Research Article

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Viral Hepatitis

Cyclosporin derivatives inhibit hepatitis B virus entry without interfering with NTCP transporter activity

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Background & Aims: The sodium taurocholate co-transporting polypeptide (NTCP) is the main target of most hepatitis B virus (HBV) specific entry inhibitors. Unfortunately, these agents also block NTCP transport of bile acids into hepatocytes, and thus have the potential to cause adverse effects. We aimed to identify small molecules that inhibit HBV entry while maintaining NTCP transporter function.

Methods: We characterized a series of cyclosporine (CsA) derivatives for their anti-HBV activity and NTCP binding specificity using HepG2 cells overexpressing NTCP and primary human hepatocytes. The four most potent derivatives were tested for their capacity to prevent HBV entry, but maintain NTCP transporter function. Their antiviral activity against different HBV genotypes was analysed.

Results: We identified several CsA derivatives that inhibited HBV infection with a sub-micromolar IC_{50}. Among them, SCY446 and SCY450 showed low activity against calcineurin (CN) and cyclophilins (CyPs), two major CsA cellular targets. This suggested that instead, these compounds interacted directly with NTCP to inhibit viral attachment to host cells, and have no immunosuppressive function.

Importantly, we found that SCY450 and SCY995 did not impair the NTCP-dependent uptake of bile acids, and inhibited multiple HBV genotypes including a clinically relevant nucleoside analog-resistant HBV isolate.

Conclusions: This is the first example of small molecule selective inhibition of HBV entry with no decrease in NTCP transporter activity. It suggests that the anti-HBV activity can be functionally separated from bile acid transport. These broadly active anti-HBV molecules are potential candidates for developing new drugs with fewer adverse effects.

Lay summary: In this study, we identified new compounds that selectively inhibited hepatitis B virus (HBV) entry, and did not impair bile acid uptake. Our evidence offers a new strategy for developing anti-HBV drugs with fewer side effects.

Introduction

Hepatitis B virus (HBV) infection is a worldwide public health problem, which is estimated to chronically infect approximately 240 million individuals [1,2]. Chronic HBV infection elevates the risk of developing liver cirrhosis and hepatocellular carcinoma [3–5]. Current clinical treatments for HBV infection include interferons (IFN)s and nucleos(t)ide analogs (NAs) [6–8]. IFNα and its pegylated form (PegIFNα) modulate host immune response to viral infection and directly inhibit HBV replication in hepatocytes. NAs, including adefovir, entecavir (ETV), lamivudine, telbivudine, and tenofovir, suppress HBV replication by inhibiting reverse transcription. These agents significantly improve the progression of HBV-associated pathogenesis; however, they rarely lead to complete elimination of HBV from infected cells. Treatment studies for human immunodeficiency virus (HIV) and hepatitis C virus

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(HCV) have shown that multidrug cocktails can greatly improve the clinical outcome [9,10]. To achieve better anti-HBV treatment in the future, the development of new anti-HBV agents targeting different steps of the HBV life cycle, and their application to multidrug treatment are needed.

Cyclosporin A (CsA), a well-known immunosuppressive agent classified as a calcineurin (CN) inhibitor, is clinically used for prevention of graft rejection after organ transplantation [11,12]. CsA primarily targets cellular peptidyl prolyl cis/trans-isomerase cyclophilins (CyPs), and suppresses their enzymatic activity. The resulting CsA/CyP complex subsequently binds CN to inhibit its phosphatase activity, which in turn inactivates NF-AT, an essential transcription factor for immune responses. In addition to this primary activity of CyPs- and CN inhibition, CsA is also reported to inhibit the transporter activity of membrane transporter families [13]. Thus far, CsA has been reported to inhibit the replication of numerous viruses, including HIV, HCV, HBV, herpesviruses, dengue virus, West Nile virus, human papillomavirus, and coronaviruses [14,15]. In most of these cases, CyP-inhibition is the principal mechanism of the antiviral activity, as CyPs play a critical role in the viral life cycles. We, and other groups, have recently reported that CsA inhibits HBV entry through targeting a membrane transporter, sodium taurocholate co-transporting polypeptide (NTCP) [16–18].

HBV enters into hepatocytes via specific interaction of the preS1 region in the HBV large surface protein with NTCP, a recently identified HBV entry receptor [19]. NTCP is specifically expressed on the hepatic basolateral membranes and functions for co-transporting bile acids with sodium ions into hepatocytes [20–25]. Identification of NTCP as an HBV receptor raises the entry process as an attractive target for drug development [26]. By inhibiting the entry step, subsequent formation of covalently closed circular DNA (cccDNA), a persistent viral reservoir that is difficult to eliminate by the current treatment, will be prevented. To date, several compounds have been reported to inhibit HBV infection by targeting NTCP, including myrcludex-B, irbesartan, ezetimibe, ritonavir, vanitaracin A, and bile acids in addition to CsA [17,18,27–31]. However, all of these drugs can also inhibit the transporter function of NTCP and impair the sodium-dependent bile acid uptake, which may induce significant adverse effects: NTCP-deficient mice, and a patient carrying a defective polymorphism mutation in NTCP, exhibit an elevated level of serum bile acids, and develop the related pathologies including growth retardation and hypercholanemia [32,33]. Thus, it is a significant challenge to identify a compound that specifically inhibits HBV infection by targeting NTCP, without any effect on bile acid uptake.

In this study, we describe the identification of new CsA derivatives that inhibit HBV infection without affecting the transporter function of NTCP. Moreover, the anti-HBV activity can be achieved by compounds having minimal inhibition activity against CyPs and CN. Importantly, these compounds are effective against entry of multiple HBV genotypes and a clinically relevant NA-resistant HBV isolate. Non-immunosuppressive CsA derivatives, such as alisporivir, NIM811, and SCY-635, have been developed as anti-HCV candidates in clinical trial to phase II and III [14,34,35]. These findings suggest that CsA derivatives constitute a useful platform for the discovery of novel anti-HBV agents with specific activity but less adverse effects.

Materials and methods

Cell culture

HepG2-hNTCP-C4, Hep38.7-Tet, and HepG2.2.15.7 cells, and primary human hepatocytes (PhoenixBio) were cultured as described previously [16,36,37]. The parental HepG2 cells were purchased from ATCC. These cells were all confirmed to be mycoplasma-negative.

HBV preparation and infection

The HBV (genotype D) used in this study was mainly derived from the culture supernatant of Hep38.7-Tet cells as described previously [38]. HBV (genotype A, C, or A carrying the mutations L180M/S202I/M204V) was prepared from the culture supernatant of HepG2 cells transfected with the corresponding expression plasmid [39]. These inoculants contained both virions and nucleocapsids without envelopes, with ratio of 53:47 in Hep38.7-Tet-derived HBV (Supplementary Fig. 1). HBV infection was performed at 6000 (Fig. 1), 2000 (Fig. 6D and 7B–D), or 1000 (Fig. 2, 6B) genome equivalents (GEq)/cell (in which HBV GEq contains HBV DNA from both virions and nucleocapsids) in the presence of 4% PEG8000 as described previously [38].

Transporter assay

Bile acid uptake activity was measured in the presence or absence of sodium using HepG2-hNTCP-C4 cells and primary human hepatocytes, essentially as described [31]. Cells were preincubated with compounds at 37 °C for 15 min and then incubated with [^{3}H]-taurocholic acid (TCA) in the presence of compounds at 37 °C for 15 min to allow substrate uptake into the cells. Radioactivity inside the cells was measured with a liquid scintillator.

Statistics

Statistical significance was determined using Student's *t* test ($^*p$ <0.05, $^{**}p$ <0.01).

Detailed materials and methods are described in the Supplementary materials.

Results

Characterization of cyclosporin derivatives

We investigated the anti-HBV activity of synthesized CsA derivatives, designated as SCY series. HepG2-hNTCP-C4 cells, overexpressing the human *NTCP* gene in HepG2 cells and susceptible to HBV infection, were exposed to HBV in the presence or absence of these compounds for 16 h. After washing out free HBV inoculum and compounds, the cells were cultured for an additional 12 days in the absence of compounds, and HBV proteins were detected as an indicator for HBV infection. Hepatitis B surface (HBs) antigen secreted into the culture supernatant (Fig. 1A) and hepatitis B core (HBc) protein in the cells (Fig. 1C and D) at day 13 post infection were greatly reduced by preS1 peptide, a N-myristoylated peptide consisting of 2–48 aa of the preS1 region of the HBV large surface protein, as a positive control. Among the CsA derivatives, SCY806, SCY446, SCY450, SCY453, and SCY995 showed significant reduction of HBs and HBc, without causing cytotoxicity (Fig. 1A–D). In contrast, other CsA derivatives including SCY582, SCY651, SCY660, SCY198, SCY506, and SCY640 did not show significant effect on HBV infection (Fig. 1A–D).

CsA is known to primarily bind to cellular cyclophilins (CyPs), in addition to the interaction with a phosphatase, calcineurin (CN), which is essential for its immunosuppressive function [15]. To characterize the activity of these new CsA derivatives,