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Identification of compounds that inhibit the interaction between core and surface protein of hepatitis B virus

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Abstract

Specific interactions of the human hepatitis B virus (HBV) surface proteins with the core protein of nucleocapsid are critical for the envelopment of virus particles, and inhibition of this process may prevent the production of infectious virus. A modified enzyme-linked immunosorbent assay (ELISA), which measured the interaction between the core protein and PreS region of the surface protein, was used to screen a chemical library for compounds that would block this interaction. Few inhibitory compounds were identified from a chemical library consisting of 5600 compounds. Among them, two compounds inhibited the production of HBV particles from transiently HBV-producing HuH7 cells. The IC_{50} values of these compounds for inhibition of HBV production in HuH7 cells were in the micromolar concentration range. These results indicate that compounds that prevent the interaction between the core protein and PreS region of the surface protein may possibly be useful as anti-HBV agents.

Keywords: HBV; HBs (surface) protein; HBc (core) protein; Anti-HBV compounds

1. Introduction

Human hepatitis B virus (HBV) infects chronically over 350 million people globally, and these chronic HBV carriers are at risk of developing cirrhosis and hepatocellular carcinoma, which is responsible for more than 1 million deaths annually (Funk et al., 2002). Although HBV infection can be prevented through vaccination, HBV has still remained as one of the most significant viral pathogens. Currently, interferon-α (IFN) and nucleosidic inhibitors such as 3TC (lamivudine) and adefovir dipivoxil have been approved for the treatment of HBV (De Clercq, 2004). IFN is effective only in less than 30% of the chronic carriers (Fattovich et al., 1988; Hoofnagle and di Bisceglie, 1997), and about 50% of IFN-treated patients experience recurrence of viremia after cessation of treatment (Fattovich et al., 1988). 3TC and adefovir dipivoxil are competitive HBV reverse transcriptase inhibitors (Doong et al., 1991). Initially, 3TC and adefovir rapidly reduce viral load, followed by a slow elimination of residual virus. However, substantial resistance to 3TC during the slow phase of HBV elimination limits its long-term treatment of HBV infection (Tipples et al., 1996). Therefore, new classes of potent antiviral compounds and/or new antiviral strategies seem highly desirable. Effective anti-HBV therapy may require the use of multiple classes of drugs in combination that block the viral life cycle at several molecular targets (Deres et al., 2003).

HBV contains a 3.2 kb partially double-stranded DNA encoding the viral envelope, core, polymerase, and X proteins (De Clercq, 1999). Core and envelope proteins mainly constitute the nucleocapsid and viral envelope, respectively. The viral nucleocapsid consists of 90–120 homodimers of the core protein (HBcAg) and forms an icosahedral shell that harbors the viral DNA and polymerase (Crowther et al., 1994). The envelope, which surrounds the nucleocapsid, contains three surface proteins (long (L), medium (M), and short (S)), which are translated from one open reading frame (Bruss and Ganem, 1991; Ueda et al., 1991). The L protein consists of PreS1, PreS2, and S regions, whereas the M protein consists of PreS2 and S regions. The L protein has two transmembrane topologies as its PreS region exposed to both the cytosolic and luminal side of endoplasmic reticulum (Bruss et al., 1994; Prange and Streeck, 1995). The

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assembly of viral particles starts at the endoplasmic reticulum, where the newly formed nucleocapsids in the cytosol interact with the envelope proteins in the membrane of the endoplasmic reticulum (Loffler-Mary et al., 2000). The enveloped virions are then transported to the Golgi apparatus and exported from the cell through the secretary pathway (Huovila et al., 1992).

Different stages of the HBV life cycle other then the viral polymerization had been investigated as potential targets of antiviral agents. The assembly of core protein to form nucleocapsid or the envelopment of nucleocapsid to form mature virus particle have been extensively examined. Expression of dominant negative core protein mutants that could not support pregenomic RNA packaging and genome maturation effectively inhibit replication hepatitis virus (Scaglioni et al., 1996; von Weizsacker et al., 1999). Polypeptides that could inhibit the assembly of core proteins were also examined for their potential application as anti-HBV agents. Intracellular single chain antibodies against HBV core protein inhibit viral replication (Yamamoto et al., 1999). Also, peptides selected for their affinity to HBV core protein could block the interaction between the core and surface protein (Bottcher et al., 1998; Dyson and Murray, 1995) or inhibit the assembly of core protein and HBV replication (Butz et al., 2001). These observations indicate that the assembly process of core protein could serve as a target for anti-HBV agents.

Once core proteins are assembled into nucleocapsids, they should interact with the envelope proteins of HBV in endoplasmic reticulum membranes to form mature enveloped virus particles. Among the three HBV surface proteins, L and S proteins are indispensable for virus formation (Bruss and Ganem, 1991). Subsequent studies showed that the border region of PreS1 and PreS2 (Bruss, 1997) and the cytoplasmic loop region of S protein interact with core particles (Poisson et al., 1997). The specific interaction between the PreS region of L protein and the core protein has been measured quantitatively using purified recombinant proteins (Choi et al., 2004). The requirement of a specific interaction between the HBV surface and core proteins for production of infectious virus suggested that the inhibition of this interaction could serve as a novel anti-HBV strategy (Yamamoto et al., 1999).

Here, we first report the identification of small molecule inhibitors against the interaction between the L protein PreS region and the core protein of HBV. The identified compounds were further found to prevent the production of HBV particles by depleting the production of mature HBV virus secreted from cells transfected with the HBV genomic DNA.

2. Materials and methods

2.1. Preparation of Trx-PreS and HBcAg proteins

The plasmids pTrx-PreS and pHBcAg were used for the expression of a His_6-thioredoxin fused with the PreS region of L protein at C-terminus (Trx-PreS) and HBV core protein (HBcAg) in *Escherichia coli* (Fig. 1). Construction of pTrx-PreS and pHBcAg has been described elsewhere (Choi et al., 2004). The PreS region of Trx-PreS represents amino acids 1–163,

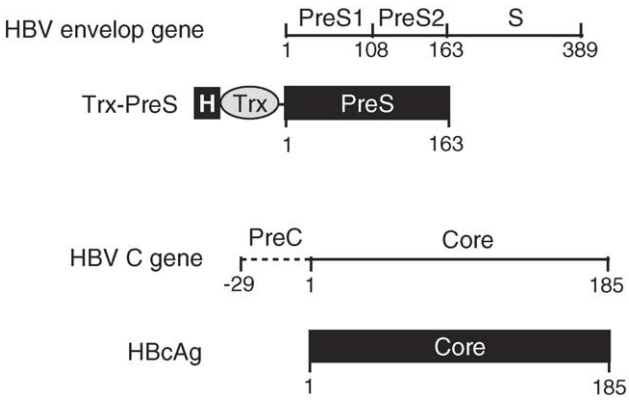

Fig. 1. Schematic representation of Trx-PreS and HBcAg proteins. Thioredoxin domain and histidine (H)-tag in Trx-PreS are indicated in gray circle and black box with bold letter **H**, respectively. HBcAg represents the core protein of HBV, consisting of 185 amino acids, without the preC region indicated as broken line.

which is identical to the previously reported sequence (GenBank accession number BAB17299), except for the Thr87 → Ala and Ser177 → Asn substitutions. HBcAg represents amino acids 1–185 which is identical to the previously reported core protein (GenBank accession number CAA96554). Trx-PreS was purified by Ni^{2+} charged His tag affinity and anion exchange chromatography, and HBcAg protein was purified by ultracentrifugation and gel filtration chromatography as described previously (Choi et al., 2004).

2.2. Measurement of the interaction between the PreS and core protein of HBV

The interaction between the PreS region and core protein of HBV was measured as described previously (Choi et al., 2004) with a small modification. Briefly, 100 μl of Trx-PreS (10 μg/ml) in 50 mM sodium phosphate, pH 8.0 was incubated in 96-well polystyrene plates (Costar-3591, Corning, NY, USA) overnight at 4 °C. After blocking the plate surface with 2% bovine serum albumin (Sigma, USA) (w/v) in PBS solution (50 mM sodium phosphate, 0.15 M NaCl, pH 7.4), 100 μl of HBcAg in PBS solution (0.2 μM) was applied, and the amount of HBcAg bound on the plate was measured using anti-HBcAb (Koma Biotech, Korea) and HRP-conjugated anti-rabbit IgG (Sigma). For the measurement of inhibitory activity, the test compounds (40 μM) in 50 μl were incubated to the Trx-PreS immobilized plated for 20 min before addition of HBcAg (0.4 μM) in 50 μl. A chemical library consisting of 5600 in-house synthetic compounds at Korea Institute of Science and Technology (KIST) was used for the screening of potential inhibitors against the interaction between PreS domain and core protein.

2.3. Transfection and immuno-capture PCR (IC-PCR) quantification of HBV DNA

HuH7 cells and HBV genomic DNA (adw R9) were kindly provided by Prof. Byung-Yoon Ahn (Korea University, School of Life Science and Biotechnology). All cell cultures were main-

tained in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 50 units of penicillin and streptomycin per ml, and 1 mM glutamine (Invitrogen). The cells were plated in 100-mm dishes and incubated at 90% confluence for 24 h. Four micrograms of HBV genomic DNA (adw R9) (Blum et al., 1991) was used for transfection into HuH7 cells with lipofectamine (Invitrogen), as described in the instruction protocol. Cells were harvested 24 h after transfection and seeded in 6-well cell culture plate. Twenty micromolars of anti-HBV compounds were added to the cell culture at the third day of transfection, and the cells were grown for more than 10 days.

The amount of HBV particles in culture medium was analyzed by immuno-capturing of HBV particles and PCR with HBV specific primers. The supernatants of the culture media (200 μl) after 8 days of transfection were transferred to a 96-well polystyrene plate (Costar, Corning, NY, USA) coated with 10 μg/ml rabbit anti-HBsAg antibodies (Sigma). After washing the plate six times with PBS-T (PBS with 0.5% Triton X-100), 25 μl/well of 0.2 M NaOH was incubated for 1 h at 37 °C to disrupt the bound HBV particles and denature the released viral DNA. The solution was neutralized by adding 40 μl of 0.2 M Tris–HCl pH 7.5, and 5 μl aliquots were used for PCR with primers Pol-SN (5′-GGATCCATGGGGACGAATCTTT CTGTTCCC-3′) and Pol-SC (5′-GGCGGTCGACT CAGG TTGGGGACTGCGAATTTTG-3′), which were specific to the coding sequence of HBV DNA polymerase. The amount of PCR product was measured either by DNA band intensity in agarose gel or by dot blot analysis. For dot blot analysis, 10 μl of PCR product was applied on Hybond$^+$ Nylon membrane (Amersham, Sweden), and visualized using enzyme-linked chemiluminescence (ECL) method (Amersham).

2.4. Cytotoxicity analysis

Cytotoxicity of chemical compounds was determined by mitochondrial toxicity testing (MTT) of cells (Carmichael et al., 1987) in 96-well cell culture plates. HuH7 cells were treated with the test compounds for 3 days, and the media were replaced with 100 μl of tetrazolium bromide (Sigma) (10 mg/ml) for 1 h at 37 °C. The supernatant was removed and placed in a 96-well plate, and the absorbance at 590 nm was measured using a Spectra Max-340 96-well plate spectrophotometer (Molecular Devices Corp).

3. Results

3.1. Screening of inhibitory compounds against the interaction between Trx-PreS and HBcAg

Previously, the specific interaction between PreS region of surface protein and core protein of HBV was measured by an enzyme-linked assay method using recombinant proteins, Trx-PreS and HBcAg (Choi et al., 2004). A modified enzyme-linked assay method using 96-well plates was used to screen potential inhibitory compounds against the interaction between Trx-PreS and HBcAg PreS. A chemical library from KIST was tested for primary inhibitory assessment. Several compounds that could inhibit more than 50% of the binding of HBcAg to immobilized Trx-PreS at 40 μM were identified from primary screening. The IC_{50} values of the primary isolates were further measured, and nine compounds with IC_{50} less than 6 μM were identified (Fig. 2A). Among them, four compounds (KSW10040, KYS00018, KKJ00626 and KYS00210) showed IC_{50} values less than 1 μM. When the cytotoxicity of these nine compounds was tested in the MTT assay, five compounds (KSW10040, KJH01C17, KSG00011, KYS00018 and KKJ00626) showed no detectable cytotoxic activity within the tested concentration range (Fig. 2B). From the primary screening and subsequent cytotoxicity assay, five compounds (KSW10040, KJH01C17, KSG00011, KYS00018, KKJ00626) were further evaluated for their inhibitory activity against HBV amplification in cells transfected with HBV DNA.

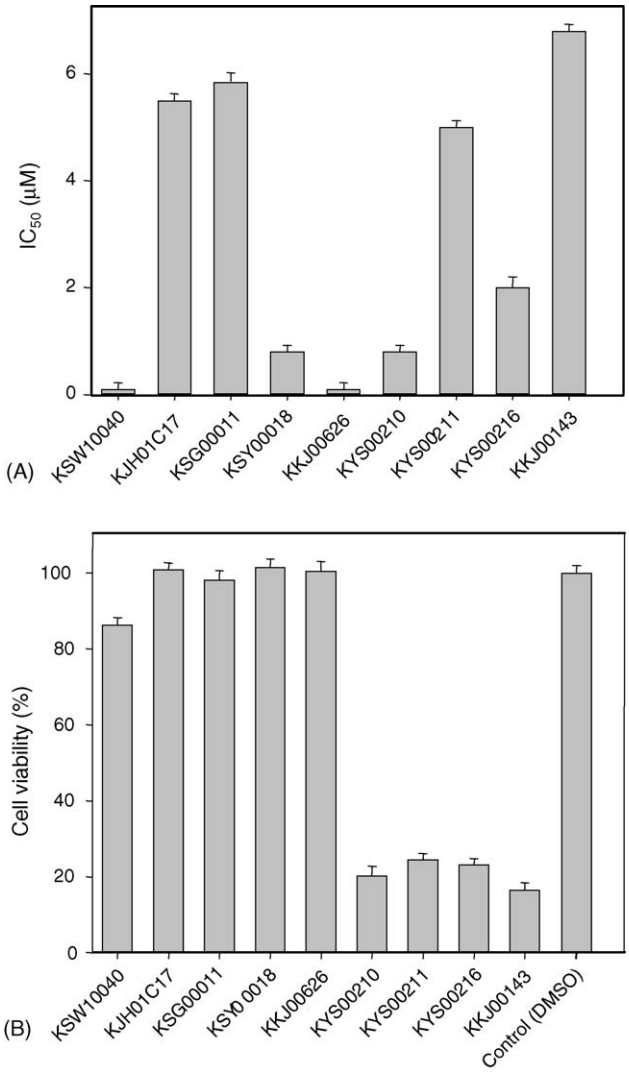

Fig. 2. (A) IC_{50} values of the compounds selected from primary screening with the ELISA method. (B) Cytotoxicity of selected compounds. The viability (%) of cells treated with various compounds (50 μM) was measured using the MTT assay. The positive control (100%) was obtained from cells treated with 0.5% DMSO.

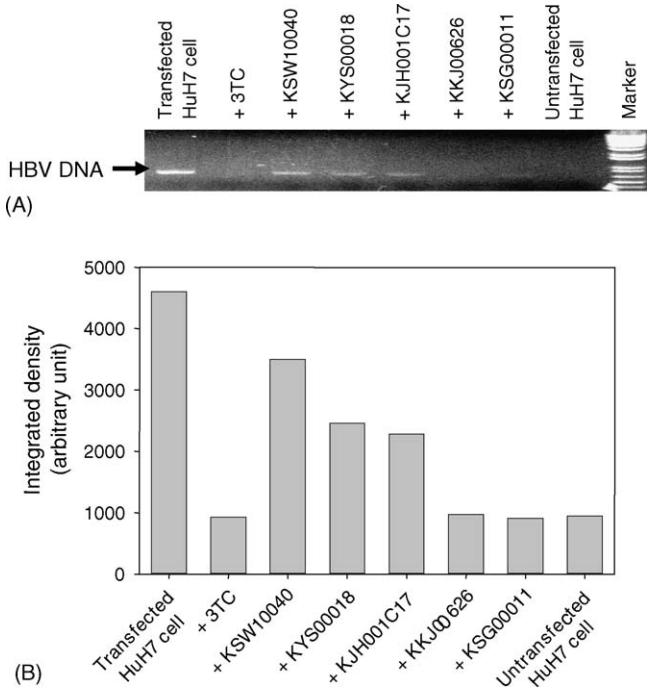

Fig. 3. Detection of HBV DNA in the culture medium of infected HuH7 cells in the presence of the compounds. (A) HBV DNA of the culture medium from cells treated with compounds was measured by IC-PCR. (B) The intensity of PCR bands was quantified by densitometry and presented as histograms. The concentration of 3TC and the test compounds in the culture medium was 100 and 20 μM, respectively.

3.2. Inhibition of HBV production by selected compounds

Selected compounds from the primary screening were examined to see whether they could prevent the production of virus particles. The level of secreted mature HBV particles from the HuH7 cells transfected with HBV genomic DNA was quantitatively determined by measuring the amount of viral DNA in the culture medium after immuno-capture with anti-HBsAg antibodies using PCR and dot-blot. In this assay method, the enveloped virus particles were captured by anti-HBsAg, and

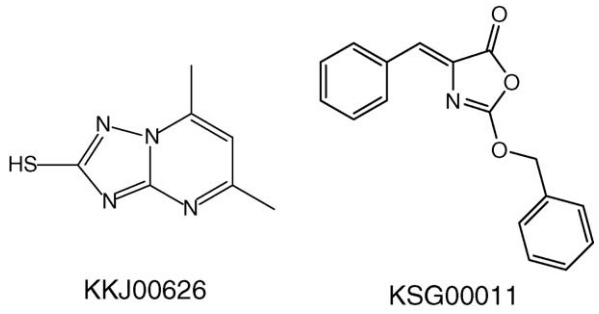

Fig. 4. Structure of KKJ00626 and KSG 00011.

then the level of viral DNA in the captured fraction was measured by PCR with primers specific to HBV polymerase. Hence, only the level of HBV particles in mature form could be measured. In the presence of 3TC, the amount of viral DNA in the culture medium was significantly reduced (Fig. 3) indicating that 3TC effectively inhibited replication of HBV. When the five compounds from the primary screening were tested for their inhibitory activities, KKJ00626 and KSG00011 as effectively reduced the level of secreted HBV particles as did 3TC (Fig. 3). Other compounds such as KSW10040, KYS00018, and KJH01C17, however, showed lower inhibitory activities. KKJ00626 is a triazolopyrimidine derivative, and KSG00011 is an oxazolidine with two benzyl groups (Fig. 4). The two selected compounds have no distinct common structural moiety suggesting that these compounds may differ in their mode of inhibition.

The inhibitory activities of KKJ00626 and KSG00011 were further examined at different concentrations, and the intensities of hybridized dot blot were quantified by densitometry. The IC_{50} values of KKJ00626 and KSG00011 from in cell based immunocapture assays were calculated as 0.23 and 4.6 μM, respectively (Fig. 5). In comparison, the IC_{50} values of KKJ00626 and KSG00011 from the modified enzyme-linked assay method were measured as 0.12 and 5.4 μM, respectively (Fig. 2A). These results indicate that KKJ00626 is about 20–40-fold more effective than KSG00011 in interfering with the interaction between HBcAg and PreS domain of L protein.

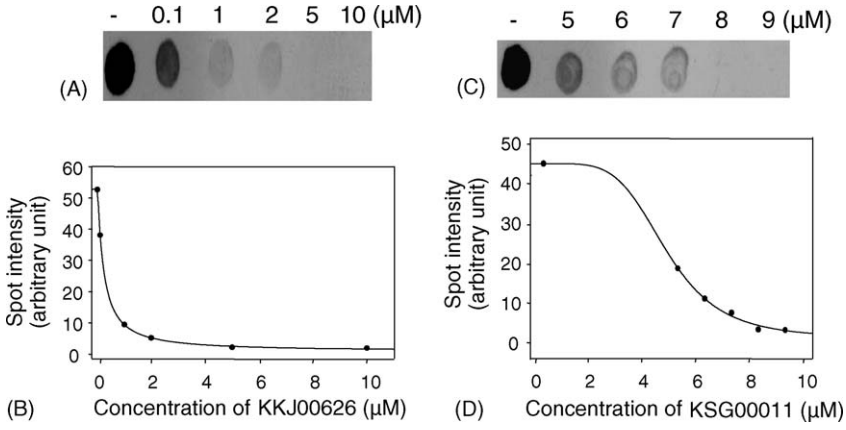

Fig. 5. Concentration-dependent inhibition of HBV production by KKJ00626 and KSG00011 in transfected HuH7 cells. Virion-associated HBV DNA from transiently transfected HuH7 cells was amplified by IC-PCR and quantified by dot blot hybridization. Transfected cells were left untreated (control) or treated with 0.1, 1, 2, 5, and 10 μM of KKJ00626 (A) or 5, 6, 7, 8, and 9 μM of KSG00011 (C) for 8 days. The intensities of dot blots were plotted against the concentrations of KKJ00626 (B) or KSG00011 (D) and the IC_{50} values were determined.

4. Discussion

Alkylated imino sugars have been found to reduce the production of HBV by disrupting the maturation of HBV nucleocapsids (Lu et al., 2003; Mehta et al., 2002). Bis-ANS that can bind to hydrophobic sites of proteins binds to the core protein of HBV and inhibits the assembly of nucleocapsid (Zlotnick et al., 2002). Heteroaryldihydropyrimidines, which were discovered as potent inhibitors of HBV replication (Weber et al., 2002), bind to HBV core protein and inhibit the assembly of nucleocapsids (Deres et al., 2003).

In this study, we have examined whether the interaction between the core and surface proteins of HBV can be exploited as a novel target site for small-molecular weight inhibitors. Specific interactions between the outer surface of core and the inner surface of envelope proteins are likely to guide the correct assembly of the virus and stabilize the resulting particle. Specifically, a boundary region between the PreS1 and PreS2 was critical for the envelopment of capsids via the interaction with HBV core protein (Bruss, 1997), and the amino acid residues of core protein around the base of spike and the area close to the pore of nucleocapsid structure were involved in the envelopment process (Ponsel and Bruss, 2003). Direct measurement of the specific interaction between the core protein and PreS region of L protein (Choi et al., 2004) further suggests that the interaction site of the two HBV proteins could be used for screening of inhibitory agents of HBV production.

A modified assay method measuring the interaction between Trx-PreS and HBcAg was applied to screen small molecules that can prevent the specific interaction between two proteins. A small size chemical library could be screened using this method within couple of weeks without high throughput screening facilities. A cell-based assay method that could detect the level of enveloped virion in culture medium by immunocapture PCR (Lu et al., 2001) was also applied to confirm the inhibitory activity of isolated compounds against the production of HBV particles. Among the primary hits from the screening of the chemical library, KKJ00626 and KSG00011 show potent antiviral activity in cells transfected with HBV genomic DNA at non-cytotoxic concentrations. Both compounds effectively reduced the yield of HBV in transfected HuH7 cell cultures in a dose-dependent manner. The IC_{50} values of KKJ00626 and KSG00011 in a modified enzyme-linked assay were similar to the values obtained in a cell based assay system.

5. Conclusions

In this report, we have explored the specific interaction between core and surface proteins that is critical for production of mature HBV particles, to screen anti-HBV agents. A few compounds identified from the screening of chemical library using this method were confirmed to have inhibitory activity against HBV production. The screening method and the identified compounds might be useful in extending the range of potential antiviral therapeutic agents.

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