Anti-hepatitis B Virus Activities of Astragaloside IV Isolated from *Radix Astragali*

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Total ethanol extract and saponins from Chinese herb *Radix Astragali* (Huangqí) have been previously shown to possess anti-hepatitis B virus (HBV) activities in vitro. To identify the active ingredients, we isolated a triterpenoid saponin that was determined to be astragaloside IV. In the human HBV-transfected liver cell line HepG2, astragaloside IV effectively suppressed secretion of HBV antigens with inhibition rates of 23.6% for the secretion of Hepatitis B surface antigen (HBsAg) and 22.9% for that of Hepatitis B e antigen (HBeAg) at 100 μg/ml after 9 d of treatment. The inhibitory activity of astragaloside IV on secretion of HBV antigens is more potent than that of 3TC without significant cytotoxicity. In duck hepatitis B virus (DHBV)-infected ducklings, astragaloside IV caused 64.0% inhibition at 120 mg/kg, 49.6% inhibition at 40 mg/kg, and 41.7% inhibition at 10 mg/kg to serum DHBVs after 10 d of treatment, and also reduced serum DHBV DNA levels. Together, our results demonstrate that astragaloside IV possesses potent anti-HBV activity.

**Keys words** astragaloside IV; *Radix Astragali*; anti-hepatitis B virus activity

Hepatitis B remains a major public health problem worldwide and hepatitis B virus (HBV) is the chief inductive factor of acute and chronic hepatitis. Several anti-viral drugs have been approved for the treatment of hepatitis B; however they cause significant complications such as dose-dependent side-effects and drug resistance. There exists a significant unmet medical need for safe and efficacious new anti-HBV drugs and finding new anti-HBV agents is still a big challenge. Due to the well-known potency of Chinese herbs in diverse disease areas, we are interested in studying their anti-HBV activities.

*Radix Astragali* (Huangqí), the dried root of *Astragalus membranaceus* (Fisch.) Bge. var. mongholicus (Bge.) Hsiao, or *A. membranaceus* (Fisch.) Bge., is one of the most widely prescribed Chinese herbs in many formulas. It has been widely used in Chinese medicine since ancient times with an excellent safety record and demonstrated efficacy in the improvement of immune disorders and liver diseases. The major active constituents of *Radix Astragali* are believed to be the total saponins and the total flavonoids. *Astragaloside IV* (AS) is a naturally occurring saponin isolated from *Radix Astragali*, and has been used for the quality evaluation of *Radix Astragali*, as listed in the 2005 edition of Pharmacopoeia of the People’s Republic of China. In recent years, as a major saponin of this herb, astragaloside IV has been shown to possess many pharmacologic activities including anti-cancer, anti-fatigue, anti-coxsackie B virus, and anti-inflammatory activities. However, there have not been many studies on its anti-viral activities. Particularly, to date there has been only one study on its anti-viral activity against HBV. In this study, further to investigate this well-known Chinese medicine for its anti-HBV activity, we isolated astragaloside IV from *Radix Astragali* by the bioactivity-guided method and evaluated its anti-HBV activity both in vitro and in vivo.

**MATERIALS AND METHODS**

**Chemicals** Lamivudine [(-)-β-L-2',3'-dideoxy-3-thiacytidine] (3TC) was purchased from GlaxoSmithKline, Inc. (U.S.A.). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (U.S.A.). Ethanol, chloroform, isopropanol, and HCl were obtained from Sinopharm Chemical Reagent Co., Ltd. (China).

**Extraction, Isolation, and Identification of Astragaloside IV** Dried rhizomes (5 kg) of *Radix Astragali*, collected in the Mongolia province of China, were cut into pieces and refluxed for 2 h with 95% EtOH for three times. The extract was filtered and concentrated under reduced pressure, then the ethanol extract concentrate was dissolved in H2O and reflushed for 2 h with 95% EtOH for three times. The extract was loaded on a D101 column, a type of macroporous resin (Shanghai Yadong Nuclear Grade Resines Co., Ltd.), and successively eluted with a gradient of EtOH–H2O solutions (30, 50, 90%). Three fractions (A—C) obtained were then assayed for their anti-HBV activity. Fraction B showed

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anti-HBV activity, and was further purified by column chromatography with an 8.0×30.0 cm column loaded with 500 g silica gel of 200—300 mesh size (Qingdao Marine Chemical Factory, China). After elution with chloroform: ethanol: H$_2$O solution (75: 25 :3), fraction #1 was subjected to subsequent chromatography with a 2.0×69 cm LH-20 column loaded with 25 g Sephadex (Sigma, U.S.A.), then eluted with chloroform/ethanol (5 :5). Compound I (105 mg) in the eluent I was determined to be the anti-HBV fraction by bioactivity-guided tests.

The purified compound I was white crystals (MeOH) with an mp 307—308 °C. [α]$^2$$^0$_20 +14.37° (c=0.5, MeOH) (JASCO P-1020). The ESI mass spectrum showed peaks at m/z 807.4 [M+Na]$^+$, 823.4 [M+K]$^+$, indicating a molecular formula of C$_{41}$H$_{68}$O$_{14}$. It was later identified as astragaloside IV based on its $^1$H-NMR (pyridine-$d_5$) and $^{13}$C-NMR (pyridine-$d_5$) data that were in agreement with those from the literature.$^9$

**Cell Culture** The human HBV-infected cell line HepG$_2$ 2.2.15$^9$ was from the Institute of Medical Technology, Peking Union Medical College and Chinese Academy of Medical Sciences. The cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l l-glutamine (all from Invitrogen, U.S.A.) at 37 °C in an atmosphere of 5% CO$_2$ and a 100% humidity. Test reagents were dissolved in DMSO and lamivudine (100 μg/ml streptomycin) at the beginning of drug treatment, blood samples were collected from the DHBV-infected ducks and sera obtained. The DHBsAg (S antigen of DHBV) in the serum samples was assayed by dot-blotting based on a reported method using anti-DHBsAg antibody.$^{12}$

All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health.

**Southern-Blot Analysis of DHBV DNA in Duck Liver** One gram of duck liver tissues was ground in 4 ml of a buffer (10 mM Tris–HCl, pH 7.6, 0.15 M NaCl, 1.27 mM EDTA, 20 mg/ml SDS, 5 μg/ml salmon sperm DNA, and 0.5 mg/ml proteinase K) at 50 °C for 3 h, and centrifuged at 13000×g for 10 min. The supernatant was extracted with phenol/chloroform and DNA precipitated with two volumes of ethanol and 1/10 volume of acetic acid. The DNA was then dissolved in 800 μl of TE buffer (10 mM Tris–HCl, pH 7.5, containing 2 mM EDTA and 100 μg/ml RNase A) and separated on a 1% agarose gel. The gel with fractionated DNA samples was then subjected to Southern blotting and hybridized with a DHBV DNA probe as previously described.$^{13}$

**RESULTS**

**In Vitro Anti-HBV Activity of Astragaloside IV in HepG$_2$ 2.2.15 Cells** The cytotoxicity of astragaloside IV was measured in cultured HepG$_2$ 2.2.15 cells. It showed no inhibitory effect on cell proliferation at a concentration up to 200 μg/ml, as analyzed by MTT assay. The 50% cytotoxic concentration was determined to be 388 μg/ml. These results were further used to determine the dose range of astragaloside IV for the following experiments.

Astragaloside IV effectively suppressed secretion of HBV antigens from virus-infected HepG$_2$ 2.2.15 cells, achieving 31.8%, 23.6%, and 19.7% inhibition to the secretion of HBsAg, and 23.3%, 22.9%, and 19.2% inhibition to that of HBeAg, respectively, at 200, 100, and 40 μg/ml after 9 d of treatment (Fig. 2). In the same experiment, 3TC at 100 μg/ml also suppressed the secretion of both HBsAg and HBeAg, at the rates of 20.1% and 19.7%, respectively (Fig. 2). Apparently the inhibitory activity of astragaloside IV on the secretion of HBV antigens is more potent than that of 3TC.

**In Vivo Anti-HBV Activity of Astragaloside IV in Ducks** During the experiments, all ducks were raised...
under the same standard conditions. The drug treatments exhibited no significant toxicity to the animals since all ducklings in the experimental and control groups grew equally well, and there were no significant weight losses or gains or abnormal behaviors observed in the animals while on the treatment scheme.

As shown in Fig. 3, astragaloside IV reduced DHBV levels in the serum of infected ducks, achieving 64.0% inhibition at 120 mg/kg, 49.6% inhibition at 40 mg/kg, and 41.7% inhibition at 10 mg/kg after 10 d of treatment. Interestingly, at day 13 (3 d after the end of treatment), the inhibition rates of astragaloside IV on serum DHBVs were 69.1%, 48.2%, 49.4%, respectively, at the three concentrations tested. At the same time, the inhibition rate of 3TC at 200 mg/kg was 68.5% on day 10 and 52.6% on day 13 (Fig. 3).

Further to investigate the in vivo anti-HBV effect of astragaloside IV in ducks, DHBV DNA levels in the liver of infected ducks obtained on day 3 after the end of treatment were examined by Southern blotting. Densitometric analysis of the autoradiographic signals showed inhibition rates of 18.3%, 16.5%, and 0% by astragaloside IV at 120, 40, and 10 mg/kg, respectively, and 40.1% inhibition by 3TC at 200 mg/kg.

DISCUSSION

The anti-HBV activity of astragaloside IV was confirmed from our in vitro experiments by its suppression of HBV secretion from HepG2 2.2.15 cells. More importantly, this activity was confirmed by its ability to reduce serum DHBV and its DNA levels in the DHBV-infected ducks.

To date, there has been only one report on the anti-HBV activity of astragaloside IV and another isolated report on the anti-HBV activity of total saponins of Radix Astragali. Previously, we isolated anti-HBV compounds from total saponins and examined the inhibitory effect of astragaloside IV on HBV in vitro and in vivo. Here, we show that astragaloside IV exhibited potent inhibitory activity against antigen secretion in HBV-transfected HepG2 2.2.15 cells, which is consistent with the previous report. In the present study, astragaloside IV inhibited 23.6% of the HBsAg expression and 22.9% of HBeAg secretion from cultured HepG2 2.2.15 cells at a concentration of 200 μg/ml. Because human HepG2 2.2.15 cells carry multiple copies of HBV DNA stably integrated into the host cell genome, they well resemble infected human liver cells, therefore offering a great cell system. Indeed, they have been widely used for the evaluation of anti-HBV drugs. It is worth noting that the potency of astragaloside IV was much higher than the known anti-HBV drug 3TC. 3TC is known to reduce HBsAg by a mechanism where it is phosphorylated inside HepG2 2.2.15 cells and subsequently incorporated into nascent viral DNA by HBV polymerase during replication.

Since a human system is not practical, we used the duck DHBV model that has been widely used for studies of in vivo activity and toxicity of anti-HBV agents, as shown in many similar studies. The present study demonstrates, for the first time, that astragaloside IV has potent anti-HBV activity in vivo in DHBV-infected ducks. Astragaloside IV reduced levels of DHBsAg in the duck sera. In addition, astragaloside IV showed 18.3% inhibition of DHBV DNA levels in the liver at 120 mg/kg, and 3TC showed 40.1% inhibition at 200 mg/kg. Inhibition of DHBV-DNA by astragaloside IV was not as high as that by 3TC. This is most likely due to its low solubility in water, leading to a relatively lower bioavailability of the drug via oral administration. Interestingly, the anti-HBV effects of astragaloside IV on DHBVs lasted for
indicating that astragaloside IV is a relatively long-lasting drug in ducks as compared with the effects of 3TC. This long duration of astragaloside IV’s activity may offer better clinical benefits and therefore supports the significance of developing astragaloside IV as an anti-HBV drug.

In conclusion, our data demonstrate that astragaloside IV has potent anti-HBV activity, and deserves to be further evaluated for the treatment of human HBV infection. Currently, astragaloside IV is under early development as an anti-HBV drug candidate.

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REFERENCES
