Anti-hepatitis B Virus Activity of 8-epi-Kingside in *Jasminum officinale* var. *grandiflorum*

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**Abstract:** Objective To evaluate the effect of 8-epi-kingside (8-Epik) derived from the buds of *Jasminum officinale* var. *grandiflorum* (JOG) on hepatitis B virus (HBV) replication in HepG2 2.2.15 cell line in vitro and duck hepatitis B virus (DHBV) replication in ducklings in vivo. Methods The concentration of extracellular hepatitis B e antigen and hepatitis B surface antigen (HBsAg) in cell culture medium was determined by ELISA, respectively. The anti-HBV effects of 8-Epik were also demonstrated in the model of DHBV. 8-Epik was ip given (20, 40, and 80 mg/kg, twice daily) to the DHBV-infected ducklings for 10 d. The isotonic saline liquid diet was ip given as negative control and Lamivudine (50 mg/kg, twice daily) was given as positive control. DHBV DNA was measured at days 0 (T0), 5 (T5), 10 (T10), and day 3 after cessation of treatment (P3) by dot blotting. Results 8-Epik effectively blocked HBsAg secretion in HepG2 2.2.15 cells in a dose-dependent manner \([IC_{50} = (19.4 \pm 1.04) \mu g/mL]\). 8-Epik (40 or 80 mg/kg, ip, twice daily) also reduced viremia in DHBV-infected ducks. Conclusion Therefore, 8-Epik is warranted as a potential therapeutic agent for HBV infection.

**Key words:** anti-hepatitis B virus activity; duck hepatitis B virus; 8-epi-kingside; HepG2 2.2.15 cell line; *Jasminum officinale* var. *grandiflorum*

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**Introduction**

The hepatitis B virus (HBV) causes acute and chronic infections of the liver and is responsible for 1.2 million deaths annually (Gish, 2005; McMahon, 2005). Approximately, 80% of HBV carriers have different levels of hepatocyte destruction, which may develop into liver cirrhosis and hepatocellular carcinoma (Park, Song, and Chung, 2006). Several antiviral drugs have been approved for the treatment of hepatitis B, including interferon-\(\alpha\) and some nucleoside analogues. Interferon-\(\alpha\) is only partially effective for clinical use and is limited by its side effects. Lamivudine, a pyrimidine nucleoside analogue, suppresses HBV through the inhibition of reverse transcriptase, but the treatment often fails due to the emergence of mutations within the catalytic site of HBV DNA polymerase, which leads to drug-resistance in patients (Yuen and Lai, 2011). Therefore, there exists a significant unmet medical need for new safe and efficacious anti-HBV drugs.

Many screening efforts have been made to find antiviral agents from natural sources. Plants have long been used as remedies, and many are now being collected and examined in an attempt to identify the possible sources of antivirals (Abad *et al.*, 2000). In the last decade, as an alternative to conventional chemical drugs, a large number of phytochemicals have been recognized as a way to control the infections caused by viruses (Kalvatchev, Walder, and Garzaro, 1997; Yamasaki *et al.*, 1998). Natural compounds, because of their structural diversity, provide a large opportunity for screening the anti-HBV agents with novel structure and distinct mechanism of action.

The plants in *Jasminum* Thunb. (Oleaceae) are widely distributed in the temperate zone and semitropical region of Asia and Africa. The flowers of many plants, such as *J. sambac* (L.) Aiton, *J. polyanthum* Franch., *J.
nudiflorum Lindl., and J. lang Gagnepain, are used as traditional or folk remedies in China for the treatment of arthritis, hepatitis, conjunctivitis, gastritis, and diarrhea (Huang, Ding, and Zhao, 2001). The buds of J. officinale L. var. grandiflorum (L.) Kobuski (JOG) were used as a folk remedy for the treatment of hepatitis, dysmenorrhea, stomatitis, and duodenitis in South China (Nanjing University of Chinese Medicine, 2006). In our primary study, we selected some kinds of Chinese materia medica (CMM), which have long been used in the folk treatment of chronic hepatitis in China, for screening novel anti-HBV agents. Among them, the hydroalcoholic extract from the buds of JOG suppressed the expression of the hepatitis B surface antigen (HBsAg) in HepG2 2.2.15 system. Phytochemical study of the extracts led to the isolation of eleven secoiridoid glycosides including 8-epi-kingiside (8-Epik) and oleuropein (Zhao, Xia, and Dong, 2007; Zhao, Yin, and Dong, 2008). Recently, we have reported the strong inhibitory effects of oleuropein against HBV replication both in vitro and in vivo (Zhao, Yin, and Dong, 2009).

In this study, we have focused on the anti-HBV activity of 8-Epik both in vitro and in vivo. Our in vitro experiments in HepG2 2.2.15 cell line revealed that 8-Epik reduced the level of HBsAg without an effect on the viability of the cells. More importantly, we have demonstrated the in vivo anti-HBV activity of 8-Epik in DHBV-infected ducks in the absence of any obvious signs of toxicity. To our knowledge, 8-Epik has not been previously reported to possess the anti-HBV activity.

Materials and methods

8-Epik and reference drug

8-Epik (Fig. 1; purity > 99%) was isolated from the dried buds of Jasminum officinale L. var. grandiflorum (L.) Kobuski (Zhao, Yin, and Dong, 2008) which were collected in Guangdong province, China, in January 2005 and identified by Prof. LI Bin in Academy of Military Medical Science of China. A plant sample has been conserved in the specimen room of Academy of Military Medical Science of China. Lamivudine (3TC), as a positive control, was purchased from GlaxoSmithKline (Suzhou, China).

Cell culture and treatment

HepG2 2.2.15, an HBV-transfect human HepG2 cell line (Sells, Chen, and Acs, 1987), was routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibico, USA) supplemented with 10% fetal calf serum (Gibico, USA) and antibiotics (100 U/mL Penicillin and 100 µg/mL Streptomycin) at 37 °C in a humidified incubator at 5% CO2 (Guo et al., 2007), and 380 µg/mL G418 in the medium was added to select the HepG2 2.2.15 cell line (Shin, Kang, and Lee, 2005). Cells at 2 × 10^4 cells per well (96-well microplates, Falcon, Oxnard, CA, USA) were treated with 8-Epik at 37 °C in duplication every 4 d for 8 d. The HepG2 2.2.15 cells as control were washed twice with phosphate-buffered saline (PBS) and re-fed with culture medium every 4 d for 8 d. Then the corresponding suspension was collected to analyze the levels of HBsAg and hepatitis B e antigen (HBeAg) (Zhou et al., 2007). The concentration of 8-Epik was 1.6, 3.2, 6.3, 12.5, 25.0, and 50.0 µg/mL, respectively.

Quantification of HBsAg and HBeAg

After incubation with various concentration of 8-Epik at 37 °C in 5% CO2 for 8 d, the concentration of HBsAg and HBeAg in culture supernatants of HepG2 2.2.15 cells was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Kewei Diagnostic Reagent Co., Beijing, China) and quantified relative to a standard curve of serial dilutions of recombinant HBsAg or HBeAg (Zhou et al., 2007). The concentration of 8-Epik was 1.6, 3.2, 6.3, 12.5, 25.0, and 50.0 µg/mL, respectively.

Cytotoxicity assay by MTT

The cytotoxicity of 8-Epik was analyzed by MTT assay. HepG2 2.2.15 cells were cultivated at 2 × 10^4 cells/well into 96-well microplate in 100 µL culture medium and grown for 48 h to reach approximate confluence conditions. Cells were treated with or without 8-Epik in duplication every 4 d for 8 d, and then 10 µL of MTT (5 g/mL) was added to each well and the solutions were further incubated in CO2 incubator at 37 °C for 4 h. The A values at 560 nm were obtained. Data were calculated as a percentage of negative control cells that were not treated with 8-Epik (Rubinstein et al., 1990). The concentration of 8-Epik was 12.5, 25.0, 50.0, 75.0, and 150.0 µg/mL, respectively.
The cytotoxicity of 8-Epik was measured to determine the treatment concentration of 8-Epik in the HepG2 2.2.15 cell culture system.

**Animals and treatment**

Ducklings at age of 1 d were purchased from Nanjing Qianjin Fowl Inc (China) and maintained under a 12 h/12 h light/dark cycle with a standard commercial diet and water *ad libitum*. All animals received human care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by National Academy of Sciences and published by National Institutes of Health.

Sera from ducks that are congenitally infected with DHBV are the sources of DHBV used for inoculation (Zoulim *et al.*, 1996). Duck serum positive for duck HBsAg (DHBsAg) at 1:1000 of dilution was used for infection. Ducklings were iv injected with DHBV at a dose of 0.2 mL DHBV (+) serum per duckling (containing $5.7 \times 10^6$ cpm of DHBV DNA equivalents). Seven days later, animals were administered with 8-Epik (20, 40, or 80 mg/kg, twice daily) for 10 d. 8-Epik was solubilized in isotonic saline solution and ig given in a liquid diet that was also given to the animals as negative control. Lamivudine (50 mg/kg, ip, twice daily) was used as positive control. DHBV DNA was measured at days 0 (T0), 5 (T5), 10 (T10), and day 3 after cessation of treatment at 10 d (P3) by dot blotting.

**Assay for DHBV DNA**

A previously reported method (Wang, 2000) was used with modifications to detect the concentration of DHBV DNA in the serum. Briefly, duckling serum (50 µL) was directly spotted on the nitrocellulose membrane, and DHBV DNA was detected with 5'-[alpha-32P] deoxycytidine labeled full-length DHBV genomic DNA. Incorporation of radioactivity was determined by Molecular Dynamics Storage Phosphor Screen Cassette (Amersham Bioscience). The phosphor screen was scanned with a Typhoon 9410 Scanner (Amersham Bioscience) and quantified by using the ImageQuant (Molecular Dynamics) software.

**Statistics**

To compare the values before and after treatment, a One-way ANOVA *F*-test was employed. Differences among study groups were tested by unpaired student *t*-test for equal or unequal variances depending on a preliminary *F*-test for the homogeneity of variance. The threshold of significance was set at $P = 0.05$ (Zhou *et al.*, 2007).

**Results**

**Cytotoxic effect of 8-Epik on HepG2 2.2.15 cell viability**

We first investigated the cytotoxic effect of 8-Epik on cell viability in HepG2 2.2.15 cells. 8-Epik exhibited a weak inhibitory effect on the proliferation of HepG2 2.2.15 cells, with about 10% and 30% inhibition at the concentration of 75 and 150 µg/mL, respectively. However, 8-Epik (50 µg/mL) showed no inhibitory effect on the proliferation of the cells. TC50 of 8-Epik, defined as the concentration that inhibited 50% cellular growth in comparison to the untreated controls, was over 150 µg/mL in HepG2 2.2.15 cells.

**Anti-HBV activity of 8-Epik in HepG2 2.2.15 cells**

Treatment of HepG2 2.2.15 cells with 8-Epik at various concentration for 8 d resulted in significant reduction of HBsAg secretion in a dose-dependent manner ($P < 0.05$), with an IC50 value of $(19.4 \pm 1.04) \mu$g/mL (Fig. 2). For HBeAg secretion, there was only a minimal inhibitory effect of 8-Epik at day 8.

**In vivo anti-HBV activity of 8-Epik in ducklings**

DHBV-infected ducklings were treated with 8-Epik at various doses or with 3TC at 50 mg/kg twice a day for 10 d. Plasma samples were taken at days 0, 5, 10, and day 3 after the cessation of 8-Epik or 3TC treatment ($n = 6$), respectively. DHBV DNA levels were measured by dot hybridization (Fig. 3). 8-Epik at dosages of 40 and 80 mg/kg significantly decreased the DHBV DNA level in duck serum. Densitometric quantity of the dots revealed a significant increase in DHBV inhibitory rate to 46.1% and 31.6% in 8-Epik-treated (80 mg/kg) ducks compared with the untreated

![Fig. 2 Effect of 8-Epik on expression of HBsAg in HepG2 2.2.15 cells (X ± s, n = 6)](image-url)

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**Fig. 3** *In vivo* inhibitory effect of 8-Epik on DHBV DNA level in duck plasma ($\bar{X} \pm s, n = 6$)

Ducks as control at days 5 and 10 ($n = 6; P < 0.05$ for day 5; $P < 0.01$ for day 10). DHBV DNA levels in the 8-Epik-treated groups (40 mg/kg) were still lower than those of control group at days 5 and 10 ($n = 6, P < 0.05$ for days 5 and 10), respectively. The inhibition declined to a non-significant level when the dose of 8-Epik was reduced to 20 mg/kg. There were no significant differences in DHBV DNA level among any of the controls. The body weights of the ducklings treated with 8-Epik or 3TC were not significantly different from those of the untreated ducklings ($P = 0.41$).

**Discussion**

Some decades ago, Western scientists started to look for novel drugs from the remedies used in traditional or folk CMM (Yao, Tang, and Huang, 2001; Chen and Chen, 1998). Several therapeutic methods, including the use of herbal therapy, were stemmed from the vast traditional Chinese medicine experience over hundreds of years and have been selected (Dhiman and Chawla, 2005). JOG is frequently used as a crude medicine for treating liver diseases in South China. In our continued investigation on searching for safe and effective anti-HBV agents from CMM, we found that the hydroalcoholic extract from the buds of JOG showed the preferable antiviral efficacy against HBV replication in HepG2 2.2.15 cell line *in vitro*. 8-Epik was a pure water-soluble iridoid glycoside isolated from the hydroalcoholic extract in the buds of JOG (Zhao, Yin, and Dong, 2008).

In this study, we have firstly evaluated the efficacious *in vitro* anti-HBV activity of 8-Epik which was extracted from the buds of JOG. In HBV-transfected HepG2 2.2.15 cells, 8-Epik effectively suppressed the secretion of HBsAg in a dose-dependent manner with an IC$_{50}$ value of $(19.4 \pm 1.04)$ µg/mL. The mechanism of how 8-Epik suppressed HBsAg gene expression in HepG2 2.2.15 cells was not yet clear. 8-Epik might incorporate into nascent viral DNA by the HBV polymerase during replication, which resulted in the termination of DNA elongation as 3TC did (Cammack *et al.*, 1992). Alternatively, 8-Epik might target the cell membrane and deliver an inhibitory signal to the nucleus to suppress HBsAg gene...
expression as insulin did (Ting, Tu, Chou, 1989; Chou et al, 1989). 8-Epik exhibited a weak inhibitory effect on the proliferation of HepG2 2.2.15 cells, with about 10% and 30% inhibition at 75 and 150 µg/mL, respectively. When the dose was reduced to 50 µg/mL, 8-Epik showed no inhibitory effect on the proliferation of the cells. TC_{50} of 8-Epik was over 150 µg/mL in HepG2 2.2.15 cells.

Furthermore, the present study has demonstrated for the first time that 8-Epik had a potential anti-HBV activity in vivo. The in vivo anti-HBV activity was investigated in DHBV-infected ducks (Guha et al, 2004; Schultz, Grjacic, and Nassal, 2004), which was a well established animal model for the pharmacological anti-HBV studies. In DHBV-infected ducks, 8-Epik (40 or 80 mg/kg, twice daily) was ip given for 5 and 10 d and the plasma DHBV DNA levels were reduced significantly. The mechanism of how 8-Epik reduced viremia in DHBV-infected ducks is not yet clear. More work is needed in our laboratory to investigate the mechanism of anti-HBV activity of 8-Epik.

Our results indicate the efficacious anti-HBV activity of 8-Epik both in vitro and in vivo. It will greatly enhance our understanding on the ethnopharmacology of JOG and other plants that contain 8-Epik. Thus, 8-Epik seems to be worth further study as an anti-HBV leading structure. These findings might suggest another therapeutic option, other than nucleoside analogues, to control the chronic HBV infection in the future.

References