Irbesartan, an FDA approved drug for hypertension and diabetic nephropathy, is a potent inhibitor for hepatitis B virus entry by disturbing Na\(^+\)-dependent taurocholate cotransporting polypeptide activity

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The liver-specific Na\(^+\)-dependent taurocholate cotransporting polypeptide (NTCP) was recently identified as an entry receptor for hepatitis B virus (HBV) hepatotropic infection. In this study, an NTCP-overexpressing HepG2 cell line named HepG2.N9 susceptible to HBV infection was established using transcription activator-like effector nucleases (TALEN) technology. Using this cell line, irbesartan, the new NTCP-interfering molecule reported recently, was demonstrated here to effectively inhibit HBV infection with an IC\(_{50}\) of 3.3 \(\mu\)M for hepatitis B e antigen (HBeAg) expression and exhibited no obvious cytotoxicity up to 1000 \(\mu\)M. Irbesartan suppressed HBV uptake weakly but inhibited HBV covalently closed circular DNA (cccDNA) formation efficiently at physiological temperature. These results suggested that irbesartan targeted HBV infection at a post-uptake prior to cccDNA formation step such as the cell membrane fusion. Based on these findings, irbesartan, an FDA approved drug for hypertension and diabetic nephropathy, could be a potential candidate for treatment of HBV infection although further in vivo experiments are required.

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1. Introduction

Despite the availability of an effective vaccine, two formulations of interferon and several nucleos(t)ide analogue inhibitors of hepatitis B virus (HBV) reverse transcriptase against it, HBV infection remains serious public health problem worldwide (Dandri and Locarnini, 2012). This is partially due to the HBV non-proofreading reverse transcriptase and its long half-life covalently closed circular DNA (cccDNA) duration (Nowak et al., 1996). Furthermore, vaccine escape and nucleos(t)ide resistant HBV strains have evolved and present a serious problem that needs to be addressed for future HBV prevention and treatment (Bian et al., 2013; Zoulim and Locarnini, 2009). Therefore, the design and development of new anti-HBV strategies and drugs interfering with different stages in the HBV life cycle are urgently needed.

Because viruses utilize host molecules to help themselves for infection and replication, host-targeted inhibitors have been shown to be a viable strategy for antiviral therapy including targeting host molecules involved in viral entry into cells (Esté and Telenti, 2007) or other steps in the viral lifecycle (Ng et al., 2005). HBV is a hepatotropic virus but currently only one cell line, HepaRG, is susceptible to infection by HBV. This cell line also requires DMSO-induced differentiation for 2 weeks before HBV infection (Gripon et al., 2002).

Recently, the liver-specific Na\(^+\)-dependent taurocholate cotransporting polypeptide (NTCP) was reported to be a functional receptor for human hepatitis B and D virus infections (Yan et al., 2012), and this discovery was validated by other three groups (Iwamoto et al., 2014; Li et al., 2014; Ni et al., 2014) very recently. NTCP mediates bile acid transport and is exclusively located on the basolateral membrane of hepatocytes, making it ideally for HBV infection. The discovery of NTCP as an HBV receptor would promote HBV entry, cccDNA formation analysis and new anti-HBV agents identification. Testing whether known or newly discovered NTCP inhibitors (Dong et al., 2013; Greupink et al., 2012; McRae et al., 2006) also interfere with HBV infection may provide new insights into the treatment of HBV.
In this study, we firstly established an NTCP-overexpressing HepG2 cell line susceptible for HBV infection using TALEN technology (Sanjana et al., 2012). Then we tested several FDA approved drugs that have inhibitory activity on taurocholate (TC) uptake by NTCP (Dong et al., 2013) for their direct effects on HBV entry. Among them, irbesartan was demonstrated to inhibit HBV infection efficiently with no obvious cytotoxicity up to 100 μM.

2. Materials and methods

2.1. Reagents and cells

Plasmids AAVS1-SA-2A-puro-pA (Hockemeyer et al., 2009), hAAVS1-1L-TALEN, hAAVS1-1R-TALEN (Sanjana et al., 2012), gRNA-AAVS1-T2 and hCAS9 (Mali et al., 2013) were purchased from Addgene (Cambridge, U.S.A.). The NTCP donor plasmid AAVS1-mCBA-NTCP was constructed in our laboratory based on the AAVS1-SA-2A-puro-pA vector. Cyclosporin A, simvastatin, ritonavir, doxazosin mesylate, irbesartan, ezetimibe, bendroflumethiazide and losartan potassium were purchased from Sigma (St. Louis, U.S.A.) and Selleck Chemicals (Houston, U.S.A.). The m47F peptide (myr-GTNLSVPNLGFHDPQDLPAFGANSNNPNWDIFPNK DHWEPEANQV-K-FITC) derived from the first 47 amino acids of HBV pre-S1 domain with N-terminal myristoylation and C-terminal fluorescein isothiocyanate (FITC) conjugation was synthesized and purified (>95% pure) by the LifeTein Company (Beijing, China). Rabbit anti-hepatitis B core antigen (HBcAg) antibody was obtained from DAKO ( Carpinteria, U.S.A.). Williams E medium, anti-rabbit-lgG conjugated with Alexa Fluor 594 and SlowFade™ Gold with DAPI. The pictures were captured with FV1000 (Olympus, Tokyo, Japan). HepG2 cells were obtained from American Type Culture Collection (ATCC, Manassas, U.S.A.).

2.2. Establishment of HepG2 cell lines persistently expressing NTCP

HepG2 cells were cultured with Modified Eagle’s Medium (MEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, U.S.A.), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO₂ in a humidified incubator except otherwise indicated. Plasmid combinations of AAVS1-mCBA-NTCP/hCAS9/gRNA-AAVS1-T2 or AAVS1-mCBA-NTCP/hAAVS1-1L-TALEN/hAAVS1-1R-TALEN were co-transfected into HepG2 cells respectively and selected using 2 μg/mL puromycin. Individual clones maintained for more than 6 months were used in the experiments described below.

2.3. Analysis of peptide m47F binding to HepG2.N9 cells

For confocal microscopy analysis, cells were incubated with 400 nM m47F at 37 °C for 1 h, washed with PBS for 3 times, and then stained with SlowFade™ Gold with DAPI. The pictures were captured with FV1000 (Olympus, Tokyo, Japan). For fluorescence-activated cell sorting (FACS) analysis, cells stained with m47F were detached with 0.5 mM EDTA/PBS, washed, resuspended in PBS and analyzed by BD FACSCalibur™ platform.

2.4. HBV production, purification and infection

Ad38 cells were used to produce HBV particles (genotype D). The cell media were collected on days 4, 6, 8, 10 and 12 after subculture and concentrated using PEG8000 as previously reported (Sureau et al., 2003). Serum from a patient positive for HBV infection (genotype C) with viral titer of 2 × 10⁶ IU/mL was also used in some of the experiments after obtaining written informed consent from the patient.

HBV infection was performed as follows: cells were cultured in 24-well plates with Williams E medium containing 2% DMSO, 18 μg/mL hydrocortisone, 40 ng/mL dexamethasone, 10 ng/mL epidermal growth factor (EGF), 5 μg/mL transferrin, 3 μg/mL insulin, 2 μM L-glutamine, 5 ng/mL sodium selenite, 100 U/mL penicillin and 100 μg/mL streptomycin for 48 h. Then the cells were inoculated with HBV at a multiplicity of genome equivalents (MGE) of 200 in the presence of 4% PEG8000 at 37 °C for 16 h. Cell medium was changed every 2 days.

In the infection inhibition assay, the tested agents were added 2 h before and 16 h during infection or at other time points as indicated. For HBV pretreatment assay, HBV particles pretreated with irbesartan for 16 h at 37 °C were concentrated via ultrafiltration and quantitated using real-time PCR, and the infectivity was evaluated using HepG2.N9 cells as described above. For HBV binding analysis, HepG2.N9 cells were treated with drugs 2 h before viral infection and 3 h during virus particles incubation at 4 °C in the presence of 4% PEG8000, followed by extensive wash with cold PBS for 5 times. For HBV uptake assay, HepG2.N9 cells were treated with drugs 2 h before viral infection and 16 h during HBV infection at 37 °C in the presence of 4% PEG8000, followed by extensive wash and trypsinization. For HBV cccDNA assay, HepG2.N9 cells were treated with drugs 2 h before viral infection and 16 h during HBV infection at 37 °C in the presence of 4% PEG8000, followed by extensive wash, and HepG2 cccDNA was detected at 3 days post-infection as illustrated before (Lucifora et al., 2013; Yan et al., 2012).

2.5. Enzyme-linked immunosorbent assay (ELISA) for HBcAg and hepatitis B surface antigen (HBsAg)

HBcAg and HBsAg were detected using 50 μL supernatants with commercial ELISA Kit (Kehua, Shanghai, China) following manufacturer’s instructions. ELISA results were presented as S/CO ratio (S = sample ratio and CO = Cutoff ratio).

2.6. Indirect immunofluorescence analysis of HBcAg

For cellular HBcAg detection, indirect immunofluorescence analysis was performed. Briefly, after fixation with 4% paraformaldehyde and permeabilization with 0.5% Triton X-100, the HepG2.N9 cells were firstly stained with anti-HBc antibody (1:750 dilution), and subsequently incubated with the second antibody with Alexa Fluor 594 (1:1000 dilution), washed with PBS 3 times and then stained with SlowFade™ Gold with DAPI. The pictures were captured with FV1000 (Olympus, Tokyo, Japan).

2.7. Real-time PCR

Total RNA from cells was isolated using Trizol reagent (Invitrogen, Carlsbad, U.S.A.) and reverse transcribed into cDNA by 2.0 First Strand cDNA Synthesis Kit (NEB Ipswich, U.S.A.). 5 μL cDNA product was used as template for real-time PCR analysis using SYBR® Premix Ex Taq™ II kit (Takara, Otsu, Japan) on an ABI 7500 real-time system instrument (Life Technologies, Carlsbad, U.S.A.). The primers used for NTCP, HBV RNA and GAPDH amplification were as follows: NTCP-F (5’-CTTTCTCCTATTGGCATA-3’), NTCP-R (5’-CTTTTGAGTGGCATTTC-3’); HBV-F (5’-GACCGACCAATCCGACAGC-3’), HBV-R (5’- AAGCCA CCAAGGGCAGAGC-3’); GAPDH-F (5’-GTCGAATGCTTCTCAGTGATTG-3’) and GAPDH-R (5’-CTGTTTCTGGTCTGCTG-3’). NTCP mRNA and HBV RNA quantification was normalized to GAPDH mRNA levels. HBV DNA was quantified using a commercial real-time PCR kit (Puruikang, Shenzhen, China). Real-time PCR for
quantiﬁcation of HBV cccDNA was carried out using primers cccDNA-F (5′-TGCACTTCGCTTCACCT-3′) and cccDNA-R (5′-AGGGG CATTGGTGGGTC-3′) (Glebe et al., 2003; Yan et al., 2012). Besides, the exacted DNAs other than cccDNA, including single-stranded and relaxed circular viral DNAs, were degraded prior to PCR ampliﬁcation using plasmid-safe ATP-dependent deoxyribonuclease DNase (Epicentre Biotechnologies, Madison, U.S.A.). Ratio of HBV cccDNA quantity per ng cellular DNA was used for comparison between groups.

2.8. Cell viability

HepG2.N9 cells were treated with different concentrations of irbesartan for up to 72 h and cell viability was determined using Cell Counting Kit-8 kit according to the manufacturer’s protocol.

2.9. Statistical analysis

The data presented here were expressed as mean ± standard deviation (SD) and statistical signiﬁcance was determined by the Student’s t test. P-values are indicated by asterisks (**P < 0.001, *P < 0.01, †P < 0.05).

3. Results

3.1. Successful establishment of NTCP persistent expressing HepG2 cell lines susceptible to HBV infection

To establish an HepG2 cell line persistently expressing NTCP, an NTCP expression cassette driven by mCBA promoter was inserted into the AAVS1-SA-2A-puro-pA vector (Fig. 1A). After 6 months of selection, 6 NTCP persistent expressing HepG2 cell lines were ﬁnally established using clustered regularly interspaced short palindromic repeats (CRISPR) and TALEN technologies. NTCP mRNA expression was determined using real-time PCR and 5 of the cell lines had NTCP expression levels similar to those of GAPDH (except HepG2.N8) (Fig. 1B). After inoculation with HBV (genotype D) at MGE 200, all 6 of the NTCP expressing cell lines secreted higher HBeAg into the supernatants compared with the parental HepG2 cells, especially HepG2.N9 (Fig. 1C). The HepG2.N9 cell line was also shown to be efﬁciently infected by serum from a patient positive for HBV infection (genotype C) at MGE 100 (Fig. 1D). The NTCP protein expression in the HepG2.N9 cell line was also demonstrated in Fig. S1.

3.2. The preS1-derivated m47F peptide binding and HBV infection in HepG2.N9 cells

Further characterization of the HepG2.N9 cell line included measurement of binding of the m47F peptide which is derived from the ﬁrst 47 amino acids of the HBV pre-S1 domain. By confocal microscopy, the m47F peptide (400 nM) was found to bind speciﬁcally to the membranes of HepG2.N9 cells (Fig. 2A). This speciﬁc binding was also demonstrated with ﬂow cytometry analyses (Fig. 2B). After inoculation with HBV (genotype D) at MGE 200, HepG2.N9 cells without the m47F peptide treating secreted large amounts of HBeAg into the media during 20 days of observation (Fig. 1C) and the percentage of HBcAg-expressing cells after infection was about 5–20% among different batches (Fig. 1D).

3.3. Irbesartan inhibited HBV infection with high potent activity and no obvious cytotoxicity up to 1000 μM

To investigate whether small molecule inhibitors of NTCP could interfere with HBV entry, 7 FDA approved drugs previously
reported as NTCP inhibitors were tested for prevention of HBV infection here (Dong et al., 2013). Among the drugs tested, cyclosporin A, irbesartan and ezetimibe inhibited HBeAg level in the cell supernatant most effectively at day 8 after HBV infection (Fig. 3A). The anti-HBV activity of cyclosporin A and ezetimibe were reported recently (Nkongolo et al., 2014; Watashi et al., 2013), although the mechanism of HBV inhibition by ezetimibe was unclear at that time (Lucifora et al., 2013).

Irbesartan (structure shown in Fig. 3B) also inhibited intracellular HBcAg and HBV RNA expression as the m47F peptide and ezetimibe did (Fig. 3C and D). The EC_{50} of irbesartan for HBeAg inhibition at day 8 was about 3.3 μM (Fig. 3E), and there was no obvious cytotoxicity in HepG2.N9 cells at the concentration up to 1000 μM (Fig. 3F). The inhibitory activity of irbesartan on HBV infection was also validated on primary Tupaia hepatocytes cells (Fig. S2).

3.4. Irbesartan inhibited HBV infection at viral entry steps before cccDNA formation

To determine at which step irbesartan inhibited HBV infection, irbesartan was administered at different times during HBV infection of HepG2.N9 cells. As shown in Fig. 4A, irbesartan administered either 2 h before HBV infection or 16 h during HBV infection produced a large reduction in HBeAg secretion on day 8, but had no effect when irbesartan was administered after HBV infection.

To preclude the possibility that irbesartan had direct binding or destructive effects on HBV envelopes, concentrated HBV particles were pretreated with irbesartan and were observed to have similar infectivity compared to HBV that had not been treated (Fig. 4B). Irbesartan inhibited HBV binding to the cell membrane of HepG2.N9 cells at 4 °C (Fig. 4C) but had only a minor suppressive effects on HBV uptake compared to controls at 37 °C (Fig. 4D), suggesting a weak inhibition on HBV binding and uptake at physiological temperature. HepG2.N9 cells were treated with irbesartan 2 h before and 16 h during infection with HBV, then the HBV cccDNA levels were measured at day 3 and found to be effectively inhibited (Fig. 4E).

4. Discussion

Currently approved therapies for HBV infection include interferon and a growing set of nucleos(t)ide analogues, but unfortunately all of them have rarely been associated with complete recovery (Vigano and Lampertico, 2011). The discovery of NTCP as a functional receptor for HBV opens new possibilities for the study of HBV infection and the development of new antiviral agents. It may be very fruitful to explore NTCP itself or other regulatory factors (Stross et al., 2010, 2013) as potential anti-HBV targets.

To set up a cell system susceptible to HBV infection, the NTCP-overexpressing cassette was inserted into the AAVS1 genome locus of HepG2 cells using the TALEN and CRISPR technologies. In our study, the TALEN method was found out to be more specific than the regular CRISPR technology (data not shown) and recently a group reported that the off-target mutagenesis of CRISPR could be reduced 50- to 1000-fold by paired guide RNAs.
to introduce targeted double-strand breaks (Ran et al., 2013). Nevertheless, several NTCP-overexpressing HepG2 cell lines were established. Among them, the HepG2.N9 cell line possessed median NTCP expression and exhibited highest rate of HBV infection, indicating that there are other host factors regulating HBV infection and replication such as peroxisome proliferator-activated receptor α (PPARα) (Hu et al., 2012).

Using the HepG2.N9 cell line, 2 small molecules, cyclosporin A (Nkongolo et al., 2014; Watashi et al., 2013) and ezetimide (Lucifora et al., 2013) reported recently as HBV infection inhibitors, were independently validated in this study, and irbesartan was demonstrated as a new inhibitor of HBV entry. Cyclosporin A is an immunosuppressive drug which may restrict its application for HBV therapy in future (Palmore et al., 2009). Ezetimibe is an inhibitor of intestinal cholesterol absorption and is widely used for the therapy of hypercholesterolemia (Ballantyne et al., 2003). Ezetimibe might be more potent to inhibit Hepatitis C virus infection other than HBV since its IC50 for HBV infection in vitro was much higher compared to HCV (Lucifora et al., 2013; Sainz et al., 2012). Irbesartan is used as an antihypertensive and to prevent kidney damage for patients with type 2 diabetes mellitus (Lewis et al., 2001). Irbesartan has also exhibited some activities suppressing hepatic fibrosis (Kim et al., 2008). With the IC50 of 3.3 μM, the plasma concentration of irbesartan should be about 1.4 μg/ml to obtain the anti-HBV effect. When irbesartan was given at up to 300 mg per day, the highest dose usually recommended, the Cmax was about 2.6 μg/ml in healthy volunteers (Qiu et al., 2014). Based on these findings, irbesartan might be a potential candidate for HBV patients’ therapy with two therapeutic goals: (1) to block HBV entry, and (2) to attenuate liver fibrosis. However, further experiments and clinical survey are needed to determine whether irbesartan will be effective against HBV infection and spread in vivo. Considering that HBV and its satellite virus hepatitis D virus (HDV) both utilize NTCP for their entry, we hypothesize that irbesartan could also exert anti-HDV effects which was reported very recently (Blanchet et al., 2014) and this might be helpful for HBV/HDV coinfection therapy.

The mechanism for inhibition of HBV entry by irbesartan was further investigated. Irbesartan showed no direct binding or lytic effects on HBV envelope, and weakly inhibited HBV uptake at physiological temperature. However, irbesartan inhibited HBV cccDNA formation effectively and had no obvious effect on HBV expression regulation (data not shown). This suggested that irbesartan targeted HBV infection at a post-uptake step such as fusion. Irbesartan is an antagonist against AT1R (Angiotensin II receptor type 1) and NTCP (Dong et al., 2013), but the shRNAs targeting AT1R and another AT1R antagonist losartan did not inhibit HBV infection (data not shown), suggesting that irbesartan interfered...
with HBV entry by inhibiting NTCP activity. We speculate that the molecular determinants of NTCP for HBV entry may be shared with those for bile salts uptake which was demonstrated very recently by others (Yan et al., 2014).

The mechanism of irbesartan could be particularly interesting considering that irbesartan targets a host factor for inhibition of HBV infection instead of the virus itself. In view of the above, NTCP as the long-sought HBV receptor is a good drug target for inhibiting HBV entry into hepatocytes, and the NTCP-overexpressing HepG2.N9 cells established here may facilitate the understanding of HBV infection mechanisms and accelerate future anti-HBV drug development. Irbesartan may be a new and excellent candidate for HBV therapy, although more animal and clinical efficacy data are needed in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2015.06.007.

References


