Antiviral Research 89 (2011) 109–114

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Artificial recombinant cell-penetrating peptides interfere with envelopment of hepatitis B virus nucleocapsid and viral production

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ARTICLE INFO

Article history:
Received 6 September 2010
Received in revised form
19 November 2010
Accepted 1 December 2010

Keywords:
Hepatitis B virus
Nucleocapsid
Cell penetrating peptide
Assembly
Antivirus

ABSTRACT

Hepatitis B virus (HBV) is a major human infectious pathogen, with over 300 million chronically infected patients worldwide. Current therapeutics for chronic HBV infection have shown only limited success. The plasma membrane represents an impermeable barrier for development of most macromolecular antiviral agents. To develop new anti-HBV macromolecules that can cross the membrane barrier, we designed a series of artificial recombinant peptides including cell penetrating sequence oligoarginine R7 and several nucleocapsid binding subunits (NBS). The anti-HBV function of these peptides was evaluated in a HBV DNA replicative cell line HepG2.2.15. Our results showed that the synthetic recombinant cell penetrating peptides retained the activity of cell penetrating in the living cells. HBV DNA in culture medium markedly decreased in cells treated with cell penetrating peptides bearing NBS for three days. Intracellular HBcAg and HBV DNA replicative intermediates increased by 2–3 fold. In conclusion, the synthetic recombinant cell penetrating peptides bearing NBS can efficiently enter into the cells; block nucleocapsid assembly and inhibit HBV release. Cell penetrating subunit presents a high efficiency tool to deliver synthetic antiviral peptides into cells.

1. Introduction

Hepatitis B virus (HBV) is a major human infectious pathogen, with over 300 million chronically infected individuals worldwide. Chronic hepatitis associated with HBV infection often leads to the development of cirrhosis, liver failure, and highly malignant liver cancer (Liaw and Chu, 2009; Te and Jensen, 2010; Yang et al., 2002). Current therapeutics for chronic HBV infections such as interferon alpha and nucleoside analogs have shown only limited success, which emphasizes the need for new therapeutic strategies (Zoulim and Locarnini, 2009; Mohanty et al., 2006).

Classified in the *Hepadnaviridae* family, HBV is a small, enveloped DNA virus with a genome size of 3.2 kb. In the HBV life cycle, the virus enters cells by a receptor-mediated process and endocytosis. After uncoating of the capsid and nuclear transport, nuclear DNA repair enzymes complete the plus and minus strands of the open circular genomic DNA, generating a covalently closed circular DNA (cccDNA) molecule that provides the template for the synthesis of viral mRNAs and the pregenomic RNA (pgRNA). pgRNA is packaged together with the viral polymerase protein into immature nucleocapsids and then reverse transcribed by polymerase protein in the lumen of the particle and finally converted to double-stranded DNA. The DNA-containing nucleocapsid can be enveloped by the HBV surface proteins (HBsAg), generating virions termed 'Dane particles' (Gerelsaikhan et al., 1996; Seeger and Mason, 2000). A few of the nucleocapsids may not assemble with HBsAg but instead return to the nucleus, where they amplify the pool of cccDNA (Kann et al., 1999; Ning and Shih, 2004). cccDNA amplification plays a key role to maintain the size of cccDNA pool, viral persistence and resistance to antiviral therapy (Sung et al., 2005).

Theoretically, compounds that interfere with any step in the HBV life cycle are likely to reduce the production of HBV. Recently, many small molecules such as nucleoside and nucleotide analogs (like lamivudine, adefovir, entecavir, telbivudine and tenofovir) that enter cells and inhibit HBV production by targeting the viral DNA polymerase are already being used clinically to treat HBV infection (Reynaud et al., 2009; Quan and Peters, 2004). Most recently, some nonnucleoside small molecules, which inhibit HBV replication by interfering with nucleocapsid assembly or encapsidation, were also reported (Deres et al., 2003; Feld et al., 2007; King et al., 1998; Stray and Zlotnick, 2006). However, the cell plasma membrane represents an impermeable barrier for most

Abbreviations: HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; pgRNA, pregenomic RNA; HPLC, high-performance liquid chromatography; NBS, nucleocapsid binding subunits; HBsAg, hepatitis B surface antigen; R7, RRRRRRR; FQPCR, fluorescence quantitative PCR.

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doi:10.1016/j.antiviral.2010.12.001

Table 1
The sequence of the peptides and the functional characterization.

Peptide	Sequence	EC50 (μM)	EC90 (μM)	GI50 (μM)	LC50 (μM)
P1	RRRRRRR	n.i	n.i.	n.i.	n.i.
P2	RRRRRRR Acp PTSNHSPTSCPPTCPGYRWMCLRRF	12.8 ± 2.0	85.6 ± 9.5	135.4 ± 22.3	457.9 ± 41.0
P3	RRRRRRR Acp GSLLGRMKGA	2.5 ± 1.0	8.6 ± 3.2	287.5 ± 27.1	828.7 ± 50.3
P4	RRRRRRR Acp LDPAFR	3.0 ± 1.0	10.9 ± 3.4	556.8 ± 43.0	n.i.
P5	RRRRRRR Acp PLSPPLRNTHPQAMQWNSTTF	6.5 ± 1.5	41.4 ± 8.7	152.2 ± 24.1	515.9 ± 43.8

Note: The EC50, GI50 and LC50 values are means ± SD of at least three independent determinations.
EC50: concentration of the peptide reducing the production of extracellular HBV DNA to 50% of controls.
GI50: concentration of the peptide resulting in inhibition of cell growth to 50% of controls.
LC50: concentration of the peptide required to reduce the initial cell number by 50%.
n.i.: no effect was observed at 1mM of peptide concentration.

macromolecules and thus greatly limits the utility of new antiviral macromolecules. In order to overcome this problem, several methods of carrier-mediated delivery systems have been developed (Malik et al., 2007). Among them, much attention has recently been given to the use of cell-penetrating peptides. These short cationic peptides, either by covalent binding or by noncovalent binding, can traverse cell membranes and deliver a variety of molecules that are otherwise unable to transit the cell membrane in their own capacity (Foged and Nielsen, 2008; Hällbrink et al., 2001). Oligoarginine was derived from the human immunodeficiency virus-1 Tat and had been reported to have the ability to bring exogenous proteins into the cells (Herce et al., 2009; Maiolo et al., 2005). Here, based on the cell-penetrating peptide oligoarginine, we introduced a series of synthetic macromolecular peptides into hepatocytes to inhibit HBV production.

2. Materials and methods

2.1. Peptide design and synthesis

The cell-penetrating peptides of human immunodeficiency virus (HIV)-1 Tat (13aa), Drosophila Antennapedia (Antp, 16aa) and oligoarginine (7-9aa) are widely used to deliver cargo into living cells. Considering that the short peptide is easier to be synthesized and the short cell-penetrating sequence has less chance to interfere with other sub-domain, we selected oligoarginine R7 as a cell-penetrating sequence.

HBV nucleocapsid is made up of 180 or 240 subunits of core proteins, which comprises 183 amino acids with a molecular mass of about 22 kDa. It consists of a dimer of two HBcAg subunits that are linked by two intermolecular disulfide bonds. Each dimer consists of a protruding spike that sticks out from the underlying shell domain. Viral envelopment and secretion depends on specific interactions between the outer surface of the nucleocapsid and the inner surface of the envelope. Previously, several peptides have been demonstrated to interfere with the interaction in a cell-free system. Böttcher et al. showed that small peptides containing the core motif sequence GSLLGRMKGA, which bind at the tips of the core particles, block interaction with L-HBsAg *in vitro* (Dyson and Murray, 1995; Böttcher et al., 1998). Poisson et al. found that the synthetic peptide PTSNHSPTSCPPTCPGYRWMCLRRF, which is derived from residues 56 to 80 in the cytosolic loop of S protein, and PLSPPLRNTHPQAMQWNSTTF which is derived from 13 C-terminal amino acids of pre-S1 plus 8 N-terminal amino acids of PreS2 domain bound efficiently to the purify HBV core in a binding affinity assay (Poisson et al., 1997). Sequence LDPAFR lying between residues 30 and 35 of the pre-S1 region is the epitope for monoclonal antibody MA18/7 (Heermann et al., 1984, Poisson et al., 1997). However, whether these peptides interact with nucleocapsid and inhibit virus secretion in living cells still remains unclear.

Hence, we synthesized a series of recombinant cell-penetrating peptides including a cell-penetrating sequence R7 subunit and a presumptive nucleocapsid binding subunit (NBS). The sequences are shown in Table 1. A flexible linker epsilon-aminocaproic acid residue (Acp) was used to link R7 and NBS. The flexibility of Acp may reduce the potential structural interference between the R7 and NBS and keep individual subunit function (Karle et al., 1997). The recombined peptides were synthesized using a solid-phase fluorenylmethoxycarbonyl chemistry strategy (Shanghai Bioengineering Ltd, China). N terminal FITC-labeled peptides were synthesized to evaluate the penetrating efficiency of the peptides. Productions were isolated by lyophilization and characterized by analytical high-performance liquid chromatography (HPLC) and MALDI-TOF mass spectrometry. The purity of the peptide powders was >95% as determined by analytical HPLC. The peptides were stored at −80 °C and dissolved in pure water for a fresh solution at concentration of 2 mM before using.

2.2. Cell culture

The HBV-DNA integrated hepatoma cell line HepG2.2.15 which produces infectious viral particles, was maintained in complete DMEM (Gibco-BRL, CA) containing 10% FBS (Hyclone, Thermo Fisher, PA), 100 units/ml penicillin, 100 mg/ml streptomycin, and 380 μg/ml G418 antibiotic (Sigma, MO). Cells were cultured at 37 °C in an atmosphere of 5% CO_2–95% air.

2.3. Cell viability test for determination of cytotoxicity of peptides

Cellular growth in the presence of different concentration of peptides (≤1 mM) for 3 days and the medium was completely replaced each day. Cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based Cell Titer 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the instruction of the Manufacturer. Trypan blue exclusion assay was used to determine the number of live/dead cells in HepG2.2.15 cultures exposed to peptides.

2.4. Detections of viral proteins and HBV DNA in culture medium

A single HepG2.2.15 cell (2×10^5/well) suspension was seeded in 6-well plates. Cells were respectively treated with the peptides at concentrations of 0 μM, 3.0 μM, 10 μM, 30 μM and 100 μM for 3 days and the medium was completely replaced each day. HBV DNA was quantified using a commercially available real-time fluorescence quantitative PCR kit (FQ-PCR, Pi-Ji, Shenzhen, China) and medium were pretreated as previous report (Pan et al., 2008). FQ-PCR was run on a LightCycler instrument (Roche, Mannheim, Germany). HBsAg in culture medium was detected using of electrochemical illuminescent immunoassay kits (Abbott Labs, IL) on an ARCHITECT i2000 automatic immunoassay analyzers (Abbott).