Original article

The FDA-approved drug irbesartan inhibits HBV-infection in HepG2 cells stably expressing sodium taurocholate co-transporting polypeptide

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Background: Little is known about the early steps of the HBV life cycle due to the lack of susceptible cells permissive for viral infection. Hence, viral entry has not been exploited for antiviral targets, but the recent seminal discovery of sodium taurocholate co-transporting polypeptide (NTCP) as the cellular receptor for HBV entry opened up many avenues of investigation, making HBV entry amenable to therapeutic intervention.
Methods: In order to exploit HBV entry, we established a HepG2-NTCP cell line that supports HBV infection. Over 70% of cells were infected at a dose of 10^4 genome equivalents (GEq) per cell. Several FDA-approved drugs with NTCP-inhibiting activity were tested for their ability to inhibit HBV infection of the cell line.
Results: Consistent with their NTCP inhibitory activities, our results showed that several of them inhibit HBV infection. In particular, irbesartan, a drug used for the treatment of hypertension, inhibits HBV infection at the 50% effective concentration value of 35 μM.
Conclusions: The observation that the pharmacological inhibitors of the NTCP transporter could block HBV entry suggests that NTCP represents an attractive molecular target for therapeutic intervention in HBV infection.

Introduction

HBV infection is a major global public health concern, with over 300 million chronically infected patients worldwide. Chronic HBV infection carries a great risk of developing severe liver diseases, including cirrhosis and liver cancer, which result in a million deaths annually [1]. HBV carries a small (approximately 3.2 kb), relaxed circular (RC), partially double-stranded DNA. Following entry into the host cells, the virion RC DNA traffics to the nucleus prior to conversion to a covalently closed circular DNA (cccDNA), which then serves as the template for the viral transcription [2].

Current treatments for chronic hepatitis B rely on monotherapy with nucleoside/nucleotide analogues (NUC) such as lamivudine (LAM), adefovir (ADV), entecavir (ETV) and tenofovir (TDF). Although LAM and ADV are associated with rapid emergence of viral resistance, viral suppression is achieved in up to 95% of patients treated with the so-called second-generation NUCs such as ETV and TDF. In other words, the second-generation NUCs exhibit high genetic barriers for viral resistance [3]. However, the loss of HBV surface antigen (HBsAg), which is a major treatment end point, is achieved in only less than 10% of patients after 5 years of NUC administration [3–5]. Thus, it is expected that the addition of one or more drugs to the current regimen, which have a novel target, should improve the response to therapy [2,6].

Studies on the entry step of HBV infection have largely relied on primary human hepatocytes (PHH) and primary hepatocytes from *Tupaia belangeri* (PTH) as well as differentiated HepaRG cell line [7]. Although PHH and PTH are valuable tools to study the entry step of HBV infection, its application remains somewhat limited due to poor cell accessibility and marked batch-to-batch variability. The HepaRG cell line, the first HBV-susceptible cell line established, has

been used to study HBV infection; however, its utility was limited due to the tedious differentiation process required [8]. With the recent discovery of sodium taurocholate co-transporting polypeptide (NTCP) as the cellular receptor for HBV entry, this obstacle has been eliminated [9,10]. Not surprisingly, HDV, which shares HBV surface proteins for envelopment, also exploits the NTCP receptor for entry [10,11]. The establishment of an NTCP-expressing HBV-susceptible cell line has now allowed for testing of selected FDA approved drugs for their inhibitory activity on HBV entry. Intriguingly, cyclosporin A (CsA) has been shown to block HBV entry via its inhibition of the NTCP transporter [12,13]. In addition, ezetimibe, a cholesterol-lowering drug, was shown to block HBV infection in HepaRG cells [14]. Furthermore, over a dozen drugs that have been FDA approved for other indications were shown to exhibit inhibitory activity, albeit modest, to NTCP-mediated bile acid uptake [15]. Here, we examined whether these drugs could inhibit HBV infection.

Methods

Cell lines and culture conditions

HepAD38 cell was kindly provided by Christoph Seeger, Fox Chase Cancer Center (Philadelphia, PA, USA) [16]. HepG2-NTCP2 cells stably expressing NTCP were generated by transfection of pcDNA6-hNTCP-C9 plasmids (kindly provided by Wenhui Li, National Institute of Biological Science, Beijing, China) into HepG2 cells (ATCC; HB-8065). HepG2-NTCP2 cells were maintained in Medium C (DMEM supplemented with 10% fetal bovine serum [FBS], 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin) [13,17]. For some experiments, two additional media were used: Medium A (Williams' E medium supplemented with 2% FBS, 5 μg/ml transferrin, 10 ng/ml epidermal growth factor [EGF], 3 μg/ml insulin, 2 mM L-glutamine, 18 μg/ml hydrocortisone, 40 ng/ml dexamethasone and 5 ng/ml sodium selenite) and Medium B (DMEM/F-12 supplemented with 10% FBS, 10 mM HEPES, 200 U/ml penicillin, 200 μg/ml streptomycin, 50 μM hydrocortisone and 5 μg/ml insulin) [11,18].

HBV preparation and infection

HBV particles were prepared from the culture supernatant of HepAD38 cells, as described previously [19]. For HBV infection, HepG2-NTCP2 cells were seeded in 12-well plates coated with collagen type I (BD Biosciences, Bedford, MA, USA). One day later, the cells were inoculated with HBV at 10^3 genome equivalents (GEq) per cell in Medium C containing 4% polyethylene glycol (PEG8000; Sigma, St. Louis, MO, USA) for 24 h [17], if not indicated otherwise. Cells were then maintained in Medium C containing 2.5% DMSO for an additional 8 days, if not indicated otherwise. For the infection inhibition experiment, the reagents were either added during the infection (that is, 24 h) or at another time point as indicated.

Analysis of viral DNA

Viral DNAs were isolated from cytoplasmic capsid and subjected to Southern blot analysis, as previously described [20].

Immunofluorescence analysis

HepG2-NTCP2 cells were seeded at 2×10^4 cells per well in collagen-coated 96-well plates and then infected with HBV at 10^3 GEq per cell. Cells were stained 9 days post-infection (dpi). For the detection of hepatitis B core antigen, the primary antibody, anti-HBc (DAKO, Carpinteria, CA, USA), was used, and Alexa-labelled 488 goat anti-rabbit antibody was used as a secondary antibody for fluorescence detection. Nuclei were stained with Hoechst 33342. Fluorescence images (magnified 10×) were taken using the Operetta High Content Imaging System (Perkin-Elmer, Waltham, MA, USA) and analysed using the in-house Imaging Mining software I.M 3.0 [21].

Western blot analysis

Western blot analysis was essentially performed as described previously [20].

Cell viability assay

HepG2-NTCP2 cells were seed at 1×10^4 cells per well in collagen-coated 96-well plates. One day after, each drug was added at a concentration of up to 200 μM for 24 h. Cell viability was determined by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA).

Reagents

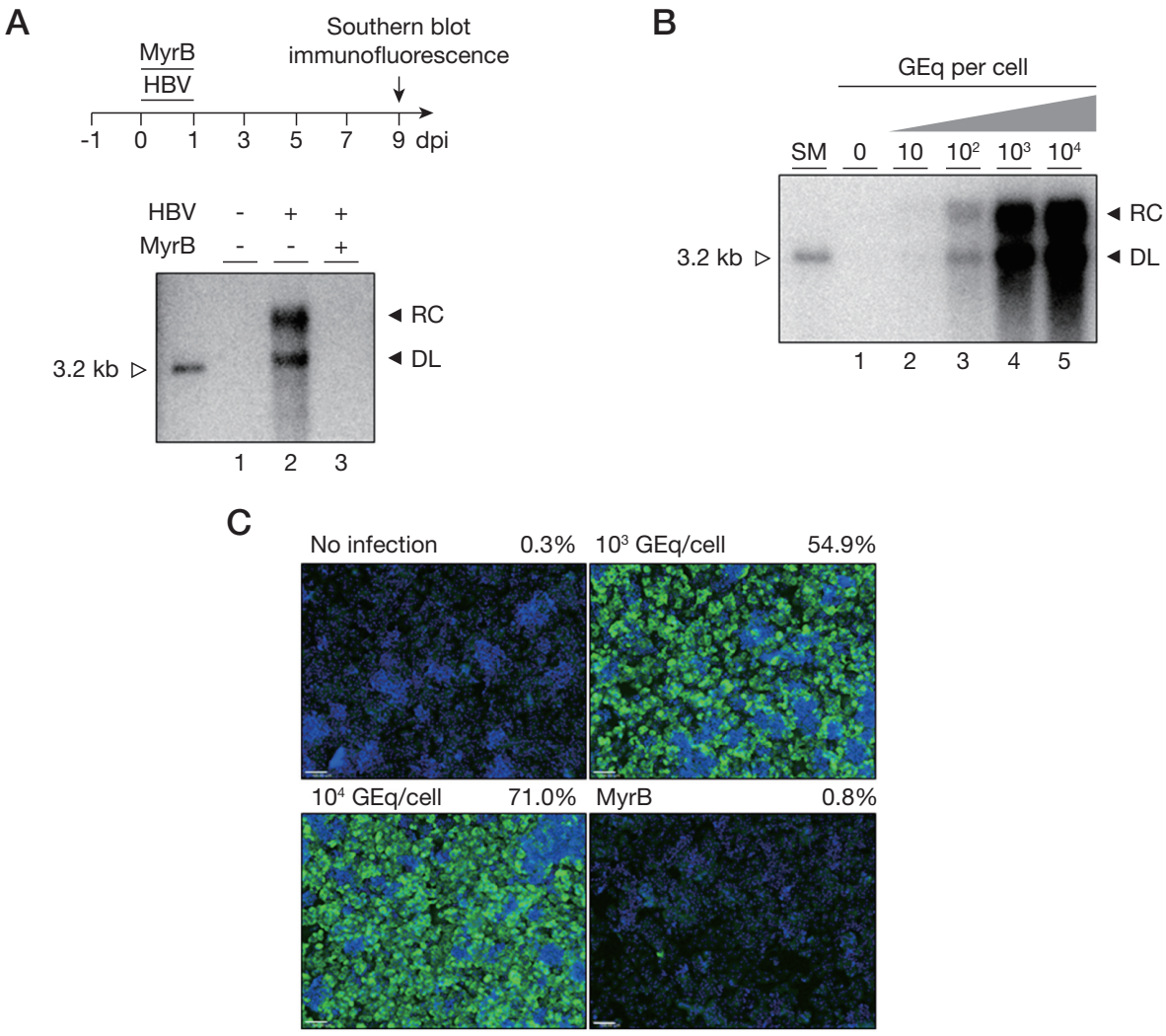
All drugs, including irbesartan, were purchased from Sigma and dissolved in DMSO to give a stock of 10 mM. Myrcludex-B (MyrB) was a generous gift from Stephan Urban (University Hospital Heidelberg, Heidelberg, Germany).

Results

Establishment of the HBV-susceptible HepG2-NTCP cell line

We established NTCP-expressing cell lines by transfecting HepG2 cells as previously described [9,10,18]. A clone (that is, HepG2-NTCP2) that expressed NTCP abundantly was chosen for further investigation (Additional file 1). In order to examine whether HepG2-NTCP2 cells support HBV infection, cells were infected at a dose of 10^2 GEq per cell, and the cytoplasmic capsid-associated DNA

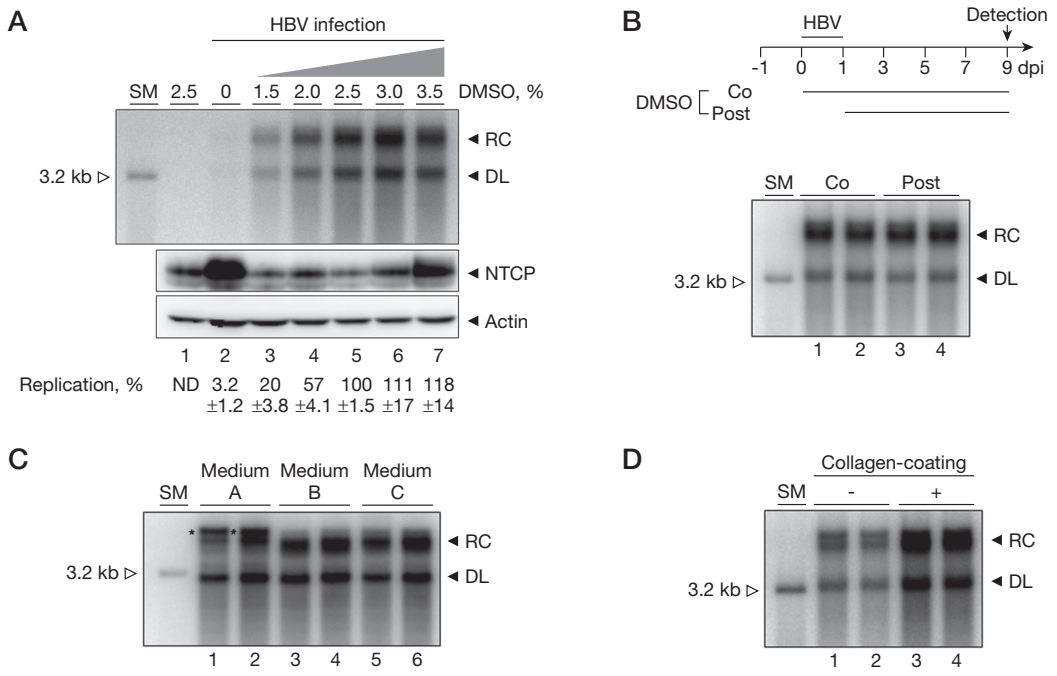
Figure 1. Stable expression of NTCP renders HepG2 cells susceptible to HBV infection



(A) Southern blot analysis to measure viral DNA replication. The upper scheme illustrates the treatment schedule with HBV inoculum (10^2 GEq per cell) and MyrB (200 nM). DMSO (2.0%) was added to the medium at 1 day post-infection (dpi). The capsid-associated viral DNAs were analysed by Southern blotting from cells harvested at 9 dpi. Viral DNA replication intermediates (relaxed circular [RC] DNA and duplex linear [DL] DNA) are noted. A restriction fragment representing one HBV genome unit, 3.2 kb in size, serves as a size marker. **(B)** Titration of HBV inoculum required for efficient HBV infection. HepG2-NTCP2 cells were infected with increasing amounts of HBV inoculum (10 to 10^4 GEq per cell). At 1 dpi, cells were maintained in the same medium containing 2.5% DMSO. The capsid-associated DNA isolated from the cells at 9 dpi was analysed by Southern blotting. **(C)** Immunofluorescence analysis. Cells were infected with an HBV inoculum of 10^3 or 10^4 GEq per cell, as indicated. DMSO (2.5%) was added to the medium at 1 dpi. Cells infected with an HBV inoculum of 10^3 GEq per cell were treated with MyrB (200 nM). At 9 dpi, the HBV core protein was immunostained with Alexa, as shown in green and nuclei were labelled with Hoechst 33342, as shown in blue. Percentage (%) of the HBV infected cells are shown above each panel. Scale bar (100 μm) is shown in lower left of each panel. SM, size marker.

were harvested at 9 dpi and analysed by Southern blot analysis. Consistent with previous reports, the viral DNAs were detectable in HBV-infected cells, but not following treatment with MyrB, an HBV entry inhibitor, which is a synthetic lipopeptide derived from the large (L) form of HBsAg [22] (Figure 1A, lanes 2 and 3). The formation of cccDNA, which is a hallmark of the HBV infection, was confirmed, as we demonstrated in our recent report [17] (data not shown). Having established the genuine HBV infection system, we next determined the viral titre in an inoculum required for efficient HBV infection. Southern blot analysis showed that a viral titre between 10^3 and 10^4 GEq per cell in the inoculum was sufficient to detect viral DNA (Figure 1B). Accordingly, we used 10^3 GEq per cell for the remainder of our study. Immunofluorescence analysis showed that over 70% of cells were infected at a dose of 10^4 GEq per cell, and the infection was nearly completely blocked by 200 nM MyrB (Figure 1C).

Figure 2. Optimization of culture conditions for HBV infection



(A) The effect of DMSO concentration on HBV infection. At 1 day post-infection (dpi), DMSO (up to 3.5%) was added as indicated. At 9 dpi, cells were harvested, and the capsid-associated DNAs were measured by Southern blot analysis, as shown in Figure 1A. The intensity of viral DNAs observed from lane 5 is set to 100, and intensity for other lanes is relative to this standard. Data represent the means ± standard deviations of results from three independent experiments. Sodium taurocholate co-transporting polypeptide (NTCP) was detected from the cells harvested above by western blot analysis: β-actin serves as a loading control. **(B)** Time of DMSO addition. The upper scheme illustrates the time of DMSO addition (2.5%); DMSO was added either during the time of infection (Co) or at 1 dpi (Post). The capsid-associated DNA was analysed as shown in panel (A). **(C)** Comparison of different cell culture media for HBV infection. Cells were cultured and infected in medium C in the absence of DMSO. At 1 dpi, the medium was replaced by three different media (medium A, B or C, as indicated), and DMSO (2.5%) was added. The capsid DNA was analysed in duplicate for each medium by Southern blot analysis. Slow-migrating relaxed circular (RC) DNA species found in medium A are denoted by asterisks (lanes 1 and 2). **(D)** Effect of collagen-coating on HBV infection. HepG2-NTCP2 cells were seeded either in collagen-coated or non-coated plates. One day after, the cells were infected with HBV (10^3 GEq/cell). The capsid-associated DNAs isolated from the cells on 9 dpi were subjected to Southern blotting in duplicate. DL, duplex linear; SM, size marker.

Optimization of cell culture conditions for efficient HBV infection

Having validated the HBV infection, we sought to optimize cell culture conditions for efficient infection. First, we tested the influence of DMSO on HBV infection. DMSO treatment (>1.5%) has also been shown to be essential for HepG2-NTCP cell lines, although the reason for this remains uncertain [10]. It was speculated that DMSO treatment may be essential for entry, either for abundant NTCP expression or for NTCP receptor function. We examined the effect of DMSO (at a concentration of up to 3.5%) on HBV infection. The results showed that the viral DNAs were barely detectable in the absence of DMSO, but levels were linearly enhanced at 1.5% to 2.5% DMSO (Figure 2A, lanes 2 to 5). On the other hand, the accumulation of HBV replication intermediates was similar at DMSO concentrations ranging from 2.5% to 3.5% DMSO (Figure 2A, lanes 5 to 7). It is notable that HBV replication levels appeared to be slightly increased upon addition of 3% to 3.5% DMSO, but it led to higher variations in HBV replication, perhaps due to cytotoxicity. Taken together, we chose 2.5% DMSO for further analysis. Unexpectedly, the NTCP expression measured in parallel was rather high in the absence of DMSO treatment, but rather lower in DMSO-treated cells, indicating that DMSO treatment did not induce the NTCP expression (Figure 2A, lane 2 versus lanes 3 to 7).

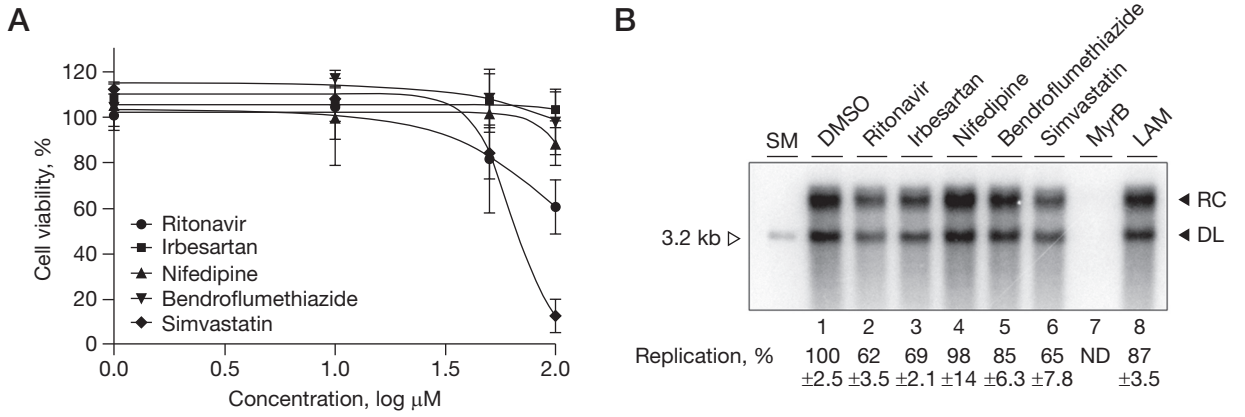
In order to examine whether DMSO treatment was required for entry, DMSO was added to the medium at the time of infection or at 1 dpi (Figure 2B). The results of Southern blot analysis showed that the viral DNA replication level was comparable regardless of DMSO treatment schedule. Overall, the time-of-addition experiment revealed that DMSO treatment is rather dispensable for the entry step itself, but instead contributes to enhancement of viral infection at a post-entry step.

Three laboratories, who reported HBV infection using HepG2-NTCP cell lines, used slightly different culture media, as detailed in *Methods*. For instance, PMM medium (Medium A, herein), which Li's group used, contains several components including EGF, insulin, hydrocortisone and dexamethasone [11], whereas the cell culture medium (Medium C) that Urban's group used does not contain any of these components [13]. Therefore, it remained unclear to what extent these medium components influence HBV infection. To address this issue, we tested HBV infection using three different media and found that all three media more or less equally supported HBV replication, suggesting that several components supplemented in Medium A are largely dispensable (Figure 2C). It was noted that a slow-migrating RC DNA (denoted by an asterisk), was markedly detected with Medium A (Figure 2C, lanes 1 and 2) for reasons that are unclear. We speculated that the slow-migrating RC DNA could represent the mature RC DNA, in which the gap region present in the plus-strand RC DNA is partly filled. The hypothesis deserves further investigation. In addition, we examined the effect of collagen coating on cell culture plates and found that collagen coating considerably enhanced viral DNA synthesis (Figure 2D). It remains to be seen, however, whether HBV infection *per se*, as opposed to viral DNA replication, is enhanced by collagen coating.

Inhibitory activity of FDA approved drugs on HBV infection

Over a dozen of the drugs that have been FDA approved for other indications were shown to exhibit a modest inhibitory activity on NTCP-mediated bile acid uptake [15]. However, it remains open to what extent these drugs may inhibit HBV entry. Drugs that exhibited EC_{50} values of less than 65 μM and are commercially available include the following: CsA (EC_{50}=7.6 μM), irbesartan (EC_{50}=11.9 μM), ritonavir (EC_{50}=18.4 μM), ezetimibe (EC_{50}=25.0 μM), simvastatin (EC_{50}=47.9 μM), bendroflumethiazide (EC_{50}= 53.0 μM) and nifedipine (EC_{50}=62.6 μM) [15]. Among these drugs, we excluded CsA and ezetimibe from our analysis, whose anti-HBV activity has already been reported [12–14]. Prior to evaluation of the antiviral activity, we first tested the cytotoxicity of selected drugs in HepG2-NTCP2 cells. As depicted in Figure 3A, all drugs did not show evident cytotoxicity at the concentration below 20 μM, although ritonavir and simvastatin showed severe cytotoxicity at 100 μM. In order to examine whether these drugs could inhibit HBV infection, the drugs were added at a concentration of 20 μM during infection (that is, for 24 h). Southern blot analysis showed that three drugs (irbesartan, ritonavir and simvastatin) resulted in reduced accumulation of HBV replication intermediates (Figure 3B, lanes 2, 3, and 6). MyrB was employed as an entry inhibitor, as shown in Figure 1A, whereas LAM was employed as a post-entry inhibitor that does not affect HBV entry, as demonstrated previously by using HepaRG cells (Figure 3B, lanes 7 and 8) [12]. Treatment of LAM slightly reduced the level of HBV replication intermediates. Our interpretation is that small amounts of residual LAM affect HBV replication after infection. CsA also efficiently inhibited the accumulation of viral DNA at a concentration of 8 μM (data not shown), as previously reported [12,13].

Figure 3. The inhibitory effect of FDA-approved drugs on HBV infection



(A) Cytotoxicity. HepG2-NTCP2 cells were seeded in 96-well plates and treated with each drug. Cell viability was determined by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA), according to the manufacturer's protocol. The 50% cytotoxic concentration (CC_{50}) value was calculated by sigmoidal fitting: ritonavir (>100 μM), irbesartan (>100 μM or 287.4 μM), nifedipine (>100 μM), bendroflumethiazide (>100 μM) and simvastatin (64.53 μM). Error bars represent the standard deviation from three independent experiments. **(B)** HepG2-NTCP2 cells were infected with HBV (10^3 GEq/cell) and treated with drugs (20 μM each) as indicated. MyrB (200 nM) and lamivudine (LAM; 1 μM) treatment were included as references. The capsid DNAs were analysed by Southern blot analysis. The representative data from at least three independent experiments is shown, and the percentage of viral replication is quantitated relative to DMSO control (lane 1; mean ±SD). DL, duplex linear; ND, not detected; RC, relaxed circular; SM, size marker.

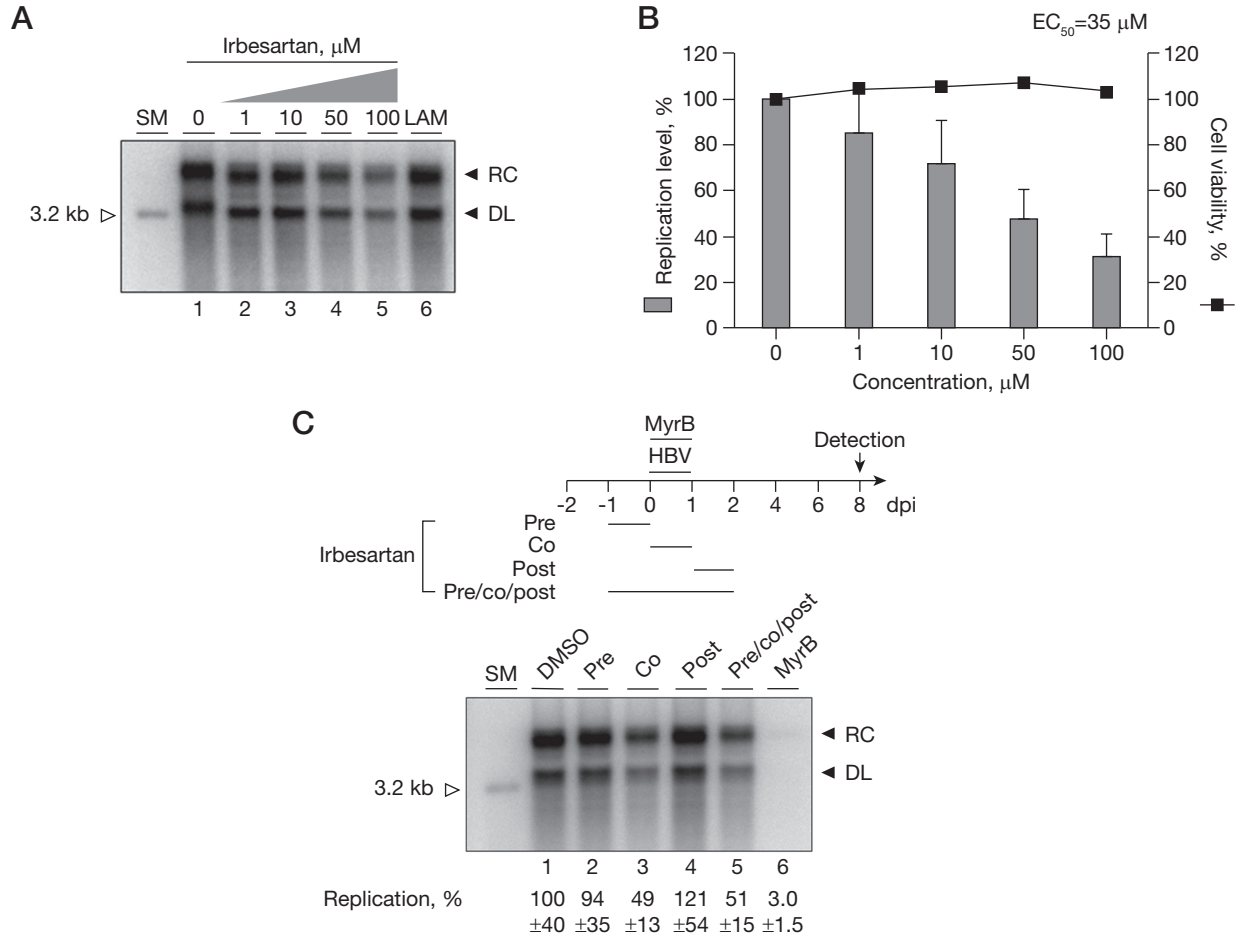
Time course of the inhibitory effect of irbesartan on HBV infection

Among three drugs, we evaluated the antiviral efficacy of irbesartan by increasing the concentration of drugs up to the concentration of 100 μM, because irbesartan did not show cytotoxicity (Figure 3A). HBV-infected cells were treated with increasing concentrations of irbesartan (up to 100 μM) during infection for 24 h. Southern blot analysis showed that viral DNA level was linearly reduced by increasing concentrations of irbesartan (Figure 4A). The quantification of the data shown in Figure 4A showed that the EC_{50} value of irbesartan is approximately 35 μM, while the 50% cytotoxic concentration (CC_{50}) was estimated to be 287.4 μM (>100 μM; Figure 4B), manifesting a modest therapeutic index (CC_{50}/50% effective concentration [EC_{50}] value =8.2). To determine the specific step of viral infection at which the drug acts, four drug treatment schedules were considered, as depicted in Figure 4C. The data revealed that HBV infection decreased only when the drug was added during the time of infection (Figure 4C, lanes 3 and 5). The result of the time-of-addition experiment indicates that irbesartan affects early stages of HBV infection.

Discussion

In this report, we examined a few FDA-approved drugs for their inhibitory activity on HBV infection using an HBV-susceptible HepG2-NTCP cell line. Our results showed that irbesartan, an angiotensin II receptor

Figure 4. Evidence that irbesartan blocks the early stages of HBV infection



(A) Cells were infected with HBV and treated with an increasing dose (up to 100 μM) of irbesartan during infection (for one day). At 9 days post-infection (dpi), the capsid DNAs were isolated and analysed by Southern blot analysis. Lamivudine (LAM; 1 μM) was included as a control. **(B)** Quantification of the results shown in panel (A). The intensity of viral DNAs observed from lane 1 of (A) is set to 100, and intensity for other lanes is relative to this standard. Error bars represent the standard deviation from seven independent experiments. Cell viability (%) was examined in parallel, as shown in Figure 3A. **(C)** The upper scheme illustrates the treatment schedule with HBV inoculum and irbesartan. Cells were treated with irbesartan (50 μM) either only for 24 h before (Pre), for 24 h during the time of infection (Co), for 24 h after infection (Post) or for 72 h (Pre/co/post). At 8 dpi, the capsid DNAs were isolated and analysed by Southern blot analysis. MyrB (200 nM) was included as a control. The experiment was repeated for three different times, and the representative data are shown with quantification (mean ±SD). EC_{50}, 50% effective concentration; DL, duplex linear; RC, relaxed circular; SM, size marker.

antagonist used for the treatment of hypertension, modestly inhibited HBV infection (Figure 4A). Significantly, the inhibitory potency of irbesartan on HBV infection parallels that of NTCP's bile acid transporter [15], suggesting that irbesartan inhibits HBV infection by inhibiting NTCP's bile acid transporter function.

Having established the NTCP-expressing cell line, we attempted to clarify the impact of DMSO on HBV infection. In the literature, DMSO treatment was shown to be essential for HBV infection both in PHH and HepaRG cells [8,23]. The observations that NTCP expression is induced after DMSO treatment in HepaRG cells suggest that DMSO is essential for the maintenance of the differentiation state of hepatocytes that entails NTCP expression [9,10]. In contrast to the belief that DMSO is required for the viral entry, our results documented here showed that DMSO treatment was largely dispensable for entry, but was required for a post-entry step (that is, after 24 h post-infection; Figure 2B). This unexpected finding led us to speculate that DMSO treatment may either induce host factors that support viral infection or suppress host factors that restrict viral infection. Experiments are currently underway to identify the DMSO-induced host factors that promote the cccDNA formation.

The main physiological function of NTCP is to uptake bile salts into hepatocytes for enterohepatic circulation [24]. Intriguingly, Li's group recently demonstrated that some bile acids, in particular taurour-sodeoxycholic acid, inhibit HBV entry with a 50% inhibitory concentration value of approximately 1.0 μM, and further revealed that the same amino acid residues in NTCP are commonly recognized by both the pre-S1 peptide and bile acids [25]. Conversely, the pre-S1 peptide was shown to inhibit the physiological bile acid cotransporter function of NTCP [26]. In this regard, whether or not irbesartan could compete with the pre-S1 peptide or bile acids for the binding site on the NTCP receptor merits further investigation.

While this manuscript was being prepared, others reported the inhibitory activity of some of these drugs (that is, irbesartan and ritonavir) on HDV entry with an EC_{50} value of 10 μM [27]. This value is slightly different with the EC_{50} value of 35 μM that we estimated for HBV entry (Figure 4B). Whether the discrepancy in the potency is attributable to the different virions (that is, HBV versus HDV) or simply to an experimental variation remains uncertain.

The efficient HBV infection obtained using the NTCP-expressing cell line will facilitate investigations regarding early steps of HBV infection including cccDNA formation, the molecular determinant of viral persistence. It is hoped that a novel drug targeting the early steps, including entry, would allow for the development of multi-drug anti-HBV regimens, together with current NUCs, which could lead to better control of viral persistence.

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Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: Western blot analysis of the HepG2-NTCP cell line can be found at http://www.intmedpress.com/uploads/documents/3503_Ko_Addfile1.pdf

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