Ting, 1997), pA3CPCAT, pXPCAT respectively, were inserted into the *NheI-HindIII* site of the pGL3-Basic vector (Promega, Madison, WI). The pSPII-Luc plasmid contains the entire major surface gene corresponding to map positions at nucleotides 2869–3180 of HBV (the accession number of GenBank: AM282986, Eco RI site as nucleotide 1). The pCP-Luc plasmid contains core promoter of the HBV pregenomic promoter (nt 1636–1851) into the pGL3-Basic vector. The pSPI-Luc plasmid was from nucleotides 2710 to 2826 and the pXP-Luc was from nucleotides 1177 to 1376 of HBV. Plasmid pHBV1.3, containing a 1.3-fold-HBV genome (ayw subtype) (Chou et al., 2005; Galibert et al., 1979), was used for transient transfection experiments.

2.9. Transient transfections and luciferase assay

HepA2 cells were transfected with various plasmids, using the calcium phosphate precipitation method (Graham and van der Eb, 1973). The cells were transfected in DMEM supplemented with 10% FCS for 16 h. After 8–10 h transfection, the cells were transferred into a fresh medium to recover for 16–18 h. The transfected cells were then incubated with serum-free DMEM and treated with HE-145 for 2 days. To prepare the total cell lysate from transfected cells for luciferase activity measurements, the medium was aspirated from the cell culture and the cells were gently rinsed with PBS. Cells were scraped from the plates and collected by centrifugation. The supernatant was collected for protein and luciferase activity measurements immediately following lysate preparation. The protein concentrations of the resultant cell lysates were measured by the Bradford method (Bradford, 1976). Using a luminometer and the Promega Luciferase Assay System as described by the Manufacturer (Promega, Madison, WI), lysates prepared from transfected cells were analyzed for luciferase activity. For all transient transfections with promoter-luciferase reporter constructs, the level of luciferase activity was determined without drug treatment. The transfection efficiency was normalized using the activity of β-galactosidase as an internal control.

2.10. Gel electrophoretic mobility shift assay

Double-stranded oligonucleotide probes were obtained by annealing equal moles of single-stranded complementary oligonucleotides. The probes corresponding to the different Sp1, FTF, HNF3 or HNF4 binding sites, identified in the core promoter of HBV, were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). The sequences of the oligonucleotide probes used for the EMSA are as follows: FTF: 5′-CAC CAA ATA TTG CCC AAG GTC TTA-3′ (nt 1631–1654). Sp1 (1): 5′-CCG TGA ACG CCC ACC AAA TAT TGC-3′ (nt 1620–1643). Sp1 (2) and (3): 5′-TGG GAG GAG TTG GGG GAG GAG ATT-3′ (nt 1733–1756). HNF4 (1): 5′-AAG AGG ACT CTT GGA CTC TCA GCA-3′ (nt 1658–1681). HNF3 (1): 5′-GAC TCT CAG CAA TGT CAA CGA ACC G-3′ (nt 1671–1694). HNF3 (2): 5′-TAC TTC AAA GAC TGT TTG TTT AAA-3′ (nt 1706–1729). PPAR: 5′-GAG ATT AGG TTA AAG GTC TTT GTA CT-3′ (nt 1751–1780). HNF4 (2): 5′-GAG ATT AGG TTA

AtG aTC TTT GTA CT-3′ (nt 1751–1780). Non-specific: 5′-TTG AGG CAT ACT TCA AAG ACT GTT-3′ (nt 1698–1721). Nuclear extracts were incubated with or without added unlabeled competitor oligonucleotides in a total volume of 20 μl containing 10 mM Hepes, pH 7.8, 50 mM KCl, 2.5 mM $MgCl_2$, 20% glycerol, 0.5 mM DTT, and 2.5 mg of poly (dI-dC). After 15 min of incubation on ice, the radiolabeled oligonucleotide was added, and the reaction mixture was further incubated for 15 min on ice and then 20 min at 30 °C. Protein–DNA complexes were separated with a 5% polyacrylamide gel.

3. Results

3.1. Antiviral activity of HE-145 in stably HBV-transfected HepG2 cells

We have shown previously that HE-145 and its analogues have been reported to inhibit both HBV replicative intermediates and mRNA in stably HBV-replicated HepG2 2.2.15 cells (Li et al., 2005). Since two other HBV genome stably transfected HepG2 cell lines have been established, we intended to examine molecular mechanism of HE-145 in these cells. HepA2 cells contain a head to tail dimer HBV genome which continually secretes HBsAg and HBeAg into the culture medium (Yeh et al., 1993). As shown in Fig. 1B, HE-145 suppressed both HBsAg and HBeAg production with an IC_{50} about 0.17 μM and 0.13 μM, respectively, in HepA2 cells.

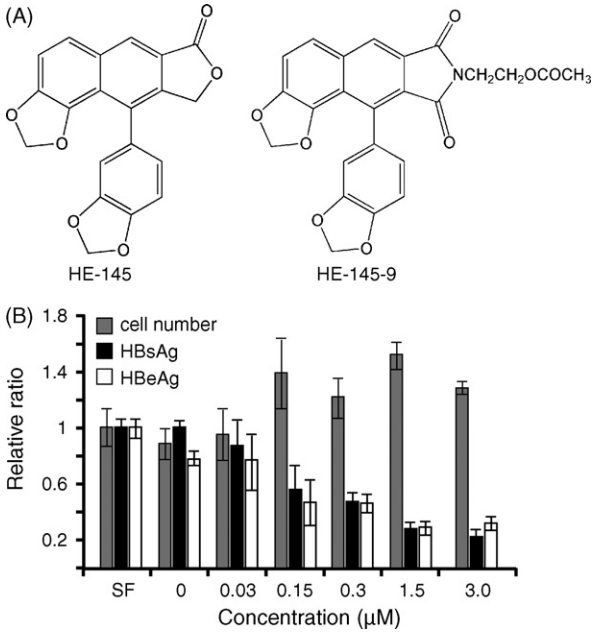

Fig. 1. (A) Chemical structures of HE-145 and HE-145-9. (B) Effect of HE-145 on the production of HBsAg and HBeAg in HepA2 cells. HepA2 cells were seeded on 24-well plates at a density of 8×10^4 cells/cm^2 in DMEM with 10% fetal calf serum and allowed to attach overnight. The cells were then washed twice with phosphate-buffered saline (pH 7.0) and treated with various concentrations of HE-145 in serum-free DMEM for 48 h. The amount of HBsAg and HBeAg production in the culture medium was determined by enzyme immunoassay. Viable cells in each well were determined by the WST-1 assay. Data are expressed as mean ± S.D. ($n = 3$).

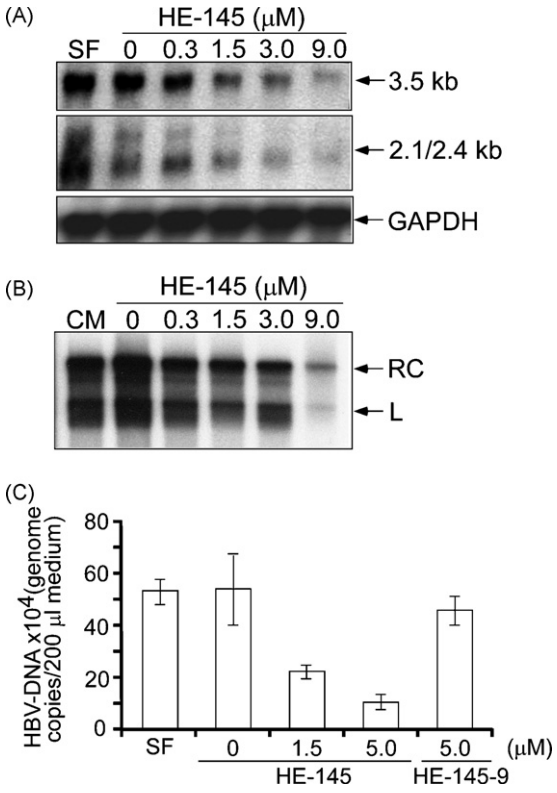

Fig. 2. Antiviral activity of HE-145 in 1.3ES2 cells. (A) 1.3ES2 cells were seeded on 100-mm culture dishes and treated with various concentrations of HE-145 in serum-free DMEM for 48 h. Total RNA was extracted from serum free (SF) and HE-145-treated cells and analyzed by Northern hybridization with HBV DNA probe as described in Section 2. GAPDH was used as control. (B) 1.3ES2 cells were seeded. Different doses of HE-145 were added into the complete medium (CM), and the expression level of secreted viral particles was analyzed. HE-145 was supplemented at 2 days after plating (day 0) and the total viral DNA of medium were harvested every 2 days for 6 days. DNA were electrophoresed in 1.2% agarose gel. Amounts of secreted relaxed circular (RC) and linear (L) forms of HBV were reduced in the HE-145-treated cultures. (C) Quantitative real-time PCR was used to detect HBV titer in the media of 1.3ES2 cells. Cultured cells were treated with various concentrations of HE-145 (0, 1.5 and 5.0 μM) and HE-145-9 (5.0 μM) in serum-free (SF) DMEM for 72 h and media collected for real-time PCR analysis using primer pair HBV DNA as template. Data were expressed as mean ± S.D. ($n = 3$). The difference in HE-145 (1.5 μM in A and B, 1 μM in C)-treated vs. untreated serum free (SF) cells is statistically significant by t test (*$p < 0.05$).

1.3ES2 cell line was also derived from HepG2 cells by stable integration of a 1.3-fold HBV genome (Chou et al., 2005). Northern blot analysis revealed two major HBV specific RNA species with molecular sizes of 3.5-kb (HBeAg mRNA and pregenomic RNA) and 2.4/2.1-kb (large HBsAg mRNA and middle/major HBsAg mRNA) in 1.3ES2 cells. As shown in Fig. 2A, all three viral transcripts in 1.3ES2 cells were significantly decreased in a dose-dependent manner after 48 h of HE-145 treatment. HE-145 also decreased the viral particles released from 1.3ES2 cells into the culture medium measured by Endogenous HBV DNA polymerase activity assay (Fig. 2B) and Quantitative real-time PCR (Fig. 2C). Anti-HBV activity of HE-145 is structure-specific, since a synthetic analog HE-145-9, shares similar chemical structure and hydrophobicity as HE-145 but showed no anti-HBV activity at all (Fig. 2C).

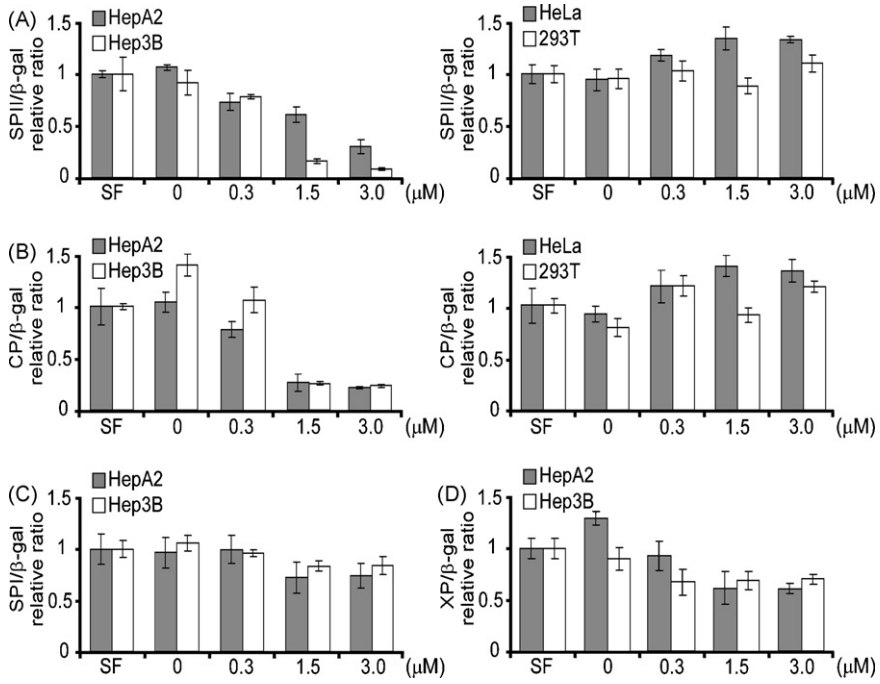

Fig. 3. HE-145 suppressed the promoter activity of HBV in HepA2 or Hep3B cells but not in HeLa or 293T cells. (A) Different cells were transfected with SPII of HBV promoter region with luciferase reported gene, using the calcium phosphate precipitation method. Left panel: Hep3B and HepA2. Right panels: 293T and HeLa. (B) Different cells were transfected with core of HBV promoter region with luciferase reporter gene. Left panel: Hep3B and HepA2. Right panels: 293T and HeLa. (C) HepA2 and Hep3B cells were transfected with SPI of HBV promoter region with luciferase reporter gene. (D) HepA2 and Hep3B cells were transfected with XP of HBV promoter region with luciferase reporter gene. After transfection, the cells were treated with serum free (SF) media (lane1), 0, 0.3, 1.5, 3.0 μM (lanes 2–5) of HE-145 for 48 h. The transfection efficiency was corrected by co-transfecting β-gal expression vector and assayed β-gal activity simultaneously. The normalized luciferase activities (CPS/OD) of core promoter were 2×10^6, 1.5×10^6, 7×10^5 and 2.5×10^6 in HepA2, Hep3B, HeLa and 293T cells, respectively. The similar expression level of SPII was also observed in both liver and non-liver cells. Data were expressed as mean ± S.D. ($n = 3$).

3.2. HE-145 selectively suppressed viral promoter activity for HBV major surface antigen and core antigen only in the liver cells

To investigate the molecular mechanism of anti-HBV activity of HE-145, we examined four HBV promoter activities for large viral surface antigen (SPI), major viral surface promoter (SPII), viral core protein (CP) and viral X protein (XP), respectively, upon HE-145 treatment, using luciferase as a reporter assay. We found that HE-145 selectively suppressed SPII and CP promoter activities but has no effect on SPI and XP activities (Fig. 3A–D) in both HepA2 and Hep3B cells. Interestingly, the suppressive activity of HE-145 against HBV promoters is cell type-specific. Neither SPII nor CP promoter activity was suppressed by HE-145 in non-human liver cell HeLa or 293T cells (Fig. 3A and B).

3.3. Effect of HE-145 on the DNA-binding activity of nuclear extract of HepA2 cells to the cis element of Sp1, FTF, HNF4, HNF3 and PPAR in HBV core promoter

HBV preC/C promoter/Enh II region contains many transcriptional factor binding sites and many of these *cis* elements have been shown to play critical role in viral gene expression (Gilbert et al., 2000; Li and Ou, 2001; Yu and Mertz, 2003). We designed twelve primer pairs to cover all the putative *cis* elements in this region to perform electrophoretic mobility shift assay (EMSA) to evaluate which transcriptional factor binding site is responsible for anti-HBV activity of HE-145. As shown in Fig. 4, synthetic DNA probe containing the Sp1, FTF, HNF4, HNF3 and PPAR binding site could be bound by the nuclear extracts of HepA2 cells to form a complex with a slower electrophoretic mobility on the EMSA gel. The binding activity of nuclear extract prepared from HE-145-treated (1.5 or 3.0 μM) HepA2 cells to Sp1, FTF, HNF4 and PPAR probes was significantly decreased. However, the binding activity of the same nuclear extract to HNF3 DNA probe did not yield any significant change. The specificity of DNA-protein complexes formation in EMSA assay was established by showing that the complex band could only be competed by unlabeled specific probe but not by the non-specific probe. Another control experiment showed that treatment of HepA2 cells with the inactive analog HE-145-9 has no effect on the DNA binding activity examined.

3.4. Ectopic expression of PPARγ and HNF4α could relieve the suppression of HE-145 to core promoter of HBV

In order to examine the involvement of PPARγ and HNF4α in HE-145-mediated suppression of HBV genes expression, we compared the anti-HBV activity of HE-145 in control and in PPARγ or HNF4α ectopically expressed in HepG2 cells. We co-transfected pHBV1.3 with control vector or expression vector for PPARγ or HNF4α and then examined HBV transcripts

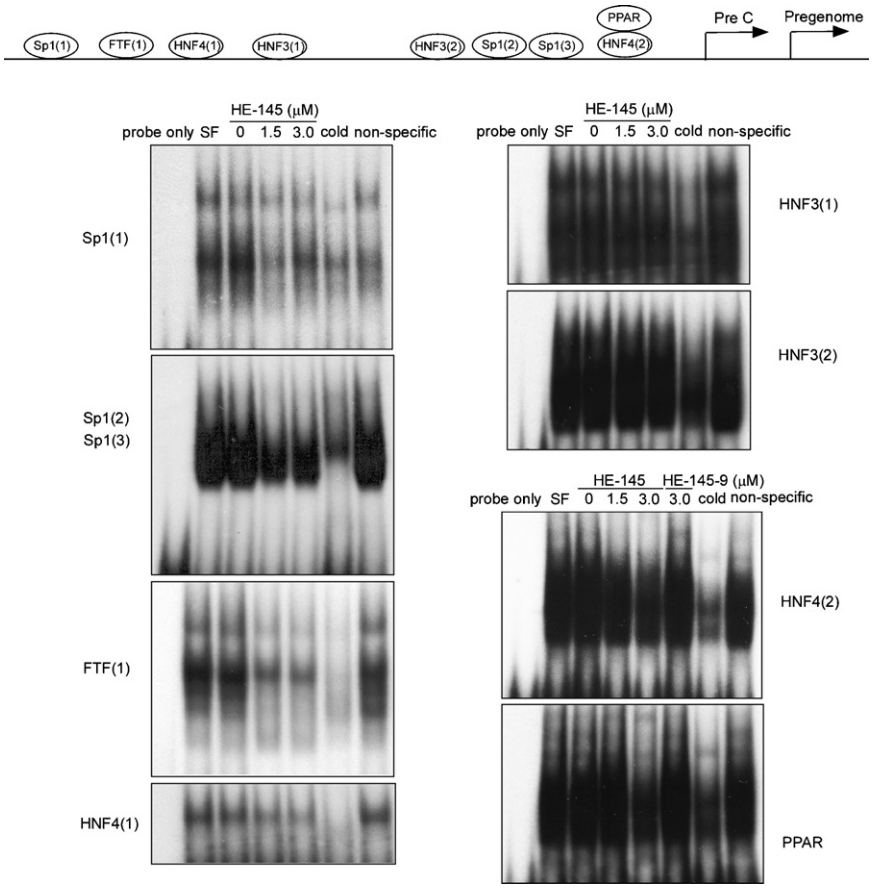

Fig. 4. HE-145 decreased the DNA-binding activity of FTF, Sp1, HNF4 and PPAR but not HNF3 in HepA2 cells. Gel electrophoretic mobility shift assays were carried out as described in Section 2. Nuclear extracts of HepA2 cells were incubated with double-stranded ^{32}P-end-labeled FTF, Sp1, HNF4, HNF3 or PPAR oligonucleotide, and treated with various concentrations of HE-145 (lanes 3, 4 and 5) or HE-145-9 (lane 6 in HNF4 and PPAR oligonucleotides) and untreated (SF, lane 2) in serum-free DMEM for 48 h. Probe incubated only without nuclear extracts is shown in lane 1. Excess unlabeled self-competitor oligonucleotide was added to confirm specific binding (cold). Non-specific element (nt 1698–1721) showed no effect on the complexes.

in these cells after HE-145 treatment by Northern blot analysis. As shown in Fig. 5, both 3.5-kb and 2.4/2.1-kb HBV transcript transcribed from pHBV1.3 were enhanced by ectopic expression of HNF4α but not by ectopic expression of PPARγ. As we expected, HE-145 suppressed both 3.5-kb and 2.4/2.1-kb HBV transcript in the control cells. However, ectopic expression of HNF4α partially relieved HE-145-mediated suppression of both 3.5-kb and 2.4/2.1-kb HBV transcript. Surprisingly, ectopic expression of PPARγ only marginally affected HE-145-mediated suppression of 3.5-kb HBV RNA but completely abolished HE-145-mediated suppression of 2.4/2.1-kb HBV RNA.

4. Discussion

To develop an antiviral drug against HBV infection with a novel mode of action to overcome the problems associate with current approved drugs is still a major challenge. Previously, we have shown that a new class of lignans, Helioxanthin (HE-145), isolated from *Taiwania cryptomerioides,* has potent inhibitory activity on HBV gene expression and replication in cultured human hepatoma cells (Li et al., 2005). In this study, the mode of action of HE-145 on HBV transcription and the involvement of several nuclear receptors such as HNF3, HNF4, and PPAR were examined.

In this study, all three HBV viral transcripts in 1.3ES2 cells were significantly decreased under HE-145 treatment (Fig. 2A). 1.3ES2 cells have been shown to carry both chromosomal integrated 1.3 copy of HBV genome and the ccc form of HBV DNA (Chou et al., 2005, 2007). Currently, we cannot distinguish the viral mRNA transcribed from integrated HBV genome from the viral mRNA transcribed from ccc DNA of HBV genome. However, HE-145 suppressed all viral transcripts expressed from the transient transfected episomal form of HBV DNA (Fig. 5). We speculate that HE-145 may suppress viral gene expression from both chromosomal integrated HBV genome and episomal cccDNA of HBV.

HBV has a very compact genome size and all four transcriptional units extensively overlap. It is difficult to dissect or to identify the *cis* element in HBV genome that is responsible for the inhibitory activity of HE-145. We therefore examined the effect of HE-145 on individual HBV promoter activity using luciferase as reporter gene.

Our studies first revealed that HE-145 is a potent transcriptional inhibitor of viral promoter for core antigen (CP) and major surface antigen (SPII), but has no effect on other two

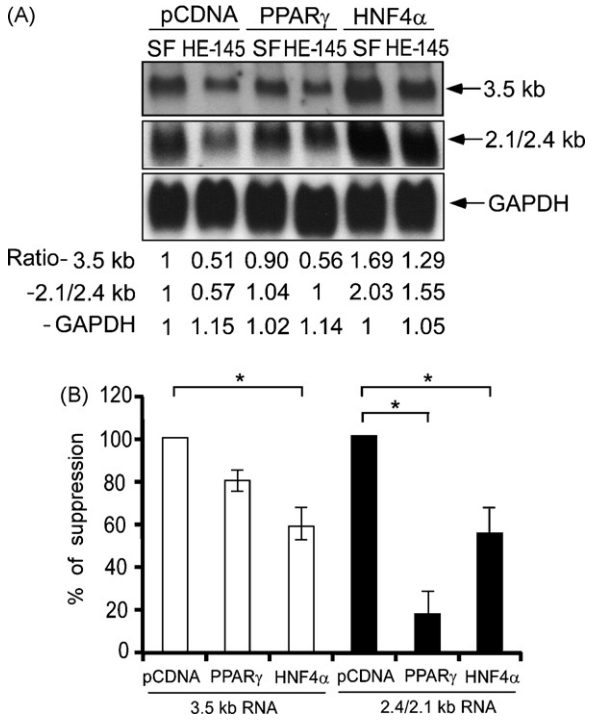

Fig. 5. PPARγ and HNF4α involved in the HE-145 suppression of HBV gene expression. (A) HepG2 cells were co-transfected pHBV1.3 with pCDNA3.1 or pPPARγ and pHNF4α, respectively. The transfected cells were cultured in serum-free (SF) medium or treated with HE-145 3 μM, and Northern blot analysis was performed as described in Fig. 2A. (B) HE-145-mediated suppression was relieved by overexpression pPPARγ and pHNF4α. The intensity of the 3.5-kb and 2.4/2.1-kb RNA was normalized with the GAPDH transcript. The suppression activity of HE-145 to the HBV viral mRNA in the control cells was assigned as 100%. The difference in HE-145-mediated suppression of HBV mRNA between pCDNA- and PPARγ or HNF4α-overexpressed cells are statistically significant ($*p < 0.05$).

viral promoters for pre-S antigen and X protein in human hepatoma cells. This observation suggests that the reduction of 3.5 kb HBV pregenomic RNA and 2.1 kb major surface antigen RNA introduced by HE-145 treatment may be mainly situated at the transcriptional level. Even though HE-145 has no effect on viral promoter for pre-S antigen (Fig. 3), we consistently observed the reduction of 2.4 kb pre-S RNA after HE-145 treatment (Fig. 2). One possibility is that the regulation of the pre-S promoter activity in the intact HBV genome is different from that in the cloned HBV DNA fragment. Pre-S promoter activity may be influenced by several regulatory *cis* elements such as viral enhancer I (Doitsh and Shaul, 2004) or II (Fu et al., 1996; Su and Yee, 1992) which are located thousand base pairs downstream of the Pre-S promoter. Several studies have reported that the Pre-S promoter activity is indeed influenced by viral enhancer I or II (Faktor et al., 1988). Our results also showed that HE-145 reduced the activities of HBV enhancer I when viral enhancer I is linked to the viral promoter for X gene (data not shown). Therefore, HE-145 reduced 2.4 kb pre-S RNA and may act indirectly through other control elements such as enhancer I or II in the HBV genome. This explanation deserves further investigation in the future.

Another interesting finding is that the inhibitory activity of HE-145 against CP or SPII appears to be liver cell-specific. HE-145 selectively inhibited CP and SPII activity in liver derived HepG2 or Hep3B cells but not in non-liver derived HeLa or 293T, even though both CP and SPII exhibited significant activity in either HeLa or 293T cells (Fig. 3A and B). The liver tropism of HE-145-mediated activity suggests that the target of HE-145 may be the liver-specific transcriptional machinery that is also responsible for liver tropism of HBV infection and replication.

The liver-specific inhibitory activity of HE-145 towards CP may provide a mechanistic explanation on how HE-145 reduced 3.5 kb HBV pregenomic RNA transcribed either from integrated HBV DNAs in the host genome (Fig. 2) or from transiently transfected HBV DNA (Fig. 5). The 3.5 kb HBV pregenomic RNA not only encoded the core and polymerase protein, but also served as template for reverse transcription. Therefore, HE-145 reduced the 3.5 kb HBV pregenomic RNA production through blocking viral CP activity, which is consistent with our observations that HE-145 decreased intracellular replicative intermediates of HBV DNA (data not shown) and HBV viral particle production by 1.3ES2 cells (Fig. 2).

Recent studies by Ying et al. on the anti-HBV activity of one analogue of HE-145 in human hepatoma cell line, HepG2.2.15 have reached similar conclusions as ours (Ying et al., 2007). However, two discrepancies between their conclusions and ours have been noticed. First, they suggested that HE-145 inhibits CP activity may be due to the decrease of HNF4α and HNF3β protein. In our study, we did not observe any changes in the protein levels of HNF4α, PPARγ, C/EBPα, and C/EBPβ in HepG2 cells after HE-145 treatment (data not shown). Secondly, Ying et al. reported that HE-145 suppresses all four viral promoter activities, whereas we found that HE-145 selectively suppresses CP and SPII without affecting the SPI and XP promoters in the HepG2 and Hep3B cells. One possible explanation is that we examined protein level of HNF4α and viral promoter activity in cells treated with HE-145 only for 48 h in serum-free medium. In contrast, Ying et al. treated cells with HE-145 in the medium with 10% FCS for 6 days. We observed no changes of the abundance of HNF4α protein, while both CP activity and binding activity of nuclear extract to HNF4 response element were significantly reduced in HE-145-treated HepG2 cells. Therefore, the primary effect of HE-145 may be the direct interference of specific transcriptional complex formation *in vivo*. The reduction of HNF4α protein and the inhibition of SPI or XP promoter activity may be the consequence of the long-term treatment of HE-145.

How exactly HE-145 inhibits HBV viral CP activity is still not clear. The CP consists of a basal core promoter (BCP) and a core upstream regulatory sequence (CURS) that is overlapped with the enhancer II and contains several binding sequences of different nuclear receptors. EMSA analysis revealed that the HE-145 treatment reduced binding activity of nuclear extract to the response elements of Sp1, HNF4, FTF and PPAR, respectively. However, HE-145 has no effect on the binding of HNF3 to its response element in the CP. We proposed that HE-145 may not reduce protein level of transcriptional factors but interfere with the transcriptional complex formation in viral core promoter *in*

vivo. Alternatively, HE-145 may directly associate with the CP specific transcriptional machinery *in vivo* and reduce its affinity to the *cis* element *in vitro*. Our current study cannot distinguish between these two possibilities. A quantitative comparison of the abundance and affinity of the specific transcriptional complex to CP in the control and HE-145-treated cells may solve this critical question. HE-145 treatment has no effect on the protein level of PPARγ and HNF4α. Then, why can overexpression of either PPARγ or HNF4α partially reduce the suppression activity of HE-145 towards the production of both 3.5 and 2.4/2.1 kb HBV transcripts in HepG2 cells? All attempts to show specific physical interaction between HE-145 and different nuclear receptors failed (data not shown). One possibility is that overexpression of PPARγ or HNF4α may shift the less active transcriptional complex induced by HE-145 to a more active state. We are currently investigating this possibility by a quantitative CHIP assay.

We have shown that HE-145 suppressed transcriptional complex formation in HBV core promoter *in vivo*. However, HE-145 neither reduces binding of transcriptional complex to HBV core promoter *in vitro*, nor binds to naked DNA directly (data not shown). We could not rule out the possibility that HE-145 may be modified or degraded by hepatic CYP450 enzymes *in vivo* and that the metabolites of HE-145 possess anti-HBV activity.

In conclusion, our results strongly suggest that HE-145 is an anti-HBV agent with a novel mode of action. It may act through liver-specific transcriptional machinery for viral CP to inhibit HBV viral gene expression and viral particle production.

Acknowledgments

The authors thank Dr. Ling-Pai Ting for providing pA3SPI-CAT, pA3CP-CAT and pA3SPII-CAT plasmids, and Dr. Chung Ming Chang for providing the pXp-CAT plasmid. We are grateful that this work was supported by grants NSC 96-3112-B-010-005 and CMRPD140103.

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