Original Article
Inhibition of hepatitis B virus in HepG2.2.15 by indirubin through suppression of a polypyrimidine tract-binding protein

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Abstract: Chronic hepatitis B virus (HBV) infection continues to be a public health burden worldwide. With the current treatment strategy, a limited number of patients achieve “functional cure”. There is a need for novel therapeutic approaches that enable resolution of HBV. In our study, HepG2.2.15 cell lines were used as an in vitro cellular model to assess the antiviral activity of indirubin. The cytotoxicity of indirubin was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. HBsAg and HBeAg in the cell culture were determined by enzyme-linked immunosorbent assay, and HBV DNA secretion was measured by real-time fluorescence quantification PCR. The intracellular HBsAg was detected. To demonstrate the mechanisms underlying the anti-HBV activity of indirubin, western blotting and qRT-PCR were performed to detect the expression levels of a polypyrimidine tract-binding protein (PTB). The role of indirubin from Isatisindigotica extracts presented low cytotoxicity. The results demonstrated indirubin effectively reduced the secretion of HBV antigen (HBsAg and HBeAg), and suppressed HBV DNA secretion. In addition, indirubin significantly reduced intracellular HBsAg. Furthermore, indirubin significantly reduced the protein level of PTB. This study is the first to demonstrate that indirubin possesses anti-viral ability and the mechanism may be through down regulating the polypyrimidine tract-binding protein. It could be used as a potential treatment against HBV infection targeting the host factor.

Keywords: Hepatitis B virus, anti-viral activity, indirubin, PTB

Introduction
Chronic hepatitis B virus (HBV) infection continues to be a major health burden worldwide [1]. Current first-line drugs for chronic hepatitis B include pegylated interferon alpha2a (PEG-IFN) or nucleotide analog (NAs) [2]. Data supporting a long-term clinical benefit of combination therapies for patients are lacking. Monotherapy with PEG-IFN or NAs remains the therapy of choice. HBsAg loss is considered a “functional cure”, but does not mean viral eradication. Only a limited number of patients achieve HBsAg loss with the current treatment approaches. There is a need for novel therapeutic approaches that enable not only suppression of viral replication, but the resolution of HBV infection [1].

During the past few decades, bioactive compounds from numerous plant species have been investigated to show potential antiviral activities, such as extracts of Phyllanthus urinaria L. (Euphorbiaceae), which presented potent anti-infection effects against HSV-2 [3]. We proved that pretreatment of I. indigotica extracts, indigo, greatly inhibit Japanese Encephalitis Virus (JEV) replication in vitro [4]. Since ancient times, Isatisindigotica has been used in traditional Chinese medicine. Daqingye and Banlangen are the leaves and roots of I. indigotica, respectively. It is frequently used as the anti-inflammatory, anti-tumor such as anti-leukemia and antipyretic agents [5]. The Isatisindigotica extracts are also widely used for clinical treatment of viral diseases like hepatitis, encephalitis, foot-and-mouth disease, influenza and severe acute respiratory syndrome (SARS). And the extracts are also frequently used for clinical treatment of virus infection.
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Indirubin is found in indigo-producing plants, such as Isatisindigotica, which has frequently been used as TCM to treat the respiratory viral infection. It was firstly identified as the active ingredient of a TCM preparation Danggui Longhui Wan. Indirubin is one of the biologically active compounds of Isatisindigotica. This chemical compound not only showed potent antiviral activities but also exhibits strong anti-inflammatory and antitumor such as anti-leukemic effect [8]. In influenza virus infection, the initial sites of infection are airway epithelial cells, which participated in airway inflammatory response by expressing various cytokines, including RANTES. The signal pathway involved inducing p38 MAP kinase activation and NF-kB translocation by indirubin [9]. Indirubin derivatives also have the immunomodulatory effects on pulmonary endothelium. They can suppress H9N2-induced cytokines production through MAPKs and STAT3 signaling pathways [10].

Polyhyridine tract-binding protein (PTB, 57-kD) preferentially binds to the pyrimidine-rich sequences of RNA [11]. The protein can shuttle between the cytoplasm and nucleus [12, 13]. This protein is able to act as both a repressor and activator of RNA metabolism, through acting as an RNA chaperone [14], which is known to be important for the life cycle of many viruses [15].

It has been reported that indirubin from the Isatisindigotica extracts plays its role in several virus replications. We speculated that indirubin might inhibit HBV replication. The aim of the present study was to investigate the anti-HBV ability of indirubin. We used a stably HBV-transfected cell line, HepG2.2.15, as a cell model. HBsAg, HBeAg, and HBV-DNA in the cellular supernatant were detected. And the intracellular HBsAg were detected by immunofluorescence. Our results indicate that the mechanism of HBV replication inhibition may be through inhibiting PTB.

Materials and methods

Compounds and cell culture

Stock indirubin (Chengdu Herbpurify CO., LTD) was prepared in dimethylsulfoxide (DMSO) and stock antiviral positive control lamivudine was prepared in phosphate-buffered saline (PBS). Lamivudine was obtained from pharmacy department in the First Affiliated Hospital of Medical School of Zhejiang University. They were stored at -20°C. All the drugs in the study are endotoxin free.

For the antiviral analyses, HepG2.2.15 cells were maintained on 96-well flat-bottom tissue culture plates in DMEM medium with 2% fetal bovine serum [16]. Working dilutions of antiviral compounds were prepared in medium containing 2% FBS. Lamivudine was used as positive control.

Analysis of cellular toxicity

The survival rates of HepG2.2.15 cells treated with compounds were detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays. Briefly, the cells were seeded at a density of 5 × 10³ cells per well on 96-well plates and treated with media containing indirubin or lamivudine of different concentrations: indirubin 64, 32, 16, 8, 4, 2 and 1 μg/mL; lamivudine 0.3 μM. 3 or 6 d later, the supernatants were discarded and 20 μl MTT (5 g/l) was added per well. After incubation at 37°C and 5% CO₂ for 4 h, the supernatants were discarded and 150 μl of DMSO was added to dissolve the purple formazan of MTT. The survival ratio of HepG2.2.15 cells (%) = (A570 of compound group/A570 of negative control) × 100% [17].

Antiviral assays for cellular supernatant

Cultures (three per each test concentration) with three independent experiments were treated with three or six consecutive daily doses of the test compounds. HBV DNA levels were measured 3 d or 6 d after the last treatment. Extracellular (virion) HBV DNA levels were assessed by real-time fluorogenic quantitative PCR with a commercial kit (Acon, Hangzhou, China) according to the manufacturer’s protocols.

The assays of HBsAg and HBeAg from culture supernatants were performed as described previously using ELISA kits (Architect HBsAg, Abbott, Abbott Park, IL; AXSYM HBeAg, Abbott, Abbott Park, IL, USA) [18].
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Immunofluorescence for HBsAg

HepG2.2.15 cells were divided into two groups: the control group and the test compound group. The cells were seeded on glass cover slips (BD Biosciences, San Jose, CA, USA) overnight. After the 72 h exposure time, the cells were washed with PBS, and fixed with 4% paraformaldehyde (30 min, RT) followed by permeabilization with 0.3% Triton X-100. Then the cells were blocked with 1% BSA-PBS for 1 h at RT. A primary mouse monoclonal anti-HBsAg antibody (AbcamInc., Cambridge, MA, USA) was incubated overnight at 4°C. The cells were washed and treated with Alexa Fluor 488-conjugated secondary Antibody (Product No, A21206, Molecular Probes, Invitrogen) for 1 h at RT. The nucleus was further stained with DAPI (Molecular Probes, Invitrogen) for 1 h at RT. The every imaged HepG2.2.15 cell was observed using a confocal microscope (Olympus Inc., Center Valley, PA, USA), and analyzed using the Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD, USA) [19].

Detecting PTB mRNA by real-time fluorescence quantitative RT-PCR

Total RNAs were extracted from HepG2.2.15 cells cultured in 24-well plates with TRlzol reagent (Invitrogen) and dissolved in 30 μL RNase-Free ddH₂O. Then cDNAs were synthesized by reverse transcription with First-Strand Synthesis System for RT-PCR (Takara, Dalian, China) according to the manufacturer’s instruction. PCR was performed with gene-specific primers for PTB and GAPDH: PTB (NM_031990.3), 5'-TCATTCCAGAGAAAGCCACTT-3' (forward), 5'-CGGATACCAGCAAGTGTAACCT-3' (reverse); GAPDH (NM_008084.2), 5'-CCATGGTGTCATGGGTGATG-3' (forward); GAPDH (NM_008084.2), 5'-GCCATGAGCGGATGATG-3' (reverse). qPCR was performed on the ABI 7900 Sequence Detection System using the SYBR Green kit (Takara, Dalian, China). Results of RT-PCR were normalized with the housekeeping gene GAPDH as control [20]. For each sample, qRT-PCR was performed in duplicate.

Western blot analysis for PTB

The following antibodies or reagents were used: antibody to PTB and antibody to β-actin (Santa Cruz, Dallas, TX, USA). The cell lysates were prepared using RIPA lysis buffer with protease inhibitors after the cells were washed with PBS and centrifuged. The protein of cell extracts was determined by a BCA method (Product No.23225, Thermo Scientific, Rockford, USA). 20 μg protein was separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF, Millipore, Billerica, MA, USA). The membrane was blocked in TBST containing 1% BSA and incubated with primary antibodies at 4°C overnight. After washing, the membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit or mouse immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1 h at room temperature and visualized with enhanced chemiluminescence reagents (Thermo Scientific, Wal-
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Figure 2. Inhibition of HBV replication in HepG2.2.15 treated with indirubin for 3 d and 6 d. After HepG2.2.15 cells were treated with indigo for 3 d, the culture supernatant was harvested. HBsAg (A and D) and HBeAg (B and E) was determined by ELISA assay, and HBV DNA (C and F) was detected by qPCR. The data were analyzed using a two-tailed t test. The results represent the mean data from three independent experiments.

Statistical analyses

The data were analyzed using a two-tailed t-test by SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) [23]. Each in vitro experiment was repeated at least three times. The data are presented as means ± SEM and P<0.05 was considered significant.

Results

Cellular toxicity of indirubin

The cellular toxicity of the compound indirubin (Figure 1A) on the HepG2.2.15 cells was determined with MTT method by serial 1:2 dilutions. The results showed no cytotoxicity to HepG-
2.2.15 cells with below concentration 64 μg/ml for 3, 6 d (Figure 1B and 1C).

Inhibition of HBV replication and HBsAg, HBeAg secretion by indirubin

Following treatment of HepG2.2.15 cells with indirubin for 3 or 6 days, the antiviral protein (HBsAg and HBeAg) and HBV-DNA were detected in the medium (Figure 2). As shown in Figure 2A, 2D and 2B, 2E, when the concentration of indirubin was above 4 μg/ml for 3 d, the HBsAg, and HBeAg percent was below 50%. However, the percent of HBsAg and HBeAg with lamivudine was above 80% at the concentration of 0.3 μM. To investigate the activity of inhibiting HBV viral replication, HBV DNA was isolated from treated 2.2.15 cells in the supernatant. As shown in Figure 2C and 2F, the results revealed that indirubin could markedly inhibit the HBV replication. The percent of HBV-DNA was below 20% at 32 μg/ml indirubin, while the percent of HBV-DNA was above 30% at 0.3 μM lamivudine.

Anti-HBV activity of indirubin for HBsAg in HepG2.2.15

HBV surface antigen (HBsAg) was detected by confocal microscopy. As shown in Figure 3, compared with the control cells treated with solvent DMSO, the signal of HBsAg was obviously decreased in the HepG2.2.15 cells treated with indirubin (Figure 3A and 3B).

Expression of PTB in HepG2.2.15 treated with indirubin

PTB is a PRE-interacting protein, which is involved in the process of the nuclear transport of pre-S/S RNAs of HBV. We evaluated the expression of PTB in HepG2.2.15 treated with indirubin for 3 d by Western blot analysis. As shown in Figure 4A, indirubin significantly decreased the levels of PTB. Further, we investigated the effect of indirubin on PTB mRNA levels. As shown in Figure 4B, indirubin did not significantly decrease the levels of PTB mRNA.

Discussion

In our study, we used HepG2.2.15 stably transfected with four copies of HBV genome to study the anti-HBV effects of indirubin in vitro. The cell line, which could synthesize viral DNA and secrete HBsAg, core particle and virion into the culture medium, could produce hepatitis in chimpanzees [24]. By analyzing HBsAg, HBeAg and DNA produced in the culture media, and the intracellular HBsAg, we confirmed indirubin possesses anti-viral activity against HBV. What’s more, the anti-viral mechanism of indirubin was through decreasing the expression of PTB.

Figure 3. The immunofluorescence for HBsAg in two group HepG2.2.15 cells, which were treated with indirubin (32 μg/ml) for 3 d. A and B. Cells were harvested, fixed and immunostained using monoclonal anti-HBsAg (green) antibody, DAPI (blue) was used to counterstain nuclei. The representative fluorescence images are shown (data from 3 independent experiments). The HBsAg fluorescence intensity was shown, which demonstrated fluorescence intensity (green color) in indirubin group significantly decreased. The data were analyzed using a two-tailed t test. (*, P<0.05).
Many compounds, including indigo, indirubin, isatin, trytanthrin, purin, isaindigo-tidione and so on, have been isolated from Isatisindigotica. Among them, the biological activity of Indigotin and indirubin are reported and used as the markers for quality control [25]. Indirubin, a 3,20 bisindole isomer of indigo, originates from the root of herbal plant Isatisindigotica. It has been used to treat respiratory viral infection and flu, some other diseases with inflammatory nature in TCM for a long time. With poor solubility and bioavailability of indirubin, several derivatives have been chemically synthesized to improve its functional activities. Our cytotoxicity analysis showed that indirubin (64 μg/ml) has no cytotoxicity to HepG2.2.15 cells even treated for 6 d. The results indicate that indirubin with low cytotoxicity, which may be the basis for the safety anti-viral drug development.

Studies have shown that indirubin and its derivatives are potent antiviral [6, 8]. The indirubin derivatives delay the virus replication, and strongly suppress the pro-inflammatory cytokines, on primary human culture models during influenza A virus infection. They may be used for the treatment of severe human H5N1 disease as an adjunct to antiviral therapy [8]. Indirubin derivatives have the immunomodulatory effects on pulmonary endothelium and the therapeutic potential of Influenza A virus (IAV) infection. Indirubin was found to reduce both the expression and production of RANTES in influenza virus-infected cells [9]. On the basis of the HBV antigen secretion analysis, our results indicate that indirubin inhibits HBsAg and HBeAg secretion efficiently. Indirubin also inhibited HBV-DNA efficiently. The results of immunofluorescence suggest that intracellular HBsAg was also decreased by indirubin.

PTB is primarily localized in the nucleus, as a ubiquitous RNA-binding protein. In the nucleus, its main function is to regulate the alternative splicing of many pre-mRNAs [26, 27]. However, in response to specific signals, such as viral infection, the protein can be shuttled from the nucleus to the cytoplasm [28]. The role of cytoplasmic PTB in the regulation of IRES-dependent translation of mRNAs has been extensively studied. In our study, the results indicated that the protein expression level of PTB was significantly reduced by indirubin. We considered that PTB might be a target against HBV for indirubin and beneficial for drug discovery. However, indirubin had no effect on the mRNA expression level of PTB.

The limitation of the study was the lack of animal study, which might weaken the evidence of HBV inhibition by indigo. The research was limited by the lack of reliable laboratory animals susceptible to HBV infection [29].

In conclusion, our study showed that indirubin not only could inhibit the release of HBsAg, HBeAg, and HBV DNA production, but also decreased the intracellular HBsAg. The inhibition mechanism of anti-HBV may be through
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decreasing the expression level of PTB. Our study indicates indirubin possess the potential inhibiting ability of HBV in vitro. However, the anti-HBV ability in animal model needs further study.

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Disclosure of conflict of interest

None.

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References


