In Vitro Antiviral Activity of 1,2,3,4,6-Penta-*O*-galloyl- β -D-glucose against Hepatitis B Virus

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This study examined the antiviral activity of the root of *Paeonia lactiflora* PALL. Among the solvent fractions of the crude drug, the ethyl acetate fraction showed anti-hepatitis B virus (HBV) activity (IC₅₀, 8.1 μ g/ml) in an HBV-producing HepG2.2.15 cell culture system. The active anti-HBV principle was isolated and identified as 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (PGG) from the crude drug by activity-guided fractionation. PGG isolated from *P. lactiflora* was examined for the inhibition of HBV multiplication by measurement of HBV DNA and hepatitis B surface antigen (HBsAg) levels in the extracellular medium of HepG2.2.15 cells after 8-d treatment. PGG decreased the level of extracellular HBV (IC₅₀, 1.0 μ g/ml) in a dose-dependent manner. PGG also reduced the HBsAg level by 25% at a concentration of 4 μ g/ml. The gallate structure of PGG may play a critical role in the inhibition of anti-HBV activity. These results suggest that PGG could be a candidate for developing an anti-HBV agent.

Key words *Paeonia lactiflora*; 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose; hepatitis B virus; hepatitis B surface antigen; HepG2.2.15 cell; anti-HBV activity

Hepatitis B virus (HBV) causes acute and chronic hepatitis, which affects nearly 350 million people worldwide. Moreover, HBV plays a critical role in the development of chronic hepatitis and hepatocellular carcinoma.¹⁾ Since the non-human host range of HBV is restricted to a few animals, such as the chimpanzee, the development of new therapeutics for HBV has been hampered.²⁾ A HepG2-derived HBV producing cell line was developed to identify potential therapeutics against HBV infection.³⁾ The HepG2.2.15 cell line, derived through transfection of a cloned HBV gene into the human hepatoblastoma cell line HepG2, was established for potential anti-HBV active agents.^{4–6)} In this system, extracellular levels of viral DNA and intracellular viral DNA replication intermediates were used as reliable markers for HBV DNA replication, which could be analyzed in a quantitative manner using the blot hybridization technique.⁴⁾

The root of *Paeonia lactiflora* PALL. is used in many traditional prescriptions in China and Korea. It is commonly used in nourishing blood, activating circulation, alleviating pain, regulating menstruation, and treating liver disease and cancer. We examined the antiviral activity of the root of *P. lactiflora* and identified 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (PGG) from the crude drug by activity-guided fractionation. PGG is a major bioactive constituent of the root of *P. lactiflora*, the root cortex of *Paeonia suffruticosa* ANDR.,⁷⁾ and the root of *Sanguisorba officinalis* L.⁸⁾ It has been reported that PGG has several activities such as anti-mutagenicity in the Ames test of benzo(α)pyrene,⁹⁾ chemoprevention in tumor promoter-mediated oxidative processes,¹⁰⁾ anti-invasive activity in highly metastatic mouse melanoma,¹¹⁾ antiviral effects against respiratory syncytial virus (RSV) multiplication.¹²⁾

In the present study, PGG from the root of *P. lactiflora* was examined for the inhibition of HBV replication, and HBsAg and HBsAg production in HepG2.2.15 cells.

MATERIALS AND METHODS

Plant Species The roots of *P. lactiflora* PALL. were purchased from an herb market in Seoul and voucher specimens (NPRI-A025) were deposited at the Natural Products Resources Depository of the Natural Products Research Institute, College of Pharmacy, Seoul National University.

Extraction and Purification The roots of *P. lactiflora* (8 kg) were extracted three times with methanol and the methanol extracts were partitioned into *n*-hexane, ethyl acetate, and water fractions. The ethyl acetate fraction was chromatographed over a silica gel column eluted with a gradient of cyclo-hexane/isopropyl alcohol mixtures (100:0 $\rightarrow$ 0:100) and then PGG (53 mg) was purified on a reverse-phase C18 column using water/acetonitrile mixtures (100:0 $\rightarrow$ 0:100). The structure of PGG was identified based on ¹H-, ¹³C-NMR, and electron ionization mass spectrometer spectral data together with published data.¹³⁾

Cell Culture The HepG2.2.15 cell line (kindly supplied by Choongwae Pharma Corporation, Korea) which is stably transfected with HBV DNA, was grown in RPMI-1640 culture media containing geneticin (G418) 380 μ g/ml with 5% fetal bovine serum (FBS), glutamine 2 mM, gentamycin sulfate 50 μ g/ml. Cells were incubated at 37 °C in a 5% CO₂.

Cell Viability Assay PGG and 3TC, an inhibitor of HBV reverse transcriptase,¹⁴⁾ were evaluated for cell viability by measuring cell survival at the end of the each experiment using the crystal violet staining and MTT methods. For crystal violet staining, after incubation for 8 d, culture medium was removed and 0.2 ml of crystal violet solution (0.2% crystal violet in 2% EtOH) was added to each of 24 wells. After incubation for 10 min, free crystal violet dye was removed by washing with PBS. The bound dye was solubilized with the addition of 50% ethanol solution containing 0.5% SDS. After gentle shaking for 1 h, the absorbance at 610 nm was measured using an ELISA reader. Each experiment was performed in duplicate.

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Cell viability also was measured based on the mitochondrial-dependent reduction of MTT to formazan. Briefly, cells were seeded at a density of 5×10^4 cells/ml in 96-well plates. After incubating for 24 h, cells were treated with test samples. The cells were incubated for an additional 48 h. A 50 μ l aliquot of the MTT solution (5 mg/ml) was added to each well of the assay plate, which was then incubated for a further 4 h at 37 °C. The medium was then removed by aspiration and MTT-formazan production was solubilized in DMSO 200 μ l. The extent of MTT reduction to formazan within the cells was quantified by measuring absorbance at 540 nm using an ELISA reader. Each experiment was performed in triplicate.

Anti-HBV Assay Cultured HepG2.2.15 cells were seeded into 24-well plates at a density of 3×10^4 cells/well and grown to confluence (approximately 4 d). During the 8-d treatment period, the culture medium was removed and test compound was added to the cultures in fresh culture medium every 2 d. An additional 2 d of incubation was performed without samples. On the 14th day, 400 μ l of culture medium was collected. The collected medium was stored at -70 °C for later analysis of extracellular viral DNA.

DNA Extraction and Slot Blot Hybridization From the culture medium, the extracellular viral DNA was analyzed using the slot blot hybridization technique.⁵⁾ For the analysis of extracellular HBV DNA, 0.4 ml of culture medium was incubated for 1 h at 25 °C in 1 N NaOH/10 $\times$ SSC (1 $\times$ SSC is NaCl 0.15 M/Sodium Citrate 0.015 M, pH 7.2) and then directly applied to a nylon membrane using a Bio-Dot SF apparatus. Samples were neutralized by washing twice with 500 μ l of Tris-HCl 1 M (pH 7.2)/ NaCl 2 M and once with 500 μ l of 20 $\times$ SSC. The wet membrane was crosslinked with UV (254 nm) without prior washing. The membrane was briefly rinsed in double-distilled water and allowed to air dry. The HBV probe (677 bp) was amplified from viral DNA in the HepG2.2.15 cells using a PCR amplifier and the blot was hybridized with digoxigenin-labeled probes. Hybridization was performed at 42 °C for 16 h. The anti-HBV activity of a treated sample was determined by comparison with the control group (DMSO) using chemiluminescence in a bioimaging analyzer LAS-1500 (Fujifilm). In each experiment, DMSO solution (final 0.5%) was used as negative control and 3TC [(-)- β -L-2',3'-dideoxy-3-thiacytidine] was used as a positive control.

HBeAg and HBsAg Analysis HBV antigen levels in culture medium were measured using an enzyme immunoassay kit (Green Cross Inc., Korea). HepG2.2.15 cells were treated with test compounds as described above. Culture media were collected for HBeAg and HBsAg assays. The assay was performed according to the manufacturer's instructions.

RESULTS

Effects of the Root of *P. lactiflora* PALL. on HBV DNA Replication The antiviral effects of the solvent fractions from the root of *P. lactiflora* PALL. were evaluated in the HepG2.2.15 cell culture system. As shown Table 1, the ethyl acetate fraction showed strong anti-HBV activity (IC_{50} , 8.1 μ g/ml) but the other fractions showed no inhibition of HBV DNA level at a concentration of 20 μ g/ml. PGG was

Table 1. Inhibition of HBV DNA Replication in HepG2.2.15 Cells by the Solvent Fractions from the Root of *Paeonia lactiflora* PALL.

Fraction (20 μ g/ml)	Inhibition of HBV DNA replication (%)	Cell viability (%) (crystal violet)	Cell viability (%) (MTT)
Hexane	7.5 $\pm$ 2.7	96.2 $\pm$ 3.1	96.7 $\pm$ 4.3
Ethyl acetate	72.5 $\pm$ 5.3**	95.5 $\pm$ 4.3	94.2 $\pm$ 5.4
Aqueous	1.4 $\pm$ 2.1	99.7 $\pm$ 4.1	98.9 $\pm$ 4.2

Each value represents the mean $\pm$ standard deviation obtained from 3 repeated experiments. ** Significantly different from control ($p < 0.01$).

Fig. 1. Structures of PGG, Gallic Acid, and Gallacetophenone

identified from the crude drug by activity guided fractionation. The chemical structures of PGG, gallic acid, and gallacetophenone used in this study are shown in Fig. 1.

Effects of PGG on HBV DNA Replication We examined the effect of PGG on cell viability using crystal violet staining. Cell viability was not changed in the presence of PGG up to the concentration of 4 μ g/ml, as previously reported.¹⁵⁾ Thus the concentration of the PGG in the range of 0.16–4 μ g/ml was chosen in the subsequent experiments.

In next experiment, we examined the effects of PGG and 3TC on the HBV DNA levels in the culture supernatant of HepG2.2.15 cells transfected with the HBV gene. As shown Fig. 2, treatment with PGG and 3TC inhibited the replication of HBV DNA dose dependently 8 d after treatment. In this experiment using HepG2.2.15 cells, the IC_{50} value of PGG against HBV was 1.0 μ g/ml. The positive control, 3TC-treated group had almost completely suppressed HBV replication as previously reported.¹⁶⁾ In this study using HepG2.2.15 cells, the IC_{50} value of 3TC against HBV was determined to be 0.06 μ g/ml.

Since PGG, which has five gallic acids, showed potent inhibitory effects, we examined the effect of gallic acid and gallacetophenone. As shown Fig. 2, gallic acid showed weak

Fig. 2. Effects of the PGG, Gallic Acid, and 3TC on HBV DNA Replication in HepG2.2.15 Cells

The HepG2.2.15 cells were treated with various concentrations of 3TC as a positive control, PGG, and gallic acid for 8 d. After incubation, HBV DNA in culture medium was harvested and analyzed by slot blot hybridization as described in the Materials and Methods. Data represent three independent experiments each performed in duplicate.

inhibitory effects (IC₅₀, 10 μg/ml) and gallacetophenone showed little inhibition of the viral DNA levels up to the concentration of 20 μg/ml (data not shown).

Effects of PGG on HBeAg and HBsAg Protein Production In the next experiment, the effects of PGG on HBeAg and HBsAg secretion in the culture media of HepG2.2.15 cells were determined. As observed for the replication of viral DNA, PGG also reduced the HBsAg, not HBeAg, level in HepG2.2.15 cell culture medium by 25% at the concentration of 4 μg/ml (Fig. 3). The assay results for HBsAg level confirmed the inhibitory effects of PGG on the levels of HBV DNA released from HepG2.2.15 cells. However gallic acid and gallacetophenone did not reduce HBeAg and HBsAg (data not shown).

DISCUSSION

Chronic HBV infections can be followed using several serologic markers, including HBV DNA, HBsAg, anti-hepatitis B core antigen (anti-HBcAg), HBeAg, and alanine aminotransferase. Particularly, HBV DNA levels have been monitored when assessing the efficacy of anti-HBV therapy. Thus we examined the effects of PGG on the HBV DNA levels in the culture supernatant of the HepG2.2.15 cell line transfected with HBV gene.

PGG, which has five gallic acids, showed strong inhibitory effects (IC₅₀, 1.0 μg/ml), but gallic acid showed weak inhibitory effects (IC₅₀, 10 μg/ml), and gallacetophenone showed little inhibition of the viral DNA levels up to the concentration of 20 μg/ml. It is reported that tannins with galloyl

Fig. 3. Effects of PGG on HBV Surface Antigen (HBsAg) Level in HepG2.2.15 Culture Medium

The HepG2.2.15 cells were treated with various concentrations of PGG for 8 d. After incubation, HBV protein level in culture medium was measured using an enzyme immunoassay kit. The values are expressed as mean ± S.D. of triplicate tests. **p* < 0.05 vs. control values.

groups have a potent anti-herpes simplex virus activity and that gallic acid showed little activity.¹⁷⁾ This suggests that the gallate structure of PGG, not gallic acid moieties, may play a critical role in the inhibition of HBV DNA replication.

3TC, an inhibitor of HBV reverse transcriptase, also was found to be inactive in the assay for HBeAg and HBsAg secretion, as previously reported.¹⁸⁾ These results show that there is no correlation between the inhibition of HBeAg and HBsAg levels and the inhibition of HBV DNA level. It was suggested that HBV DNA level better reflects the inhibitory effects of different drugs and can be used as an important index for evaluation of anti-HBV effects *in vitro*.¹⁸⁾

The HBV genome encodes for three related envelope proteins termed L (large), M (middle), and S (small) HBsAg. The envelope proteins play an essential role in virion morphogenesis and secretion. L and S proteins are necessary for virion secretion,^{19,20)} although the role of M is in doubt.^{19,21)} The current model of virion formation involves nucleocapsids budding into the lumen of the endoplasmic reticulum, after acquiring membranes that have been appropriately modified with virion glycoproteins.²¹⁾ Glycosidases including glucosidases are essential not only to carbohydrate digestion but also to the processing of glycoproteins and glycolipids in virus. It is reported that glucosidase inhibitors are potent inhibitors of HIV replication and HIV-mediated syncytium formation *in vitro*,²²⁾ and secretion of HBV particles.²³⁾ It is reported that PGG shows inhibitory effect on rat intestinal α-glucosidase.²⁴⁾ Therefore α-glucosidase inhibitors may be useful for the screening of anti-HBV inhibitors.

PGG exists in the root of *S. officinalis* L., the root of *P. lactiflora* PALL., and the root cortex of *P. suffruticosa* ANDR. Recently, it was reported that aqueous extract from the root of *S. officinalis* L. has anti-HBV activity in HepG2.2.15 cell culture system²⁵⁾ and against duck HBV.²⁶⁾ Methanolic extract of *P. suffruticosa* prevented the process of herpes simplex virus attachment and penetration.²⁷⁾ Therefore PGG may be responsible for the anti-viral activity of these crude drugs.

In summary, the results of the present study indicate that PGG decreases the level of extracellular HBV and surface protein HBsAg in a dose-dependent manner. Although further studies are needed to elucidate the molecular mechanisms, these results suggest that PGG could be a candidate

for developing an anti-HBV agent.

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