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Scutellariae radix suppresses hepatitis B virus production in human hepatoma cells

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1. ABSTRACT

The traditional Chinese medicine Xiao-Chai-Hu-Tang (HD-7) has been widely used to treat liver diseases in China and Japan. HD-7 consists of seven different ingredients, but which one provides the therapeutic benefits is still unknown. Here, we identified the "Minister herb" *Scutellariae radix* (HD-1S), but not the "King herb" *Bupleuri radix* (HD-1B) in HD-7, as the active component that suppresses HBV gene expression and virus production in human hepatoma cells. We have found that an aqueous extract of HD-1S not only suppressed wild-type virus but also lamivudine-resistant HBV mutant in human hepatoma cells. We show that HD-1S selectively suppresses HBV core promoter activity. Electrophoretic mobility shift assay analysis has revealed that HD-1S treatment decreases the DNA-binding activity of nuclear extract of HepA2 cells to a specific *cis*-element of the HBV core promoter, which includes the peroxisome proliferator-activated receptor binding site and HNF4. Furthermore, ectopic expression of PGC-1 abolished the suppression of HD-1S on HBV core promoter activity suggesting that HD-1S may act through modulating hepatic transcriptional machinery to suppress HBV viral gene expression and virus production.

2. INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic, non-cytopathic 3.2 kb partially double-stranded DNA virus with four open reading frames. Approximately 350 million people worldwide are infected (1). Patients who have chronic HBV have a 100-fold elevated risk of developing cirrhosis and hepatocellular carcinoma (HCC) (2, 3). Even though the effective hepatitis B vaccines have been available, HBV infection remains global health problem responsible for more than one million deaths annually.

Currently, most of the approved chemotherapeutic agents, such as lamivudine (3TC)(4, 5), adefovir dipivoxil (bis-POMPMEA)(6), penciclovir (PCV)(7), and lobucavir (LBV)(8), target the HBV viral DNA polymerase (9). All of these agents are facing the problem of emergence of drug resistant HBV variants after a prolonged antiviral therapy (10). Therefore, the development of a new generation of anti-HBV agents with novel modes of action is urgently needed.

The Chinese herbal medicine Xiao-Chai-Hu-Tang (HD-7) was prescribed in the Chinese medical archive, Shang-Han Lun, by Zhang Zhong-jing (150-219

A.D.) for liver diseases nearly two thousand years ago. It is still commonly used at the present time to treat chronic hepatitis in China and Japan. Even though the pharmacological mechanism of action of HD-7 is unknown, HD-7 has been shown to be effective in attenuating progression of liver fibrosis and chronic hepatitis in both human subjects and animal models (11-15).

HD-7 consists of seven different herb preparations: *Bupleuri radix* (HD-1B), *Scutellariae radix* (HD-1S), *Radix codonopsitis pilosulae, Rhizoma pinelliae ternatae, Radix glycyrrhizae uralensis, Rhizoma zingiberis officinalis and Fructus zizyphi jujubae.* Among these seven ingredients, *Bupleuri radix* (HD-1B) was claimed as the "King herb" that provides the major therapeutic benefits. *Scutellariae radix* (HD-1S) was claimed as "Minister herb" that provides complementary therapeutic benefits. The other herbs are proposed to be the "Assistant" and the "Servant herbs". They are thought to counteract any potential adverse effects or to harmonize the actions of the other ingredients.

In this study, we examined the anti-HBV activity of the major components of HD-7 and identified the "Minister herb" *Scutellariae radix* (HD-1S), but not the "King herb" *Bupleuri radix* (HD-1B), as the active component that suppresses HBV production and gene expression in cultured human hepatoma cells.

3. MATERIALS AND METHODS

3.1. Preparation of herbal remedy

Bupleuri radix, *Scutellariae radix*, *Radix codonopsitis Pilosulae, Rhizoma pinelliae ternatae, Radix glycyrrhizae uralensis, Rhizoma zingiberis officinalis and Fructus zizyphi jujubae* were purchased from a local wholesale distributor (Taipei, Taiwan, R.O.C.). The experimental herbal preparation was prepared as follows. 84 g *Bupleuri radix* (HD-1B), 36 g *Scutellariae radix* (HD-1S), 36 g *Radix codonopsitis pilosulae,* 60 g *Rhizoma pinelliae ternatae,* 24 g *Radix glycyrrhizae uralensis,* 12 g *Rhizoma zingiberis officinalis and* 36 g *Fructus zizyphi jujubae; or* 84 g *Bupleuri radix* (HD-1B) alone or 36 g *Scutellariae radix* (HD-1S) alone were extracted by boiling in 3600 ml water at 100^{o}C until the total volume was reduced to 1000 ml for HD-7, or HD-1B and HD-1S respectively. Extracts were filtered through layers of gauze, residues were discarded and the filtrates were kept at −30°C. Samples were then lyophilized in a vacuum freeze-dryer (VirTis freeze mobile, VirTis Co., Gardiner, USA).

3.2. Cell culture

The human hepatocellular carcinoma (HCC) HepA2 cell line was derived from HepG2 cells by transfecting a tandem repeat of full-length HBV DNA which yielded cells that continually secrete HBsAg and HBeAg into the culture medium (16). The 1.3ES2 cell line is a clone derivative of HepG2 cells in which 1.3 copies of the entire HBV genome was stably integrated in the genome (17). 1.3ES2 cells therefore continually produce HBV viral particles into the culture medium. The lamivudine-resistant cell line M33 is also a hepatoblastoma HepG2 cell line stably transfected with 1.3-copies of the entire HBV genome, which contains the L515M/M539V double mutation (18). This stable cell line secretes lamivudine-resistant HBV particles containing viral DNA and DNA polymerase activity. Stock cultures of human hepatoma cells HepA2, 1.3ES2, M33 and human kidney epithelial cell line 293T (19) 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.

3.3. Detection of HBsAg

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 48 h. The HBsAg in the culture medium were measured by enzyme immunoassay (EIA) kit (Bio-Rad, CA, USA). The viability of cells was determined with the MTT assay (20).

3.4. RNA isolation and northern blot analysis

Total cellular RNA was isolated using TRIzol solution (Invitrogen, Carlsbad, CA), which was followed by phenol/chloroform extraction and isopropanol precipitation. The RNA (20 microgram) was denatured by 2.2 M formaldehyde, separated on a denaturing formaldehyde 1.2% agarose gel, and transferred to a nylon membrane (Amersham Hybond-N^{+}; GE Healthcare). 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 a probe for glyceraldehyde-3-phosphate dehydrogenase.

3.5. Plasmids

The plasmids were constructed by standard DNA cloning procedures (21) and polymerase chain reaction (PCR) methods (22). The HBV sequence used in this study is an *ayw* subtype (GeneBank accession number: V01460, where the Eco RI site marks nucleotide 1) (23). To generate pXP-Luc, the *Xbal-HindIII* fragments containing the X promoter from pXP-CAT were inserted into the *MluI-HindIII* site of the pGL3-Basic vector (Promega, Madison, WI). The pSIP-Luc, pSIIP-Luc, pCP-Luc and C/EBP-Luc plasmids were generous gifts from Dr. Chungming Chang (National Health Research Institutes, Taiwan). The PGC1-Luc and PPARs-Luc plasmids were generous gifts from Dr. Chin-Wen Chi and An-Na Chiang (National Yang Ming University, Taiwan) respectively.

3.6. Transient transfections and luciferase assay

Cells were transfected with various plasmids using Polyplus-transfection's jetPEI transfection reagent (24). The cells were transfected in DMEM supplemented with 10% FCS for 16 h. After 24 h transfection, the cells were transferred into a fresh medium to recover for 16-18 h. The transfected cells were changed to a serum-free

DMEM and treated with HD-1S for two days. To prepare total cell lysate from transfected cells for luciferase activity measurements the medium was aspirated and the cells were gently rinsed with PBS. Cells were scrapped from the plates and collected by centrifugation. The supernatant was collected and luciferase activity measurements were executed immediately following lysate preparation. Protein concentrations of the cell lysates were measured by the Bradford method (25). Using a luminometer and the Promega Luciferase Assay System as described by the manufacturer (Promega, Madison, WI, USA), 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 was set to one. The transfection efficiency was normalized using the activity of beta-galactosidase as an internal control. The values are means plus and minus the standard error of the mean of at least three independent experiments.

3.7. 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 5 x 10^6 cells/well in DMEM containing 10% fetal calf serum. After 24 h of incubation, the cells were washed with phosphate-buffered saline (PBS, pH 7.0) and treated with various concentrations of HD-1S in serum-free DMEM for 5 days. 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 from a plasmid 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' (GenBank accession number: AY128092, nt 1168-1182) and HBV Reverse: 5'-TGCGGGATAGGACAA-3' (nt 1359-1345). The PCR cycling program consisted of an initial denaturing step at 95C for 10 min, followed by 45 amplification cycles at 95C for 12 s, 54C for 20 s.

3.8. Gel electrophoretic mobility shift assay

Preparation of the nuclear extracts was carried out using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, USA). Double-stranded oligonucleotide probes were obtained by annealing equal moles of single-stranded complementary oligonucleotides. The probes corresponding to the different C/EBP/FTF (1), HNF4 (1) or HNF4 (2)/PPRE binding sites, identified in the core promoter of HBV, were labeled with [alpha-^{32}P]ATP using T4 polynucleotide kinase (Promega). The sequences of the oligonucleotide probes used for the Electrophoretic mobility shift assay (EMSA) are as follows: C/EBP/FTF (1): 5'-CAC CAA ATA TTG CCC AAG GTC TTA-3' (nt 1631-1654). HNF4 (1): 5'-AAG AGG ACT CTT GGA CTC TCA GCA-3' (nt 1658-1681). HNF4 (2)/ PPRE: 5'-GAG ATT AGG TTA AAG GTC TTT GTA CT-3' (nt 1751-1776). 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 microliter 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 30C. Protein-DNA complexes were separated with a 5 % polyacrylamide gel with 0.5 x TBE for 3 h. Gels were dried and subjected to autoradiography.

4. RESULTS

4.1. HD-1S but not HD-1B is the active component in HD-7 that suppresses HBsAg production by HepA2 cells

The human hepatoma cell line HepA2 was derived from HepG2 cells by transfecting a tandemly repeated full-length HBV DNA (16). The HepA2 cells continuously synthesize and secrete HBsAg as well as HBeAg, a marker for viral replication. When HepA2 cells cultured in serum free medium were treated with different concentrations of HD-7, HD-1S and HD-1B for 48 h, the production of HBsAg in the media were determined by ELISA assay. As shown in Figure 1, HD-7 only showed moderate activity to suppress HBsAg production by HepA2 cells. On the other hand, we observed that HD-1S displayed dose dependent suppression of HBsAg production with a half maximal dose around 200 microgram/ml. HD-1B did not show any suppressive activity against HBsAg production up to 420 microgram/ml. Interestingly, unlike HD-1B, which showed cytotoxicity at high concentrations, HD-1S slightly stimulated cell proliferation of HepA2 cells in a dose dependent manner.

4.2. HD-1S inhibited both wild type and lamivudine resistant HBV viral particle production by human hepatoma 1.3ES2 and M33 cells

To examine whether HD-1S shares the same mode of action as conventional anti-HBV drugs such as lamivudine (3TC), we examined the anti-HBV activity of HD-1S on wild type HBV and 3TC resistant HBV viral particles produced in both 1.3ES2 cells and M33 cells, respectively. 1.3ES2 cells were derived from the HepG2 cell line by stably transfecting with a 1.3 fold wild type *ayw* strain HBV DNA (17). On the other hand, M33 cells contain a tandemly repeated full-length lamivudine-resistant HBV DNA with the L515M/M539V double mutation in the DNA polymerase region (18). 1.3ES2 and M33 cells were treated with various concentrations of HD-1S. After 5 days of treatment, the amount of HBV DNA viral particles that were secreted into the culture medium was determined by quantitative real-time PCR. Interestingly, HD-1S was better inhibitory against the M33 cells than the 1.3ES2 cells. As shown in the Figure 2, HD-1S reduced HBV particle production in both 1.3ES2 and M33 cells in a dose-dependent manner with a half maximal dose around 237 microgram/ml and 112 microgram/ml against 1.3ES2 cells and M33 cells respectively. In contrast, lamivudine only inhibited wild type HBV production in 1.3ES2 cells but not lamivudine resistant HBV viral particle production in M33 cells.

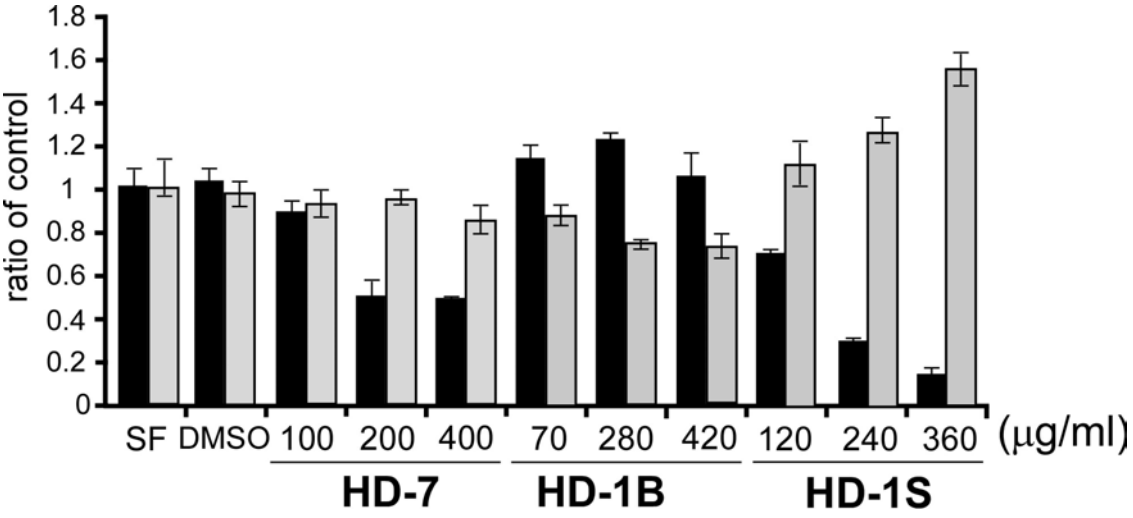

Figure 1. HD-7 and HD-1S, but not HD-1B, suppresses HBsAg production in HepA2 cells. HepA2 cells were seeded on 24-well plates at a density of 8×10^4 cells/cm^2 in DMEM with 10% fetal calf serum and allowed to attach overnight. The cells were then washed twice with phosphate-buffered saline (pH 7.0) and treated with various concentrations of drugs in serum-free (SF) DMEM for 2 days. The amount of HBsAg production in the culture medium was determined by enzyme immunoassay. Viable cells in each well were determined by MTT assay. Data are expressed as mean ± S.D. (n=3). HBsAg: Black column; Cell number: gray column.

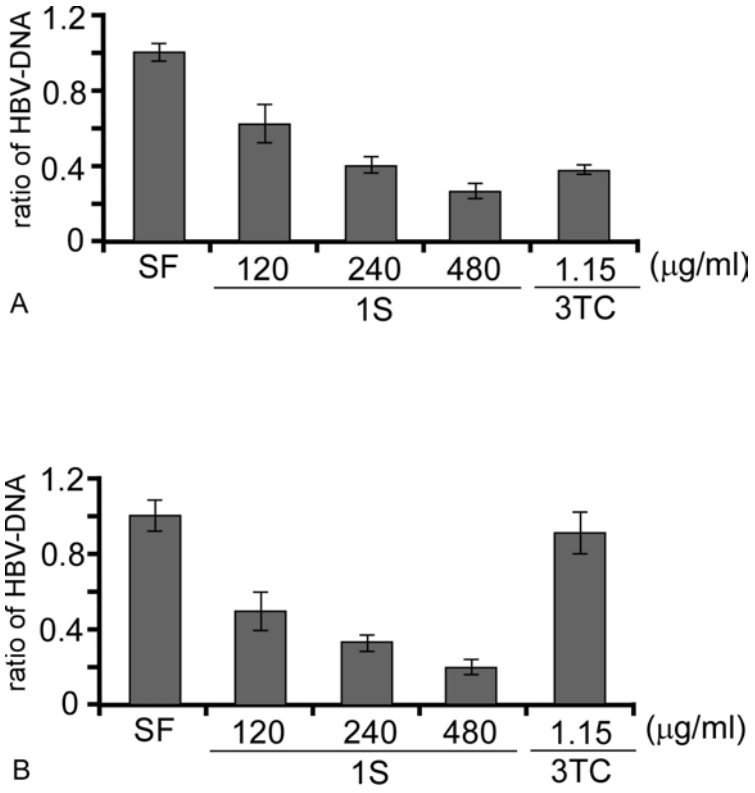

Figure 2. HD-1S suppresses both wild type and lamivudine-resistant HBV virus production by 1.3ES2 and M33 cells, respectively. Quantitative real time PCR was used to detect HBV viral titer in the media of HBV wild (1.3ES2) cells (A) and 3TC-resistant mutant (M33) cells (B). Cultured cells were seeded on 100 mm culture dishes and treated with various concentrations of HD-1S (120, 240 and 480 microgram/ml) or 3TC (1.15 microgram/ml) in serum-free DMEM for 5 days. Media were collected for real-time PCR analysis using primer pair designed HBV DNA as template. Data are expressed as mean ± S.D. (n = 3).

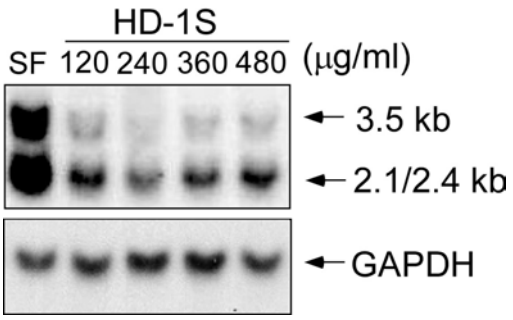

Figure 3. HD-1S suppresses the steady-state level of HBV transcripts in 1.3ES2 cells. 1.3ES2 cells were seeded on 100 mm culture dishes and treated with various concentrations of HD-1S in serum-free DMEM for 2 days. Total RNA was extracted from serum free (SF) and HD-1S treated cells and analyzed using Northern hybridization with the HBV DNA probe as described in the Materials and Methods. Each lane represents 15 microgram of total RNAs. The mRNA of the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was used as an internal marker. The experiment was performed three separate times with similar results. The figure represents one set of data.

4.3. HD-1S treatment reduced all four HBV viral transcript levels in 1.3ES2 cells

To investigate molecular mechanism of HD-1S suppression of HBV viral particle production, we examined expression of four HBV viral transcripts in HD-1S treated 1.3ES2 cells by Northern blot analysis using [alpha-^{32}P] dCTP labeled HBV DNA as a probe. As shown in Figure 3, the amount of the major HBV transcript corresponding to the precore/pregenomic RNA species (3.5 kb) and the mRNA for large and small HBsAg (2.1-2.4 kb) were all significantly decreased in HD-1S treated 1.3ES2 cells.

4.4. HD-1S selectively suppresses viral promoter activities of the large viral surface antigen and the core antigen in liver cells

To further investigate how HD-1S suppresses HBV viral transcript production in human hepatoma cells, we examined the HBV promoter activity of the four following promotors: large viral surface antigen (SIP), major viral surface promoter (SIIP), viral core protein (CP) and viral X protein (XP). We performed this analysis in control and HD-1S treated HepA2 cells. We found that HD-1S selectively suppressed the SIP and CP promoter activities but had no effect on the SIIP and XP activities (Figure 4A). Interestingly, we found that the suppressive activity of HD-1S on the CP promoter is cell type specific. CP promoter activity was not suppressed by HD-1S in non-liver cells such as human kidney 293T cells (Figure 4B).

4.5. HD-1S reduces DNA-binding activity of nuclear extract to the C/EBP/FTF and PPAR/hepatocyte nuclear factor-4 binding sequences in the HBV core promoter

The HBV preC/C promoter/Enh II region contains many transcription factor binding sequences and some hepatic transcriptional factors have been shown to play a critical role in viral gene expression (26-29). We set out to determine whether HD-1S suppresses HBV core promoter activity by altering host transcription factor activity. To this end, we synthesized several double-stranded oligonucleotides containing putative *cis*-elements, prepared nuclear extracts from control and HD-1S treated cells and performed electrophoretic mobility shift assays (EMSA). As shown in Figure 5, nuclear extract bound to synthetic DNA probes containing the C/EBP/FTF (1), HNF4 (1) and PPRE/ HNF4 (2) binding sites. The binding activity of nuclear extract prepared from HD-1S treated (120, or 240 or 480 microgram/ml) HepA2 cells was significantly reduced compared to the control extract for the HNF4 (2)/PPRE probes. On the other hand, the binding activity of HD-1S treated nuclear extract showed similar activity as control nuclear extract to C/EBP/FTF (1) and HNF4 (1). The specificity of the DNA-protein complex formation in the EMSA assay was established by showing that the mobility shifts could only be competed by specific unlabeled probe but not by the non-specific unlabeled probe.

4.6. Overexpression of PGC-1 overrides the suppressive activity of HD-1S on HBV core promoter activity

We then set out to identify which host transcription factor or coactivator might be responsible for HD-1S mediated suppression of the HBV core promoter activity. We ectopically expressed different hepatic transcription factors including C/EBP, PPARalpha, PPARgamma or PGC1 in HepA2 cells. As shown in Fig 6, PGC1 is the only transcription factor that can override HD-1S mediated suppression of HBV core promoter activity. This result strongly suggests that PGC1 or PGC1 mediated transcriptional activity is the target of HD-1S for the suppression of HBV core promoter activity in human hepatic cells.

5. DISCUSSION

Traditional Chinese Medicine (TCM) has been a rich source of biologically active substances with defined pharmacological activity. The first pure chemical identified from Chinese herbal remedies to enter the Western pharmacopoeia was ephedrine, an amphetamine-like stimulant. Ephedrine was isolated from mahuang (*Ephedra sinica*) by Japanese scientists in 1880s. Mahuang was first prescribed in the Chinese medical archive, Shang-Han Lun, to treat congestion over two thousand years ago. The next most significant advance was the isolation of artemisinin from qinghao, a relative of the sweet wormwood found in North America. Qinghao was also prescribed in Shang-Han Lun as beneficial for fever. Later, artemisinin was found to kill chloroquine resistant strains of *Plasmodium*, the parasite that causes malaria. In the present study, we first demonstrated that Xiao-Chai-Hu-Tang, a seven-ingredient formulation prescribed in Shang-Han Lun two thousand years ago, has anti-HBV activity. Our study further identified the "Minister herb" *Scutellariae radix* (HD-1S) but not the "King herb" *Bupleuri radix* (HD-1B) of Xiao-Chai-Hu-Tang is the active component to suppress HBV gene expression and virus production in cultured human hepatoma cells.

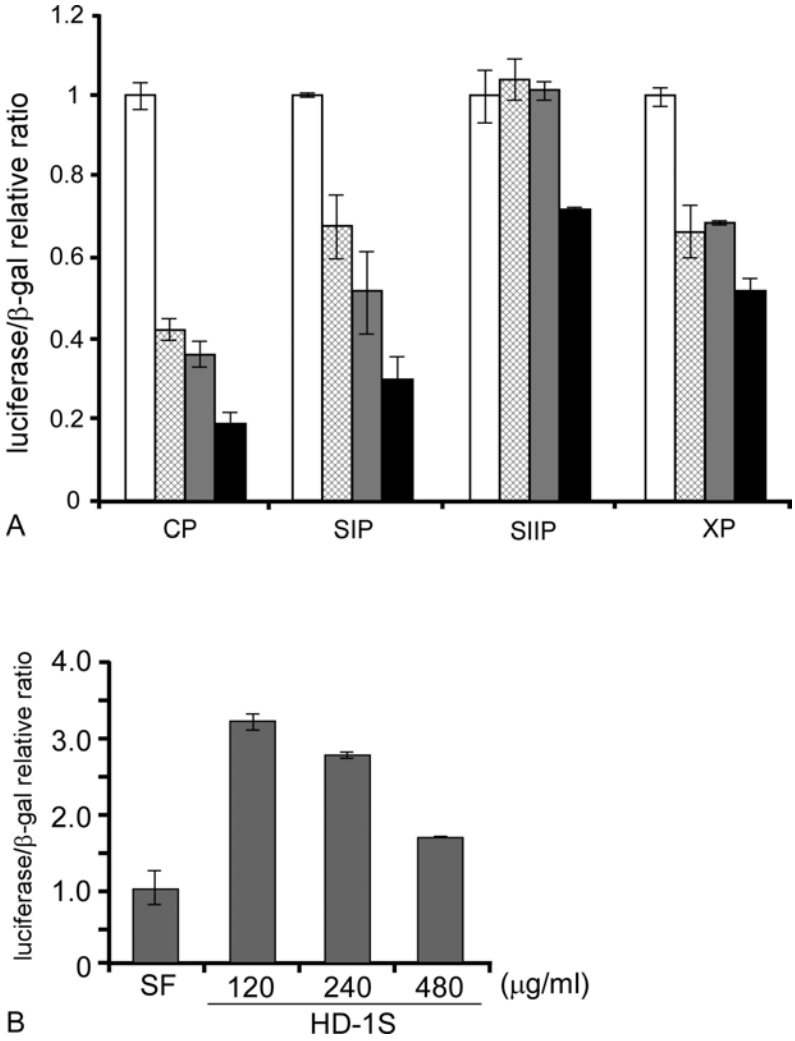

Figure 4. HD-1S selectively suppressed the pre-S and core promoter activity of HBV in HepA2 cells but not in 293T cells. Different HBV promoter regions were cloned into a promoter-less mammalian expression vector. (A) HepA2 cells were transfected with SIP, SIIP, CP, and XP of the HBV promoter region with a luciferase reporter using Polyplus-transfection's jetPEI transfection reagent. SF: white column; HD-1S 120 microgram/ml: cross line column; HD-1S 240 microgram/ml: gray column; HD-1S 480 microgram/ml: black column. (B) 293T cells were transfected with CP. After transfection, HepA2 cells were treated with serum free (SF) media alone, or with HD-1S (120, 240, 480 microgram/ml) in serum-free DMEM for 2 days. The transfection efficiency was corrected by cotransfecting a beta-gal expression vector and assaying beta-gal activity simultaneously. Data were expressed as mean ± S.D. (n = 3).

Our study has revealed several unique characteristics of the anti-HBV activity associated with *Scutellariae radix* (HD-1S). First, the mode of action of HD-1S is distinct from conventional nucleoside/nucleotide inhibitors. This conclusion is supported by the results shown in Figure 2. HD-1S not only suppressed wild-type HBV (ayw) virus production by 1.3ES2 cells but also a lamivudine-resistant mutant HBV production by M33 cells. This lamivudine-resistant mutant HBV contains the L515M and M539V double mutations in the viral polymerase protein. Therefore, HD-1S has potential to develop as a new class of anti-HBV therapeutics to overcome the problem of nucleoside/nucleotide inhibitor resistant mutants of HBV.

Secondly, the mechanism of anti-HBV activity of HD-1S may be mainly due to its ability to suppress HBV core and pre-S promoter activity. This conclusion is consistent with the observations of all following viral products: HBsAg, HBeAg, viral transcripts and virus particles produced by human hepatoma cells. HBV core promoter plays a crucial role in HBV viral production since the major 3.5kb pregenomic RNA transcribed from HBV core promoter is used to make the new copies of viral genome and to make the viral capsid core protein and the viral DNA polymerase. We clearly demonstrated that HD-1S treatment in 1.3ES2 cells indeed reduced 3.5kb HBV pregenomic RNA (Figure 3). Western blot analysis revealed that HBV core protein in 1.3ES2 cells was also

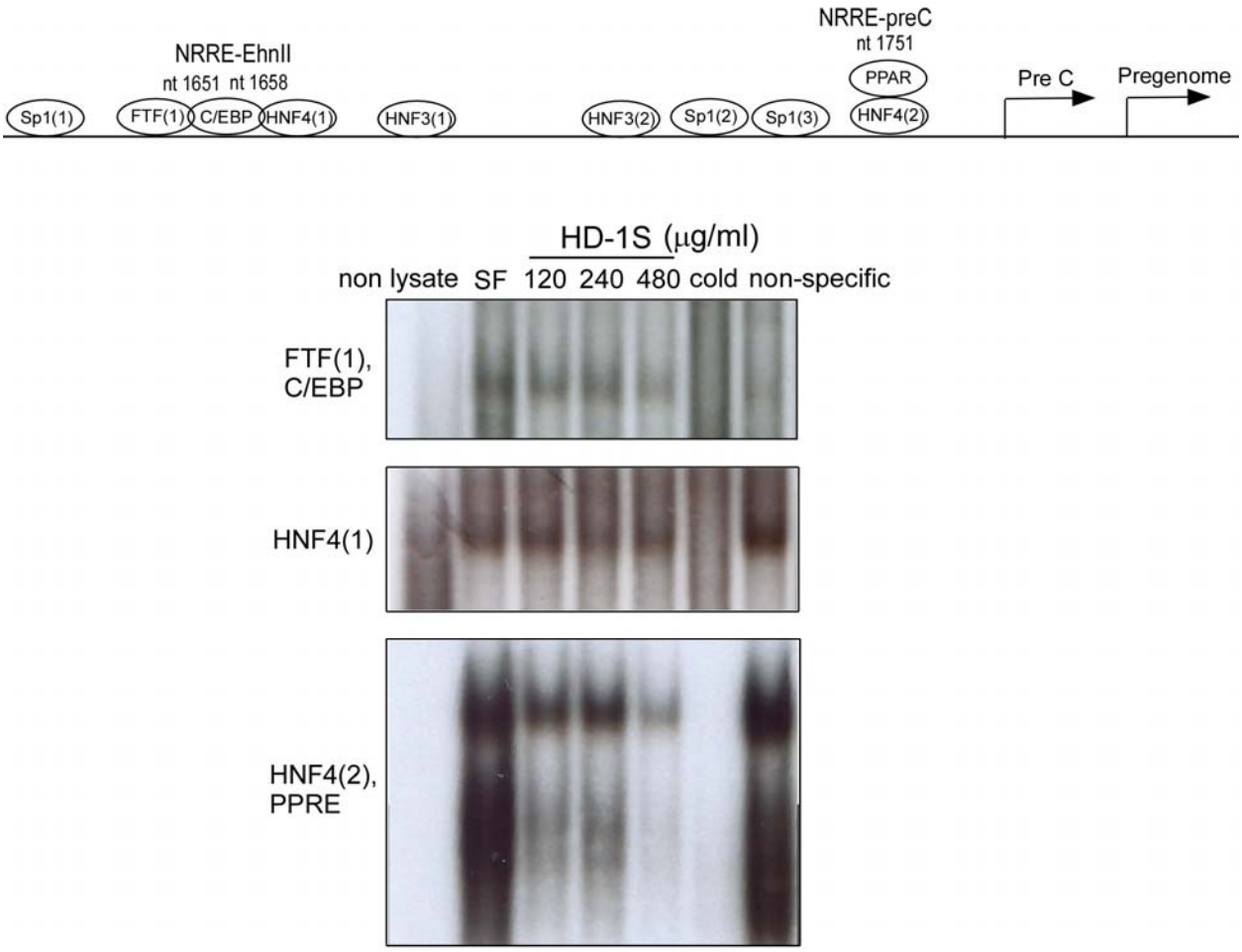

Figure 5. HD-1S decreased the DNA-binding activity of a nuclear extract to an oligonucleotide containing HNF4(2) but not C/EBP/FTF(1), or HNF4 (1) sequences. Gel electrophoretic mobility shift assays were carried out as described in Materials and methods. Nuclear extracts of HepA2 cells were incubated with double-stranded ^{32}P-end-labeled C/EBP/FTF(1), HNF4 (1) or HNF4(2) oligonucleotides and treated with various concentrations of HD-1Ş(lanes 3, 4 and 5) and untreated (SF, lane 2) in serum-free DMEM for 48 h. Probe only without any nuclear extract is shown in lane 1. Excess unlabeled self-competitor oligonucleotide was added to confirm specific binding (cold). A non-specific element (nt 1698-1721) showed no effect.

suppressed by HD-1S treatment in a dose dependent manner (data not shown)

Thirdly, the inhibition of HBV core promoter activity by HD-1S is only observed in cells with a liver origin but not in non-liver cells such as 293T cells. The cell content dependent activity of HD-1S suggests that HD-1S may act through one or a few liver specific target proteins to suppress HBV core promoter activity. This hypothesis is supported by the result of the EMSA analysis. HD-1S treatment decreased the DNA-binding activity of nuclear extract from HepA2 cells to specific a *cis*-element of the HBV core promoter (1751-1776). This sequence can be recognized by hepatic rich transcriptional factors including: RXRalpha/PPARgamma, COUP-TF1, TR2 and HNF4. There are many possible explanations for reducing DNA binding activity of HD-1S treated nuclear extract. HD-1S may directly reduce protein level of target transcriptional factors. Or, HD-1S may alter affinity of transcriptional factors to specific DNA sequence or other protein partners. Combining DNase1 footprinting analysis and chromatin immunoprecipatition assay for specific transcriptional factors and coactivators should resolve this complicated issue.

Fourth, ectopic expression of PGC-1 overrides the suppressive effect of HD-1S on the HBV core promoter activity suggesting that PGC-1 and HD-1S may act through the same pathway to regulate HBV core promoter activity. PGC-1 plays a critical role on regulating expression of a few key enzymes such as PEPCK and G-6-Pase in hepatic gluconeogenesis pathway. Previous studies have linked PGC-1, which is robustly induced during starvation and turns on the gluconeogenic program in the liver, to the activation of HBV gene expression in HBV transgenic mouse model (33). The interplay of PGC-1 and HD-1S on both HBV gene expression and hepatic gluconeogenesis program prompts us to propose that "HBV highjack

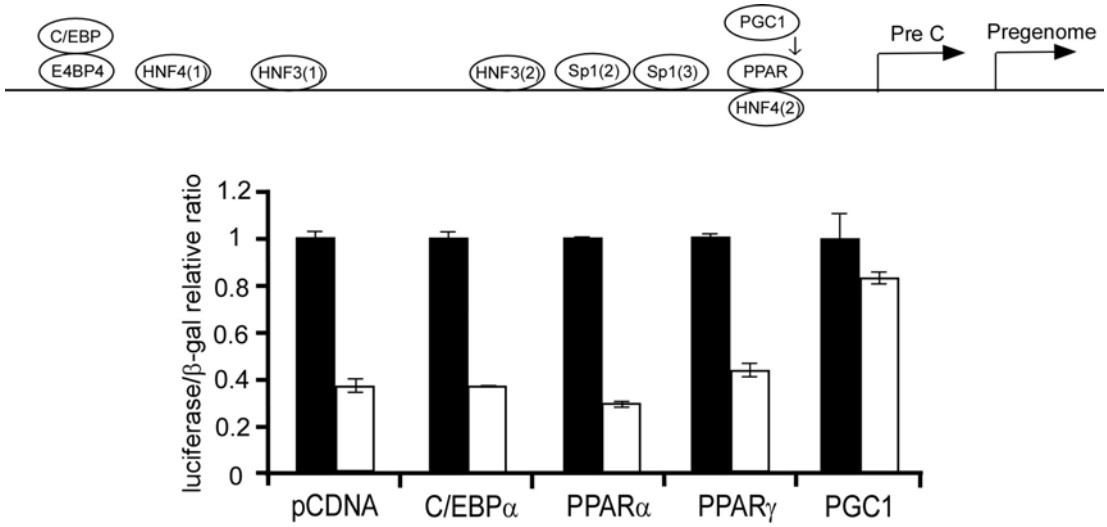

Figure 6. Overexpression of PGC-1 overrides the suppressive activity of HD-1S on HBV core promoter activity. HepA2 cells were co-transfected with the core promoter with pCDNA3.1 or a different core promoter reporter construct. The transfected cells were cultured in serum free (SF) medium or treated with HD-1S at 240 microgram/ml and luciferase assays were performed as described in Figure 4. The transfection efficiency was corrected by cotransfecting a beta-gal expression vector and then assaying beta-gal activity simultaneously. Data are expressed as mean ± S.D. (n = 3). SF: black column; HD-1S: white column

transcription machinery of hepatic gluconeogenesis for it own gene expression regulation".

This working model tightly couples two liver specific biological events, HBV gene expression and gluconeogenesis, together and immediately raises several important questions related to novel biological activity of HD-1S. For example, if HD-1S blocks HBV gene expression through suppression of the hepatic gluconeogenic program, HD-1S should be able to suppress hepatic gluconeogenesis *in vivo*. Our preliminary data indicated that HD-1S treatment indeed reduced mRNA level of PEPCK and G-6-Phosphatase in HepG2 cells (data not shown).

Several other important predictions are worthwhile to pursue in the future are listed as follows:

1. The target of HD-1S should play important role in both activation HBV core promoter activity and regulation gene expression of key gluconeogenesis enzyme such as PEPCK and G-6-Pase. PGC-1 and HNF4 are two best candidates since they are key transcriptional factors to regulate PEPCK, G-6-Phosphatase and HBV gene expression, respectively.

2. Any agents can stimulate hepatic gluconeogenesis pathway should also stimulate HBV core promoter activity. Currently, we are examining synergistic effect of cyclicAMP and glucocorticoid hormone on regulation of gluconeogenesis and HBV gene expression.

3. Any agent can suppress hepatic gluconeogenesis such as insulin or TPA should also abolished cAMP plus dexamethasone induced HBV core promoter activation.

Our study has revealed that HD-1S has a novel mode of action to suppress HBV gene expression. Based on these observations, we proposed a working model to suggest that the anti-HBV activity of HD-1S may act through targeting on hepatic gluconeogenesis machinery. This working model will lead several lines of investigation and will open a new avenue to explore novel pharmacological activities associated with HD-1S and other TCM formulations in the future.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

1. JH Kao, DS Chen: Global control of hepatitis B virus infection. *Lancet Infect Dis*, 2(7), 395-403 (2002)

2. RP Beasley: Hepatitis B virus: The major etiology of hepatocellular carcinoma. *Cancer*, 61(10), 1942-56 (1988)

3. YF Liaw: Therapy of chronic hepatitis B: current challenges and opportunities. *J Viral Hepat*, 9(6), 393-9 (2002)

4. HO Akbar: Lamivudine in chronic hepatitis B. virus. *Saudi Med J*, 23(8), 929-33 (2002)

5. HL Chan, AK Chui, WY Lau, FK Chan, ML Wong,, CH Tse, AR Rao, J Wong, JJ Sung: Factors associated with

viral breakthrough in lamivudine monoprophylaxis of hepatitis B virus recurrence after liver transplantation. *J Med Virol*, 68(2), 182-7 (2002)

6. K.M. Walsh, T Woodall, P Lamy, DG Wight, S Bloor, GJ Alexander: Successful treatment with adefovir dipivoxil in a patient with fibrosing cholestatic hepatitis and lamivudine resistant hepatitis B virus. *Gut*, 49(3), 436-40 (2001)

7. WB. Offensperger, S Offensperger, A Keppler-Hafkemeyer, P Hafkemeyer, HE Blum: Antiviral activities of penciclovir and famciclovir on duck hepatitis B virus *in vitro* and *in vivo*. *Antivir Ther*, 1(3), 141-6 (1996)

8. EV Genovesi, L Lamb, I Medina, D Taylor, M Seifer, S Innaimo, RJ Colonno, JM Clark: Antiviral efficacy of lobucavir (BMS-180194), a cyclobutyl-guanosine nucleoside analogue, in the woodchuck (Marmota monax) model of chronic hepatitis B virus (HBV) infection. *Antiviral Res*, 48(3), 197-203 (2000)

9. J Franco, K Saeian: Role of antiviral therapy in the prevention of hepatocellular carcinoma. *J Vasc Interv Radiol*, 13(9 Pt 2), S191-6 (2002)

10. GL Davis: Update on the management of chronic hepatitis B. *Rev Gastroenterol Disord*, 2(3), 106-15 (2002)

11. H Tajiri, K Kozaiwa, Y Ozaki, K Miki, K Shimuzu, S Okada: Effect of sho-saiko-to(xiao-chai-hu-tang) on HBeAg clearance in children with chronic hepatitis B virus infection and with sustained liver disease. *Am J Chin Med*, 19(2), 121-9 (1991)

12. H Oka, S Yamamoto, T Kuroki, S Harihara, T Marumo, SR Kim, T Monna, K Kobayashi, T Tango: Prospective study of chemoprevention of hepatocellular carcinoma with Sho-saiko-to (TJ-9). *Cancer*, 76(5), 743-9 (1995)

13. I Shimizu, YR Ma, Y. Mizobuchi, F Liu, T Miura, Y Nakai, M Yasuda, M Shiba, T Horie, S Amagaya, N Kawada, H Hori, S. Ito: Effects of Sho-saiko-to, a Japanese herbal medicine, on hepatic fibrosis in rats. *Hepatology*, 29(1), 149-60 (1999)

14. SM Akbar, K Yamamoto, M Abe, T Ninomiya, K Tanimoto, T Masumoto, K Michitaka, N Horiike, M Onji: Potent synergistic effect of sho-saiko-to, a herbal medicine, during vaccine therapy in a murine model of hepatitis B virus carrier. *Eur J Clin Invest*, 29(9), 786-92 (1999)

15. JS Chang, KC Wang, HW Liu, MC Chen, LC Chiang, C Lin: Sho-saiko-to (Xiao-Chai-Hu-Tang) and crude saikosaponins inhibit hepatitis B virus in a stable HBV-producing cell line. *Am J Chin Med*, 35(2), 341-51 (2007)

16. SF Yeh, CY Hong, YL Huang, TY Liu, KB Choo, CK Chou: Effect of an extract from Phyllanthus amarus on hepatitis B surface antigen gene expression in human hepatoma cells. *Antiviral Res*, 20(3), 185-92. (1993)

17. YC Chou, KS Jeng, ML Chen, HH Liu, TL Liu, YL Chen, YC Liu, CP Hu, C Chang: Evaluation of Transcriptional Efficiency of Hepatitis B Virus Covalently Closed Circular DNA by Reverse Transcription-PCR Combined with the Restriction Enzyme Digestion Method. *J Virol*, 79(3), 1813-23. (2005)

18. RL Huang, Q Huang, CF Chen, TT Chang, CJ Chou.: Anti-Viral Effects of Active Compounds from Antrodiacamphorata on Wild-Type and Lamivudine-Resistant Mutant HBV. *The Chinese Pharmaceutical Journal*, 55, 371-379 (2003)

19. RB DuBridge, P Tang, HC Hsia, PM Leong, JH Miller, MP Calos: Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol Cell Biol*, 7(1), 379-87 (1987)

20. F Denizot, R Lang: Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods*, 89(2), 271-7 (1986)

21. NJ Proudfoot, FE Baralle: Molecular cloning of human epsilon-globin gene. *Proc Natl Acad Sci U S A*, 76(11), 5435-9. (1979)

22. K Mullis, F Faloona, S Scharf, R Saiki, G Horn, H. Erlich: Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol*, 51(Pt 1), 263-73. (1986)

23. F Galibert, E Mandart, F Fitoussi, P Tiollais, P Charnay: Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. *Nature*, 281(5733), 646-50 (1979)

24. FL Graham, AJ van der Eb: A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, 52(2), 456-67. (1973)

25. M M Bradford: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-54 (1976)

26. L Fu, X Wu, YY Kong, Y Wang: Regulation of Hepatitis B Virus Gene Expression by Its Two Enhancers. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*, 28(6), 590-599 (1996)

27. J Li, JH Ou: Differential regulation of hepatitis B virus gene expression by the Sp1 transcription factor. *J Virol*, 75(18), 8400-6 (2001)

28. S Gilbert, L Galarneau, A Lamontagne, S Roy, L Belanger: The hepatitis B virus core promoter is strongly activated by the liver nuclear receptor fetoprotein transcription factor or by ectopically expressed steroidogenic factor 1. *J Virol*, 74(11), 5032-9 (2000)

29. SJ Lin, PY Shu, C Chang, AK Ng, CP Hu: IL-4 suppresses the expression and the replication of hepatitis B

virus in the hepatocellular carcinoma cell line Hep3B. *J Immunol*, 171(9), 4708-16 (2003)

30. KH Schlemmer, D Haebich, H Rubsamen-Waigmann: Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. *Science*, 299(5608), 893-6 (2003)

31. TM Tan, Y Chen, KH Kong, J Bai, Y Li, SG Lim, TH Ang, Y Lam: Synthesis and the biological evaluation of 2-benzenesulfonylalkyl-5-substituted-sulfanyl-[1,3,4]-oxadiazoles as potential anti-hepatitis B virus agents. *Antiviral Res*, 71(1), 7-14 (2006)

32. D Janmanchi, YP Tseng, KC Wang, RL Huang, CH Lin, SF Yeh: Synthesis and the biological evaluation of arylnaphthalene lignans as anti-hepatitis B virus agents. *Bioorg Med Chem*, 18(3), 1213-1226 (2010)

33. A Shlomai, N Paran, Y Shaul: PGC-1alpha controls hepatitis B virus through nutritional signals. *Proc Natl Acad Sci U S A*, 103(43), 16003-8 (2006)

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