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KNK437 Inhibits Replication and Transcription of the Hepatitis B Virus

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Abstract: During replication of the hepatitis B virus (HBV) in liver cells, the reverse transcription of pregenomic RNA (pgRNA) is initiated by protein priming at an RNA packaging signal ε located near the 5′ end of pgRNA. Heat-shock proteins (Hsps) such as Hsc70, Hsp40, and Hsp90 have been reported to be involved in the reconstitution of HBV polymerase (P protein) and ε. The P–ε complex initiates the reverse transcription and assembly of nucleocapsids. Hence, blockade of P–ε interactions is an attractive target for drug intervention. We explored the influence of the Hsp inhibitor KNK437 on replication and transcription of the HBV. Three working models were applied: HepG2.2.15 cell line; Huh7 cells transfected transiently with the 1.05 × HBV (pCH9-3091) plasmid; Huh7 cells transfected transiently with the 1.3 × HBV (pGEM-1.3 × HBV) plasmid. Cytotoxic effects of KNK437 were detected by the CCK-8 method. Levels of hepatitis B surface antigen (HBsAg) and hepatitis B virus e antigen (HBeAg) in the media secreted from cells were measured using an ELISA. Intracellular HBV DNAs within nucleocapsids were measured by quantitative polymerase chain reaction (q-PCR), and intracellular HBV RNAs by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Transcription of Hsps in cells was determined by qRT-PCR. Data suggested that KNK437 reduced the extracellular secretion of HBsAg and HBeAg in most cases; it downregulated expression of intracellular HBV DNAs within nucleocapsids and RNA transcripts. The lowest rate of viral DNAs in KNK437-treated hepatocytes for all experimental groups was about 1.5% (control, 100%), while that for RNAs was about 30%. Western blotting revealed KNK437 to inhibit intracellular core expression in HepG2.2.15. As a general inhibitor, KNK437 suppressed transcription of Hsp70, Hsp90b, and Hsp40. These data suggest that KNK437 may be a potent anti-HBV inhibitor for future therapy against chronic hepatitis. **DOI:** 10.13242/j.cnki.bingduxuebao.003090-en

Keywords: Hepatitis B virus (HBV); Heat-shock protein (Hsp); KNK437; Inhibitor

Hepatitis B virus (HBV) is a micromolecule DNA virus which is replicated by reverse transcription, and chronic hepatitis B is caused after host is infected [1]. HBV infection is worldwide public health problem. About 2 billion people were infected with HBV in the global. Among them, about 350 million people suffered from chronic hepatitis [2]. Besides, chronic infection results in hepatic fibrosis, hepatic cirrhosis and liver cancer [1–3]. At present, there are two methods to treat chronic hepatitis B: injection of α interferon and taking nucleoside (nucleotide) with analogues such as entecavir, lamivudine and adefovir. But these two methods have obvious disadvantages. For example, high-dose α interferon has low host response rate and serious side effects, while nucleoside (nucleotide) analogue drugs can result in drug resistance and rebound after drug withdrawal [3–6]. Therefore, it is in urgent need of finding new anti-HBV drugs.

Heat-shock proteins (Hsps) are a kind of chaperonins resulted from cells under stress conditions. During replication of HBV, Hsp is important for interaction between virus reverse transcriptase (P protein, also DNA polymerase) and RNA packaging signal (ε) on pregenomic RNA (pgRNA) [3]. P-ε forms RNA–protein complex (RNP) whose exertion of active effect depends on Hsps (such as Hsc70 and Hsp40) and ATP in host hepatocytes, and its activated state is stabilized by Hsp90 [3, 7–11]. KNK437 is a compound synthesized by Kaneka (Japan). The full name is N-formyl-3,4-methylenedioxybenzyl-γ-butyrolactone which belongs to benzylidene lactam [12, 13]. The chemical structure is shown in Figure 1. KNK437 inhibits Hsp mRNA transcription by influencing interaction between heat-shock factors HSF and HSE, thereby downregulating protein expression and intracellular accumulation [14]. Reports suggested that drugs make culture cells of HeLa cells, colon cancer, squamous cell carcinoma and glioblastoma sensitive to the temperature and heat resistant function reduced by downregulating

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intracellular Hsps [12–15]. KNK437 also can inhibit production of virus particles in baculovirus infection cycles [16]. In recent years, there are reports about Hsp90 inhibitor, Geldanamycin (GA) and Hsp70 inhibitor, oxymatrine (OMTR) inhibiting HBV replication successively [17, 18]. Against this background, in this study, we investigate whether KNK437 affects HBV replication.

Three liver cancer cell line models were applied in this study: HepG2.2.15 cell line, Huh7 cell line transfected transiently with 1.05 × HBV (pCH9-3091) plasmid and Huh7 cell line transfected transiently with 1.3 × HBV (pGEM-1.3 × HBV) plasmid. After cells were treated with KNK437 at appropriate concentration, cytotoxicity was determined with CCK-8 method and extracellular secretion of hepatitis B surface antigen (HBsAg) and hepatitis B virus e antigen (HBeAg) were determined by ELISA. Intracellular HBV DNAs synthesis level within nucleocapsids was measured by quantitative polymerase chain reaction (q-PCR) and intracellular HBV RNAs transcription level by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The influence of HepG2.2.15 intracellular KNK437 on expression level of capsid subunit (core) was measured by Western blotting, and finally the effect of KNK437 on Hsps RNA transcription level was analyzed.

Materials and methods

1 Cell culture and transfection

HepG2.2.15 cells and Huh7 cells were purchased from China Center for Type Culture Collection (CCTCC). DMEM culture medium involving 10% fetal calf serum and 1% double-antibody (100 U/mL penicillin and 100 μg/mL streptomycin) was prepared, and then HepG2.2.15 cell line and Huh7 cell line were cultured with this culture medium in 5% CO_2 cell culture incubator at 37 ℃.

In this study, 1.3 × HBV (pGEM-1.3HBV) plasmid which involved a 1.3 × HBV genomic fragment (4 195 bp, serotype: *adw*) [19] was given by Professor Yosef Shaul of the Weizmann Institute of Science as a gift, while 1.05 × HBV (pCH9-3091) plasmid which involved a 1.05 × HBV genomic fragment (3 375 bp, serotype: *ayw*) [20] was given by Professor Michael Nassal of University of Freiburg as a gift. Huh7 cells were inoculated into 24-well cell culture plate at the density of 1.5×10^5 cells/cm^2. After 24 h, according to operation instruction of lipo2000 reagent, 0.8 μg pGEM-1.3HBV plasmid and pCH9-3091 plasmid were transfected. Although 1.05 × plasmid and 1.3 × plasmid both could express HBV in cells, they had their own different characteristics. High-efficient human cytomegalovirus (hCMV) IE1 promoter was involved in HBV genome upstream in 1.05 × plasmid HBV with high transcription level of pgRNA and slightly strong replication capacity. In addition, 1.3 × plasmid which did not contain any exogenous eukaryotic promoters was widely used in worldwide laboratories. HBV gene transcription was initiated by native promoter, secretion level of antigen was low and replication capacity was relatively weak.

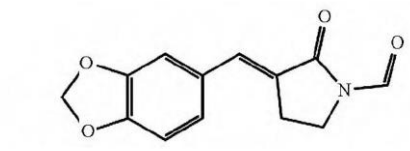

Figure 1 Chemical structure of N-formyl-3,4-methylenedioxy benzyl-γ-butyrolactone (KNK437)

2 Drug treatment

KNK437 was purchased from Calbiochem (Germany), and Entecavir-triphosphate (ETV) was bought from Biochemicals (USA). Both were used as positive control medicines. Entecavir is a kind of cyclopentane nucleotide drug and acts on P protein during reverse transcription. It only influenced HBV cDNA synthesis, but did not affect HBV RNA transcription. HepG2.2.15 and Huh7 were inoculated into 24-well cell culture plate at the density of 1.5×10^5 cells/cm^2. After 24 h, Huh7 cells were transfected transiently with 1.05 × HBV and 1.3 × HBV plasmids. After transfection for 24 h, 1 mL prepared culture medium mixed with drugs at a certain concentration was added in each well. In the experiment, 30 nM ETV was added to act as positive control. Additionally, 0.1% DMSO was added as blank control without drugs. Culture medium was replaced once every other 48 h. After cells were cultured for 2 d, 4 d and 6 d with culture solution containing KNK437 at a certain concentration, supernatant and cells were collected for following analysis. The cells were continuously incubated and treated with drugs above 6 d. Variation trend of various data was not obvious compared with data in 6 d treatment (data were not shown). Therefore, all analysis data indicated that the longest action time of KNK437 was 6 d.

3 Drug cytotoxicity detection

CCK-8 reagent was purchased from Dojindo Molecular Technologies, Inc. (Shanghai, China) and used to detect cytotoxicity. HepG2.2.15 and Huh7 were inoculated into 96-well plate at 10^4 cells/well. After 12 h, various concentrations of KNK437 were added to stimulate. Treatment time was 2 d, 4 d and 6 d respectively. Supernatant was removed in detection, and then cells were washed three times with PBS. Afterwards, fresh culture medium without serum including 10% CCK-8 solution was added, the cells were continuously incubated for 2 h. Finally, OD_{450} absorbance of each well was measured by microplate reader. OD_{450} value in blank control group without addition of drug was considered as relative value 1 of cell growth rate. The absorbance of every other well was compared with the OD_{450} value, thereby obtaining relative value of respective cell growth rate.

4 Virus antigen secretion detected by ELISA

Extracellular secretion of virus antigen level was detected by using HBsAg diagnostic kit and HBeAg diagnostic kit produced by Shanghai Kehua Bio-Engineering Co., Ltd. After the completion of the drug stimulation, the supernatant of cell culture solution in each well was collected (control group was involved). Before detection, supernatant under test was put in the incubator at 37 ℃ and incubated for 30 min. Afterwards, HBsAg and HBeAg levels in each sample were measured according to operation instruction of kit. Three duplicate wells were set for each sample. The absorbance of the control group without addition of drug was considered as relative value 1. The absorbance of every other well was compared with that of the control group, thereby obtaining relative values of HBsAg or HBeAg levels.

5 q-PCR and RT-PCR

For HepG2.2.15 after drug treatment and for Huh7 cell transfected with plasmids, 100 μL lysis buffer [2.19 g NaCl, 12.5 mL 1 M Tris-HCl (pH 8.0), 2.5 mL NP40, 0.5 mL 0.5 M EDTA] was added in each well of 24 well-plate. Then, cell residues were removed by centrifugation, and 1 M $MgCl_2$ (final concentration: 8 mM), 1 μL DNase I (70 U/μL) and 5 μL RNase A were added in the supernatant. After blending, the cells were incubated for 1 h in the incubator at 37 ℃ to completely degrade intracellular host nucleic acids and exogenous plasmid. Afterwards, 10 μL 0.5 M EDTA was added, and then the cells were treated for 10 min in water bath kettle at 60 ℃ to terminate reaction. After that, 1/10 (volume) of 10% SDS (final concentration: 1%) and 20 mg/mL Proteinase K (final concentration: 50 μg/mL) were added, and then the cells were incubated for 2 h in water bath kettle at 55 ℃. HBV nucleocapsids were split to release virus DNA. Subsequently, isovolumetric Tris-saturated phenol was added, the supernatant was taken by centrifugation, and then 1/10 (volume) of 3 M NaAc (pH 5.2) and glycogen (final concentration: 0.1 μg/μL) were added. After reverse blending, ethanol with the volume of 2.5 times was added. The mixture was blended and the kept standing at −20 ℃ overnight to precipitate DNA released from virus nucleocapsids. On the next day, the solution was centrifuged, and finally DNA precipitation was fully dissolute with 20 μL TE. In q-PCR experiment, the sequences of primers HBV-F and HBV-R in Table 1 corresponded to the HBV genome positions 3 105–3 125 and 57–77 respectively [21] (GenBank number: J02203, full length: 3 182 bp, translation initiation site of core gene was defined as 1).

Intracellular RNAs were extracted and TRIzol reagent from Invitrogen was applied, and then cell total RNAs were extracted according to instructions. Prime-Script RT reagent kit from TaKaRa was used for reverse transcription reaction, and reaction processes were conducted in strict accordance with operation instruction of kit. Finally, 20 μL cDNA solution could be obtained for each sample. In qRT-PCR experiment, HBV-F and HBV-R were adopted, HBV 57–3 125 fragments were specially amplified, and then pgRNAs about 3.4 kb and PreS1 mRNAs about 2.4 kb of HBV could be detected. SYBR Premix Ex Taq II kit (TaKaRa) was applied for PCR reaction which was conducted on LightCycler 96 Real-Time PCR amplification instrument (Roche). Reaction conditions were as follows: initial denaturation for 5 min at 95 ℃, and then amplification reactions with 45 cycles, including denaturation for 10 s at 95 ℃, annealing for 10 s at 60 ℃ and extension for 10 s at 72 ℃. After the completion of amplification, dissolve curve detection is connected to confirm amplification effects. The primers for PCR reaction are shown in Table 1.

6 Influence of KNK437 on expression of intracellular capsid subunit (core) by Western blotting

The detection was conducted according to previous method reported by our laboratory [22]. After HepG2.2.15 cells were treated with drugs for 6 d, the cells were collected by centrifugation, and washed twice with icy PBS buffer, and then lysed with cell lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% deoxycholic acid, 0.5% sodium azide, 100 μg/mL PMSF) on the ice. Afterwards, intracellular proteins were released. $MgCl_2$ at the final concentration of 10 mM was added in lysis buffer, and cells were incubated with 20U DNase I and 15U RNase A at 37 ℃ for at least 6 h to eliminate intracellular nucleic acid background. After equivalent protein samples were boiled and denatured, the samples were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation. Protein samples were printed into polyvinylidene fluoride (PVDF) membrane with semi-dry electrophoretic transfer system. After the membrane was blocked with 5% skimmed milk, this membranewas incubated with rabbit polyclonal HBcAb (1:1 000 dilution, DAKO) or with mice anti GAPDH monoclonal

Table 1 Primer names, their sequences and the used annealing temperatures for PCR

Primers	Sequences(5'→3')	Annealing temperature (℃)
Hsp40-F	CCAGTCACCCACGACCTTC	60
Hsp40-R	CCCTTCTTCACTTCGATGGTCA	60
Hsp70-F	CCGAGAAGGACGATTTGAG	60
Hsp70-R	ACAAAAACAGCAATCTTGGAAAGG	60
Hsp90b-F	CCGGTGTAGGAATGACCAGAG	60
Hsp90b -R	TTAAAAACTCGCTTGTCCCAGAT	60
HBV -F	ACCAATCGCCAGTCAGGAAG	60
HBV -R	ACCAGCAGGGAAATACAGGC	60
β-actin-F	CATGTACGTTGCTATCCAGGC	60
β-actin-R	CTCCTTAATGTCACGCACGAT	60

antibody (1:1 000 dilution, DAKO). After several rounds of PBS washing, the membrane and secondary antibodies marked with horse radish peroxidase were incubated. Protein bands were displayed by enhanced chemiluminescence (ECL). Housekeeping gene GAPDH as internal control was used for data correction.

7 Statistical analysis

At least three times of independent experiments must be carried out for all results, and histogram reflected standard deviation (SD) of experimental results.

Unpaired two-tailed t tests were used for comparison of significant difference. $*P < 0.05$ and $**P < 0.01$ meant that the difference was significant.

Results

1 Analysis on cytotoxicity of HepG2.2.15 and Huh7 with KNK437

KNK437 concentration was 0 μM, 0.1 μM, 1 μM, 10 μM, 20 μM, 50 μM, 100 μM and 500 μM respectively. Treatment time was 2 d, 4 d and 6 d, and independent repeated experiments were conducted for three times. Cytotoxicity was detected with CCK-8 method, and then absorbance of each well at OD_{450} was detected with microplate reader. As could be seen from experimental results shown in Figure 2, KNK437 had no toxicity to two kinds of cells within detection concentration of 100 μM. When cells were treated for 2 d and 4 d, relative values of HepG2.2.15 cells exceeded 95%, indicating that cell growth was not affected. Only when KNK437 concentration reached 500 μM and cells were treated for 6 d, relative values of cells decreased by 15%–22% respectively. Referring to KNK437 concentration used in other research [23, 24], in our subsequent experiments, maximum working concentration of KNK437 to be 20 μM was applied.

2 Influence of KNK437 on extracellular secretion of HBsAg and HBeAg

HBV secreted HBsAg and HBeAg antigens during infection of host cells. Secretion levels of HBsAg and HBeAg varied during different stages of HBV infection. It is clinically significant to analyze the change of these two antigens. In this experiment, the influence of KNK437 on HBsAg and HBeAg was studied with three cell models from various aspects. There were two conditions for drug treatment of cells: concentration gradient and time gradient. Under these two conditions, change of two antigens of HBV was detected respectively.

2.1 Influence of various concentrations of KNK437 on extracellular secretion of HBsAg and HBeAg

Three concentrations of KNK437 adopted in this study were as follows: 1 μM, 10 μM and 20 μM. The treatment time of cells was 6 d. This experiment included blank control group without addition of drug and positive control group with the treatment 30 nM ETV.

The experimental results showed that after HepG2.2.15 cells were treated with KNK437 at different concentrations for 6 d, HBsAg level in supernatant was not obviously changed (Figure 3A), but decrease in HBeAg in the supernatant was dose-dependent on KNK437. When KNK437 concentration reached up to the maximum value (20 μM), HBeAg decreased to 30.5% (Figure 3D). After Huh7 cells transfected transiently with 1.05 × HBV plasmid were treated with KNK437 at different concentrations for 6 d, there was no significant change in HBsAg in the supernatant. When KNK437 concentration were up to the maximum value (20 μM), HBsAg only decreased to 74.2% (Figure 3B). However, HBeAg in the supernatant was dose-dependent on KNK437: with the increase of KNK437 concentration, HBeAg was gradually decreased but decrease amplitude was not big. When the concentration was up to the maximum value (20 μM), HBeAg level decreased to 60.3% (Figure 3E). After Huh7 cells transfected transiently with 1.3 ×HBV plasmid were treated with KNK437 at different concentrations for 6 d, HBsAg in the supernatant was not dose-dependent on KNK437 at low concentrations. When KNK437 concentration was up to the maximum value (20 μM), HBsAg was obviously decreased to 43.6% (Figure 3C). Moreover, HBeAg in supernatant at lower concentrations was not obviously changed. When KNK437 concentration reached up to the maximum value (20 μM), HBeAg was obviously decreased to 45.1% (Figure 3F).

2.2 Influence of KNK437 incubation time on extracellular secretion of HBsAg and HBeAg

The above results showed that except Figure 3A, 20 μM drug could inhibit secretion of two antigens of HBV. Next, cells were treated with 20 μM KNK437 for 2 d, 4 d and 6 d respectively. Meanwhile, the experiment included blank control group without drug and positive control group with 30 nM ETV treatment.

As seen from Figure 4, after HepG2.2.15 was treated with 20 μM KNK437 for different time, HBsAg level in the supernatant was not obviously changed (Figure 4A). But decrease in HBeAg in the supernatant was time-dependent on KNK437. With the increase of treatment time of KNK437, HBeAg level was gradually reduced. When treatment time was 6 d, HBeAg level decreased to the minimum (29.6%, Figure 4D). After Huh7 cells transfected transiently with 1.05 × HBV plasmid were incubated with 20 μM KNK437 for different time, change in HBsAg in the supernatant was not obvious. When the treatment time reached up to 6 d, the HBsAg level only decreased to 75.2% (Figure 4B). Decrease in HBeAg in the supernatant was time-dependent on KNK437: after treatment for 4 d, the HBeAg level decreased to 77.7%; after 6 d, to 63.3%

(Figure 4E). After Huh7 cells transfected transiently with 1.3 × HBV plasmid were treated with 20 μM KNK437 for different time, decrease in HBsAg in the supernatant was time-dependent on KNK437: after treatment for 6 d, the HBsAg level decreased to 45.4% (Figure 4C). However, decrease in HBeAg in the supernatant was also time-dependent on KNK437. After 2 d, 4 d and 6 d, HBeAg level decreased to 69.7%, 59.3% and 45.1% respectively (Figure 4F). Cells were continuously incubated and treated with drugs for more than 6 d, compared with the data of 6 d, decrease trend would no longer continue (data were not shown, the same below).

3 Influence of KNK437 on intracellular DNA synthesis in HBV nucleocapsids

Cells were treated with KNK437 in two ways (concentration gradient and time gradient), and collected by centrifugation, and then DNAs in HBV nucleocapsids were extracted from cells. Next, change in virus DNA level was detected with q-PCR.

As seen from Figure 5, after HepG2.2.15 cells were stimulated by KNK437 at different concentrations, intracellular HBV DNA level obviously decreased and decrease degree was dose-dependent on KNK437. When treatment concentration was 1 μM, HBV DNA level was obviously inhibited, and the level decreased to 46.6%. When the concentration raised to 10 μM and 20 μM, HBV DNA level decreased to 6.3% and 1.5% respectively (Figure 5A). After Huh7 cells transfected transiently with 1.05 × HBV plasmid were stimulated by KNK437 at different concentrations, intracellular HBV DNA level decreased, and decrease degree was dose-dependent on KNK437, but inhibition degree was weaker than that of HepG2.2.15 cells. When the concentration raised to 20 μM, HBV DNA level was inhibited with the highest inhibition rate and decreased to 57.2% (Figure 5B). After Huh7 cells transfected transiently with 1.3 × HBV plasmid were stimulated by KNK437, decrease degree of intracellular HBV DNAs was dose-independent on KNK437. When treatment concentration was 10 μM and 20 μM, HBV DNAs decreased to 61.7% and 33.9% respectively (Figure 5C).

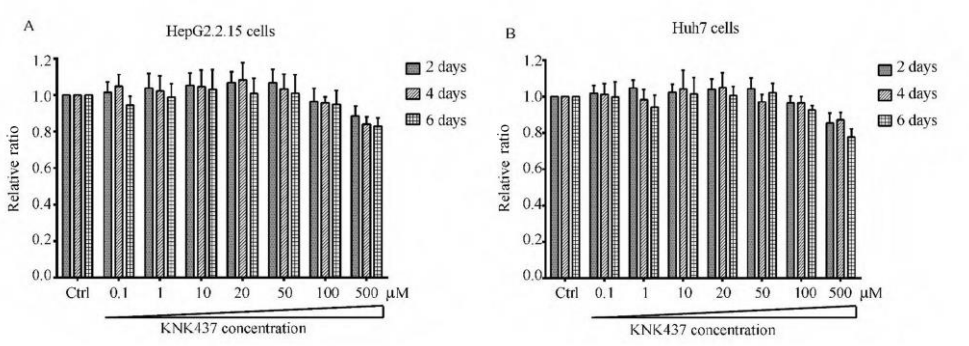

Figure 2 Cytotoxicity assays of different concentrations of KNK437 in different cell lines

(A) HepG2.2.15 cells and (B) Huh7 cells, which were incubated with the indicated concentration of KNK437 continuously for 2, 4, or 6 d. Data were compared with control (defined as 1.0). Values shown are the mean ± standard deviation of three independent experiments.

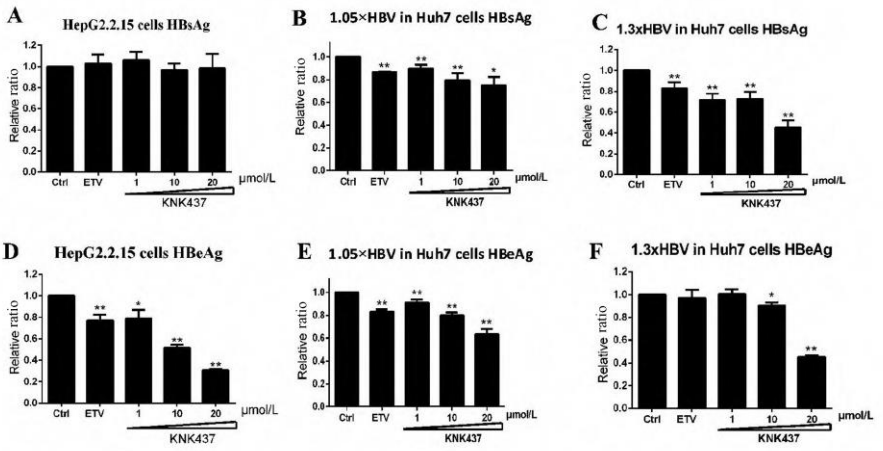

Figure 3 ELISA data of HBsAg and HBeAg in the culture media secreted from HepG2.2.15 cells (A, D), and from Huh7 cells transfected transiently with the 1.05 × HBV plasmid (B, E) or 1.3 × HBV plasmid (C, F), respectively. Cells were incubated with various concentrations of KNK437 for 6 d before analyses. Data were compared with control (defined as 1.0). Values are the mean ± standard deviation of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

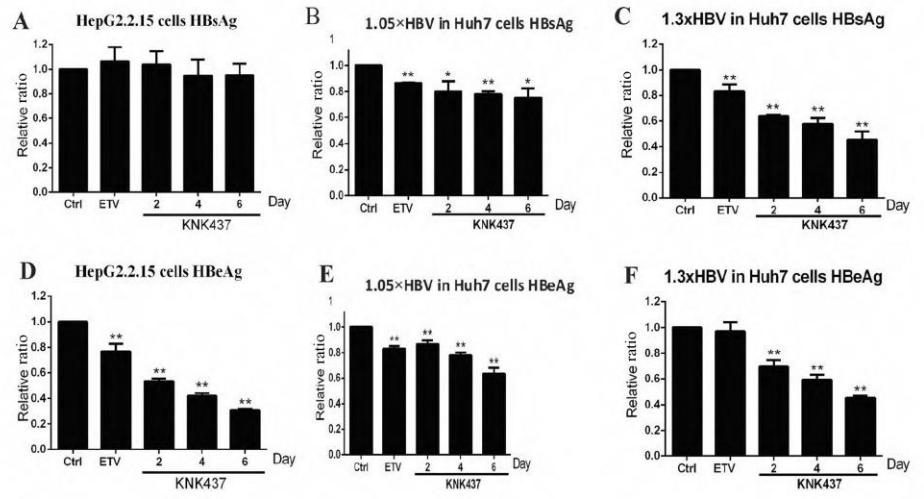

Figure 4 ELISA data of HBsAg and HBeAg in the culture media secreted from HepG2.2.15 cells (A, D) and from Huh7 cells transfected transiently with the 1.05 × HBV plasmid (B, E) or 1.3 × HBV plasmid (C, F), respectively. Cells were incubated continuously with 20 μM KNK437 for 2, 4, or 6 d before analyses. Data were compared with control (defined as 1.0). Values are the mean ± standard deviation of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

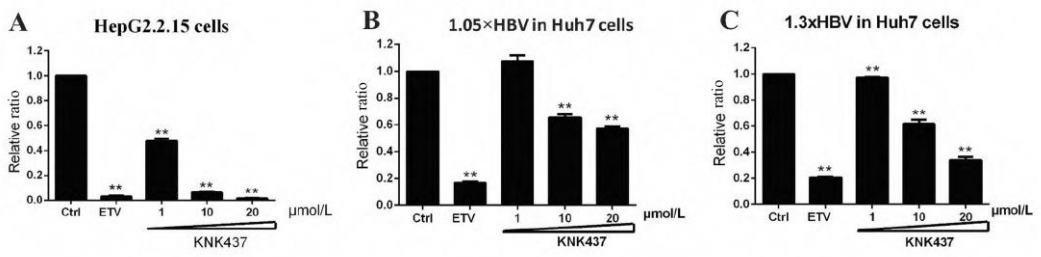

Figure 5 q-PCR data of intracellular HBV DNAs within nucleocapsids in HepG cells (A) and Huh7 cells transfected transiently with the 1.05 × HBV plasmid (B) or 1.3 × HBV plasmid (C), respectively. Cells were incubated continuously with 1, 10, or 20 μM KNK437 for 6 d before analyses. Data were compared with control (defined as 1.0). Values are the mean ± standard deviation of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

After HepG2.2.15 cells were treated with 20 μM KNK437 for different time, intracellular HBV DNA level remarkably decreased, and decrease degree was time-dependent on KNK437. After 2 d, 4 d, and 6 d, HBV DNA level reduced to 37.9%, 5% and 1.4% respectively (Figure 6A). After Huh7 cells transfected transiently with 1.05 × HBV plasmid were treated with 20 μM KNK437 for 4 d and 6 d, HBV DNAs decreased to 64.1% and 57.3% respectively (Figure 6B). Huh7 cells transfected transiently with 1.3 × HBV plasmid were treated with 20 μM KNK437, and after 4 d and 6 d, HBV DNAs decreased to 56.9% and 34.2% respectively (Figure 6C). As positive control group, two kinds of Huh7 cell models transfected transiently were treated with 30 nM ETV for 6 d, HBV DNA level was only about 20% (Figures 5B and 5C and Figures 6B and 6C).

4 Influence of KNK437 on intracellular HBV RNA transcription

Cells were treated with KNK437 in two ways (concentration gradient and time gradient), and collected by centrifugation, and then HBV RNA was extracted from cells. Intracellular HBV RNA level was detected by qRT-PCR.

As seen from Figure 7, HepG2.2.15 cells were treated with KNK437 at different concentrations, intracellular HBV RNA level decreased significantly, and decrease degree was dose-dependent on KNK437. When treatment concentration was 1 μM, 10 μM and 20 μM, HBV RNA level decreased to 80.6%, 51.4%, 34.3% respectively (Figure 7A). After Huh7 cells transfected transiently with 1.05 × HBV plasmid were treated with KNK437 at different concentrations, intracellular HBV RNA level obviously decreased, and the decrease degree was dose-dependent on KNK437. When treatment concentration was 1 μM, 10 μM and 20 μM, HBV RNA level reduced to 73.3%, 46.8% and 27% respectively (Figure 7B). After Huh7 cells transfected transiently with 1.3 × HBV plasmid were treated with KNK437 at different concentrations, the result was the same as that of cells transfected transiently with 1.05 × HBV plasmid. When drug concentration was 1 μM, 10 μM and 20 μM, HBV RNA level was reduced to 82.6%, 46.4% and 27% respectively (Figure 7C).

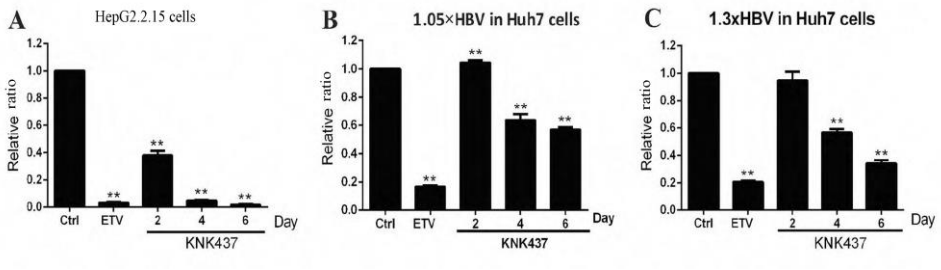

Figure 6 q-PCR data of intracellular HBV DNAs within nucleocapsids in HepG2.2.15 cells (A) and Huh7 cells transfected transiently with the 1.05 × HBV plasmid (B) or 1.3 × HBV plasmid (C), respectively. Cells were incubated continuously with 20 μM KNK437 for 2, 4, or 6 d before analyses. Data were compared with control (defined as 1.0). Values are the mean ± standard deviation of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

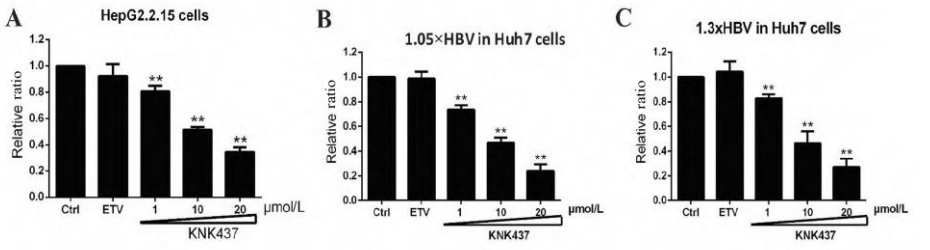

Figure 7 qRT-PCR data of intracellular HBV RNAs in HepG2.2.15 cells (A) and Huh7 cells transfected transiently with the 1.05 × HBV plasmid (B) or 1.3 × HBV plasmid (C), respectively. Cells were treated continuously with 1, 10, or 20 μM KNK437 for 6 d before analyses. Data were compared with control (defined as 1.0). Values are the mean ± standard deviation of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

After HepG2.2.15 cells were treated with 20 μM KNK437 for different time, intracellular HBV RNA level significantly decreased to 40% after 2 d. After 4 d and 6 d, the level was not reduced further (Figure 8A). After Huh7 cells transfected transiently with 1.05 × HBV plasmid were treated with 20 μM KNK437, intracellular HBV RNA level significantly decreased to 57.3% after 2 d. But as the treatment time prolonged, HBV RNA level only slightly decreased after 4 d and 6 d. After 6 d, intracellular HBV RNA level decreased to the minimum (33.3%, Figure 8B). Huh7 cells transfected transiently with 1.3 × HBV plasmid were treated with 20 μM KNK437 for different time. Intracellular HBV RNA levels decreased, and inhibitory effects of KNK437 gradually increased with the development of time course. After 2 d, 4 d and 6 d of drug treatment, HBV RNA level decreased to 67.8%, 44.7% and 29.3% respectively (Figure 8C).

As seen from Figures 7 and 8, there was no any effect of control drug ETV on HBV RNA transcription. Because ETV was synthesized by specific targeted virus reverse transcriptase inhibiting HBV DNA replication, but not by targeted virus RNA transcription level, this result conformed to the expected result.

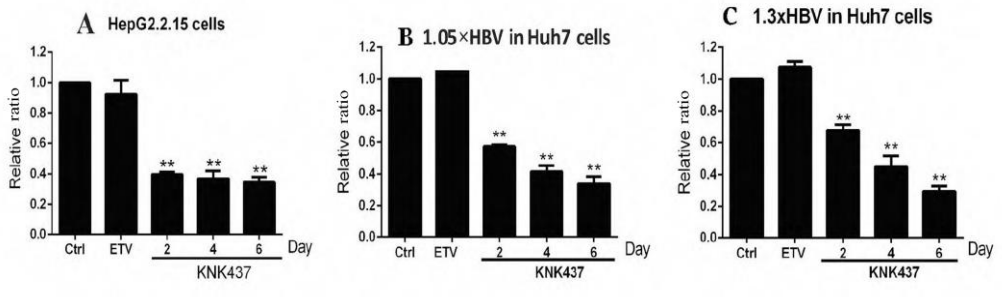

Figure 8 qRT-PCR data of intracellular HBV RNAs in HepG2.2.15 cells (A) and Huh7 cells transfected transiently with the 1.05 × HBV plasmid (B) or 1.3 × HBV plasmid (C), respectively. Cells were treated continuously with 20 μM KNK437 for 2, 4, or 6 d before analyses. Data were compared with control (defined as 1.0). Values are the mean ± standard deviation of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

5 Influence of KNK437 on expression of intracellular HBV core protein

After HepG2.2.15 cells were treated with 20 μM KNK437 for 6 d, intracellular HBV core protein expression level was measured by Western blotting. Treatment group involving 1% DMSO without addition of drug was regarded as blank control group, and 10 μM NVR3-778 (core inhibitor, provided by Johnson & Johnson (USA)) was considered as positive control group. Additionally, the other compound Quercetin was used in positive control group to inhibit core expression [25].

Results of Figure 9 showed that KNK437 obviously inhibited the expression of intracellular virus core protein. Inhibitory effects were 20 μM KNK437 > 10 μM NVR3-778 > 50 μM Quercetin.

6 Influence of KNK437 on intracellular Hsp RNA transcription

As shown in Figure 10, after treatment with KNK437, RNA transcription levels of Hsp70, Hsp90b and Hsp40 in HepG2.2.15 cells decreased (Figure 10). It indicated that KNK437 definitely inhibited transcription of Hsps. Thereinto, the influence on Hsp70 was the greatest. When KNK437 concentration was 20 μM, Hsp70 RNA level decreased to 30% (Figure 10A). At this concentration, intracellular RNA of Hsp90b and Hsp40 only decreased to 70% and 60% respectively (Figures 10B and 10C).

Discussion

Hsps are a kind of chaperonins produced from cells under stress state and its main function is to prevent misfolding and accumulation of proteins with important functions in cells. KNK437 is also called N-formyl-3,4-methylenedioxybenzyl-γ-butyrolactone which belongs to benzylidene lactam [12, 13]. Reports show that KNK437 can inhibit Hsp mRNA transcription by influencing heat-shock factors HSF and HSE, thereby reducing expression and accumulation of such proteins in cells. In addition, the drug can weaken heat resistance of culture cells of HeLa cells, colon cancer, squamous-cell carcinoma and glioblastoma by affecting Hsps [14, 15].

The replication of hepadnaviridae, with HBV as representative, is greatly different from that of other retroviruses. HBV polymerase is also reverse transcriptase, which is called P protein, including terminal protein (TP), reverse transcriptase (RT) and RNaseH. Spacer area without functional significance exists between TP and RT. Among them, TP area only belongs to hepadnaviridae, and there is no such an area in all other retroviruses, suggesting that this structure can develop into antiviral targets. Virus pgRNA enters cytoplasm from cell nucleus and then produces capsid subunits (core) and P protein after being translated. The latter combines to stem-loop structure of an RNA packaging signal (ε) located near the 5′ end of pgRNA and initiates capsid subunit polymerization assembly, and reverse transcription is initiated [3]. Before HBV minus-strand DNA is synthesized by reverse transcription, P protein synthesizes 3–4 oligonucleotides as initial primers of reverse transcription synthesis with lateral bulge in ε molecule as template, and this process is called priming [3, 27]. Previous investigation has found that RNP complex is formed by P protein and ε, which needs participation of host Hsps (such as Hsc70, Hsp40 and Hsp90) and the supply of energy by ATP (Figure 11A) [7–11].

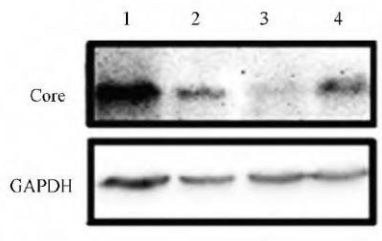

Figure 9 Western blots showing the influence of different drugs after treatment for 6 d on intracellular expression of HBV core proteins

1. Zero control without addition of drug; 2. 10 μM NVR3-778 as positive control; 3. 20 μM KNK437; 4. 50 μM quercetin as a positive control.

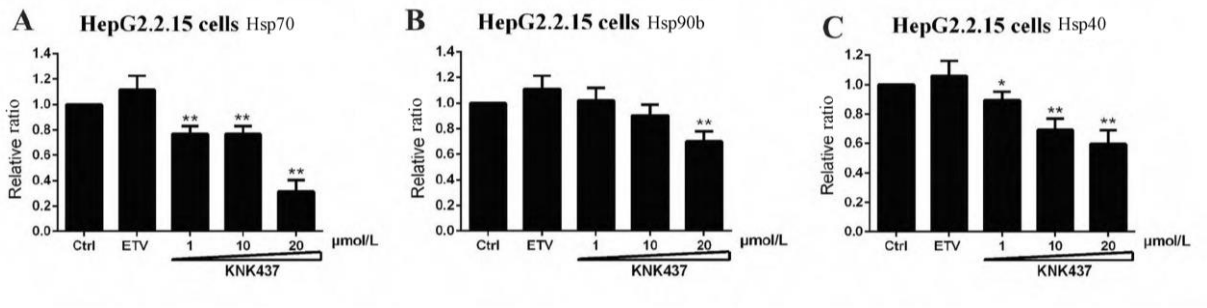

Figure 10 qRT-PCR data of intracellular Hsp70 (A), Hsp90b (B), Hsp40 (C) RNA levels in HepG2.2.15 cells. Cells were incubated continuously with 1, 10 or 20 μM KNK437 for 6 d before analyses. Data were compared with control (defined as 1.0). Values are the mean ± standard deviation of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

Blockade of P-ε complex can inhibit synthesis of HBV reverse transcription and packaging of nucleocapsids. It is an attractive and promising drug target with two functions. Currently, strategies of blocking P-ε interaction [26] include 1) Hsp inhibitor; 2) screening ε aptamer, serving as competitive inhibitor of wild-type ε; 3) blocking compound of P protein loci combined with ε. At early stage, our research group has made a series of advance in aptamer screening. *In vitro* experiment verified that aptamers could block HBV replication [22, 28, 29]. At present, we evaluate pharmacodynamics and toxicology of anti-HBV of aptamer on transgenic mice models.

In this study, influence of KNK437 on Hsp mRNA transcription was detected by qRT-PCR. As seen from Figure 10, after treatment of KNK437, RNA transcription levels of three Hsps in HepG2.2.15 cells decreased, illustrating that KNK437 was definitely a kind of Hsp inhibitor. Thereinto, we found that KNK437 was the inhibitor targeting Hsp70 through experiment. This result was consistent to that of previous report [23–24]. Considering that Hsc70 (structural type: Hsp70) played an important role in synthesis of P-ε complex [7–10], KNK437 has the potential to become anti-HBV drug.

We found that KNK437, as Hsp inhibitor, inhibited intracellular DNA reverse transcription synthesis in HBV nucleocapsids through verification with different cell models from multi-aspects. Such mechanism of inhibitory effect might be because RNP complex formed by P protein and ε lacked enough Hsps as molecular chaperones and functional conformation was maintained (Figure 11B). RNP compound lacked activity, resulting in failing to synthesize necessary primers (priming) for reverse transcription from the beginning, thereby terminating the synthesis of minus-strand DNA, and blocking assembly of nucleocapsids at the same time (Figure 11A).

KNK437 resulted in decrease in secretion levels of HBsAg and HBeAg in the supernatant, speculating that this drug reduced the expression of intracellular virus protein. Decrease in expression level was based on the drug directly downregulating corresponding RNA transcript of HBV, thereby reducing the translation, accumulation and secretion of virus proteins. But there was an exception, in HepG2.2.15, KNK437 at all concentrations would not influence HBsAg secretion. We also found this similar phenomenon in the study of screening other drugs of HBV [25]. It was speculated that in HepG2.2.15 model, virus HBsAg gene might integrate in some sites of chromosomes, thus resulting in the constitutive overexpression, and the gene was not sensitive to drugs any more.

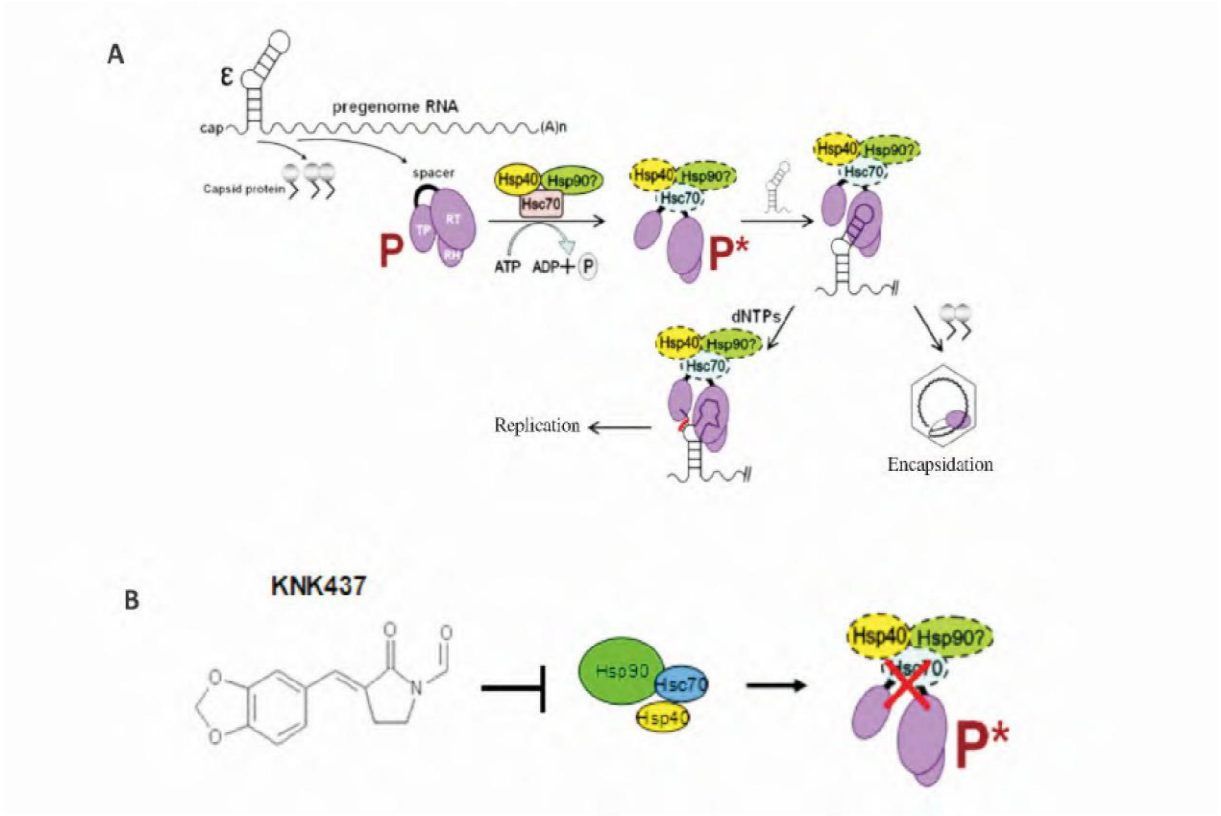

Figure 11 A: Role of Hsc70, Hsp40, and Hsp90 in formation of the P-ε complex. B: Once KNK437 enters cells, the expression and accumulation of heat-shock proteins are reduced due to down-regulation of their RNA transcription. Insufficient amounts of heat-shock proteins result in the inactivity of the RNP complex, leading to disruption of reverse transcription and suppression of assembly of nucleocapsids (modified from the literatures [3, 26]).

There are two potential mechanisms to suppress virus RNA transcription level. The first one is to direct interfere in transcriptional RNA with cccDNA as template in cell nucleus and reduce RNA transcript; the other one is to promote degradation of virus mature RNA. Because transfected intracellular 1.05 × HBV plasmid contains hCMV IE1 strong promoter, against this genetic background, KNK437 still can effectively suppress virus RNA transcription levels. We consider that KNK437 can inhibit transcription level by reducing the stability of intracellular HBV RNA, for example, promoting degradation of RNA. This conjecture needs to be further verified through experiment.

KNK437 significantly inhibits intracellular expression level of HBV core protein, which may be because core translation was influenced by the downregulation of pgRNA transcription level by drugs. Meanwhile, KNK437 inhibits Hsp70, Hsp90b, Hsp40 and other Hsps, and makes the activity of P-ε complex reduced or even lost, resulting in blockage of assembly of nucleocapsids. Virus core protein was suppressed from expression and assembly, and thus it is not difficult to understand the inhibitory effect of KNK437 on core in Figure 9. Within the scope of given concentrations, inhibition rate even exceeds that in two kinds of positive control groups.

Previous literature showed that KNK437 can inhibit Hsps transcription based at the mRNA level, and significantly reduce baculovirus DNA synthesis and production of virus particles [16]. At present, there have been reports on Hsp90 inhibitor GA and Hsp70 inhibitor OMTR inhibiting HBV replication [17–18]. These drugs inhibit wild-type HBV, and can also inhibit virus reverse transcription in strains resistant to lamivudine, adefovir dipivoxil and entecavir. Inhibitory efficiency was up to 78% [18]. In 2012, Bian et al. [30] established shRNA of targeted HBV surface protein gene and shRNA of targeted Hsc70 (siHsc70), which jointly acted on HepG2.2.15 cell lines. It was found that they could high-efficiently downregulate expression and replication levels of HBV. Hsp90 inhibitor STA-9090 has passed phase I clinical trial [31]. Results of various research groups indicated that developing Hsp inhibitors has great potential in inhibiting HBV virus replication and overcoming drug-resistant strains [32].

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