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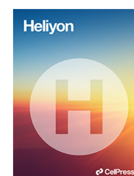
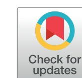


Research article

Selected ginsenosides interfere efficiently with hepatitis B virus mRNA expression levels and suppress viral surface antigen secretion



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ABSTRACT

Ginsenosides are a class of natural steroid glycosides and triterpene saponins found in *Panax ginseng*. After screening of a commercial ginsenoside compound library for low cellular cytotoxicity and the ability to mediate efficient reductions in hepatitis B virus (HBV) mRNA expression levels in HepG2.2.15 cells, three ginsenosides (Rg6, Rh4, and Rb3) are selected. Thereafter, using the same cellular model, all three ginsenosides are shown to mediate efficient, selective inhibition of HBV mRNA expression levels, and also interfere with the secretion of both HBV particles and hepatitis B surface antigen (HBsAg). Drug combination studies are performed in both HepG2.2.15 and HBV-infected HepG2-NTCPsec+ cell models with the selected ginsenosides and lamivudine (LMV), a nucleoside analogue used to treat chronic hepatitis B (CHB) infections. These studies, involving RT-qPCR and ELISA, suggest that Rh4/LMV combinations in particular act synergistically to inhibit the secretion of HBV particles and HBsAg. Therefore, on the assumption that appropriate *in vivo* data are in future agreement, Rh4, in particular, might be used in combination with nucleoside/nucleotide analogues (NUCs) to devise an effective, cost-efficient combination therapy for the treatment of patients with CHB infections.

1. Introduction

In spite of tremendous global efforts, functional cures for chronic hepatitis B virus (CHB) infections remain elusive. Current therapeutic strategies cause reductions in liver inflammation, regression of cirrhosis, and reduce the incidence of hepatocellular carcinoma (HCC) in patients with CHB infections (Duraisamy et al., 2020). However, severe side effects from drug use are common, and high levels of drug resistance develop during the treatment of hepatitis B virus (HBV) (Farrell and Teoh, 2006). This dearth of effective active pharmaceutical ingredients (APIs) against HBV has led to studies with natural products (Musarra-Pizzo et al., 2021). In addition, the development of drug combination strategies has become increasingly seen as a key strategy to ensure functional cure of CHB infections and dramatic reductions in the disease burden (Duraisamy et al., 2020).

In recent times, several medicinal plants or extracts thereof have been shown to have anti-viral properties to counter the replication cycle of different viruses such as human immunodeficiency virus (HIV) (Helfer et al., 2014), viruses causing respiratory infections (Bouredja et al., 2020; Glatthaar-Saalmüller et al., 2011; Serkedjieva et al., 2010), and others (Sanna et al., 2015; Huang et al., 2014). So too, a growing range of natural products isolated from medicinal plants and other natural sources like marine sponges have been shown to possess potentially powerful antiviral effects, at least *in vitro*, against for example, several arboviruses (Goh et al., 2020), HIV (Sagar et al., 2010), herpes simplex virus (HSV) (Sagar et al., 2010), and severe acute respiratory syndrome-associated

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coronavirus (SARS-CoV) (Ryu et al., 2010). In very recent times, the natural products baicalein and baicalin, from the Chinese medicinal plant *Scutellaria baicalensis*, and the chemically-modified natural product mixture ivermectin, derived originally from the soil bacterium *Streptomyces avermitilis*, were tested separately against severe acute respiratory syndrome-associated coronavirus-2 (SARS-CoV-2), the cause of the worldwide pandemic disease COVID-19 (Zandi et al., 2021; Huang et al., 2020; Caly et al., 2020). In the case of HBV, a range of natural products have already been tested and shown to be promising antiviral APIs effective against HBV infection, at least *in vitro* (Chou et al., 2012; Qiu and Chen, 2013; Wu, 2016; Paryez et al., 2016; Tian et al., 2010; Huang et al., 2013; Zhou et al., 2013; Li et al., 2013; Zhou et al., 2015; Wu, 2016; Cheng et al., 2015), including a ginsenoside Rg3 (Figure 1) (Kang et al., 2013; Yu et al., 2018). In general, ginsenosides are a class of natural product steroid glycosides and triterpene saponins found in *Panax ginseng*. Individual ginsenosides have been shown to exhibit various biological effects, but these are often subtle and difficult to characterize in isolation (Attele et al., 1999). Many studies have indicated that ginsenosides have antioxidant properties, and can act as free radical scavengers (Lü et al., 2009). Accordingly, ginsenosides have been suggested to be anti-proliferative with respect to cancer development and potentially neuroprotective with respect to Alzheimer's and Parkinson's diseases (Lü et al., 2009). Given such activity profiles, ginsenosides are potentially valuable broad band antiviral APIs too.

In order to study the efficacy of natural products and other potential APIs against HBV infections, a number of *in vitro* cell models have been devised over the years. One of the most popular has been the HepG2.2.15 cell line, a stably replicating cell model. This cell line was derived originally from hepatoma cells to simulate enhanced HBV DNA expression plus the packaging and secretion of HBV particles (Sells et al., 1987; Xu et al., 2021). Indeed, this has been an effective model for many to screen for anti-HBV drugs *in vitro*, and to study the structure, function, gene expression, and regulation of HBV DNA (Xiao et al., 2019). Limitations of this stably replicating cell model are that (i) cells do not support natural HBV infection, and (ii) they lack the natural sodium taurocholate co-transporting polypeptide (NTCP) receptor needed for HBV entry into hepatocyte host cells. Therefore, (iii) HepG2.2.15 cells cannot be used to study HBV adsorption, cellular entry or virus uncoating, and (iv) they cannot be used to study the development of HCC from HBV infection. Fortunately, these limitations can be addressed in large part using a second *in vitro* cell model that involves transient long-term HBV-infection of HepG2-NTCPsec+ cells. This cell line was originally created by transfection of HepG2 cells with the *NTCP* receptor gene (Yan et al., 2012), then further developed for direct infection with HBV, long-term viral spread, and the secretion of viral progeny, hence encapsulating several relevant features of CHB infections in patients (König et al., 2019). Accordingly, this cell line model is able to support the complete HBV replication cycle, and amplification of HBV infection typically as seen *in vivo* or in CHB patients.

Here, we report the initial screening of a ginsenoside library, using the HepG2.2.15 *in vitro* HBV infection model cell line, leading to the selection of three ginsenosides Rg6, Rh4, and Rb3 (Figure 1). The potential impact of these three selected ginsenosides on HBV infection was further investigated using both HepG2.2.15 and HBV-infected HepG2-NTCPsec+ *in vitro* HBV infection model cell lines, in studies also comprising drug combination studies with lamivudine (LMV), a well-established anti-HBV nucleoside/nucleotide analogue (NUC). LMV was the first approved HBV reverse transcriptase (RT) inhibitor, and LMV treatment is known to be

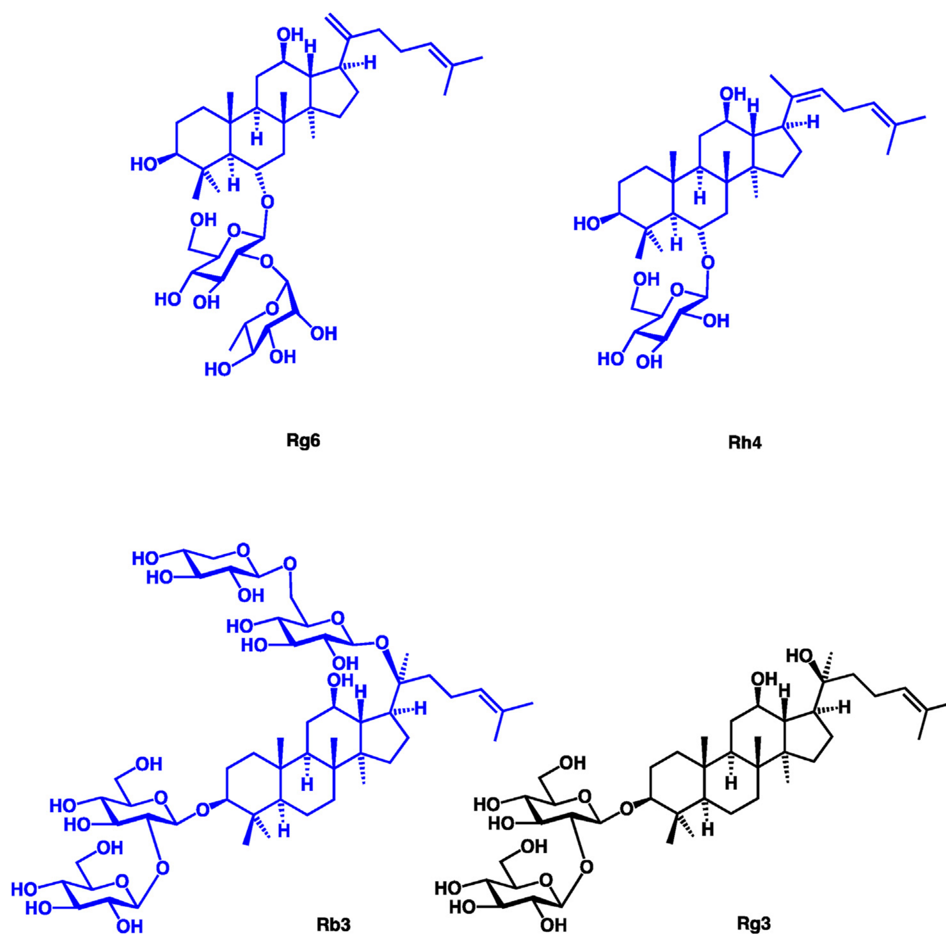


Figure 1. Structures of selected ginsenosides, Rg6, Rh4, and Rb3, plus known bioactive Rg3.

well tolerated and able to reduce HBV replication in CHB patients while keeping under control serum alanine aminotransferase (ALT) levels (León et al., 2004). Nowadays, LMV has been replaced in the clinic by more effective, recent NUCs (Duraisamy et al., 2020). Nevertheless, LMV was selected here as a proof of concept reference NUC drug, that is both inexpensive and commonly used in laboratories worldwide. In so doing, we also considered the possibility that we might be taking genuine first steps towards the potential repurposing of LMV as an effective treatment for CHB infection when used in combination with effective natural products (Chen et al., 2013; Arbab et al., 2017; Zhang et al., 2017) such as ginsenosides to maximise treatment efficacy, while minimizing the likelihood of the viral drug resistance that develops over time (Shaikh and Cooper, 2012).

2. Material and methods

2.1. Natural compounds and materials

The human hepatoma cell line (HepG2.2.15) was a gift from Dr. Jan Hodek and Dr. Jan Weber (Virology Research-Service Team, Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague). The human hepatoma cell line expressing the HBV entry receptor (HepG2-NTCPsec+) was generated, as described previously (König et al., 2019). A ginsenoside library (20 compounds) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and an antibiotic-antimycotic cocktail were purchased from Biosera Inc (Manila, Philippines). LMV and antibiotics such as penicillin and streptomycin were also purchased from Sigma-Aldrich (St. Louis, MO, USA). The QuickTiter™ ELISA kit for the detection of hepatitis B surface antigen (HBsAg) was purchased from Cell Biolabs, Inc. (San Diego, CA, USA).

2.2. Cell culture and API stock solutions

HepG2.2.15 cells were cultured at 37 °C, under 5% CO_2, using DMEM culture medium supplemented with 10% (v/v) FBS and an antibiotic-antimycotic cocktail (comprising penicillin 100 units/mL, streptomycin 100 μg/mL and G418 disulfate 250 μg/mL). HepG2-NTCPsec+ cells were cultured in DMEM supplemented with 10% (v/v) FBS and 50 U/mL penicillin. Stock solutions (10 mM) of each ginsenoside library compound, including ginsenosides Rg6, Rh4, and Rb3, were prepared in dimethyl sulfoxide (DMSO) and then diluted into various concentrations for *in vitro* experiments. Stock solutions of LMV were similarly prepared in DMSO.

2.3. HepG2.2.15 cell cytotoxicity assays

HepG2.2.15 cells (seeded at a density of 4×10^4 cells per well in 96-well plates) were cultured at 37 °C in humidified air with 5% CO_2. After cells reached 70–80% confluence, initial screening of ginsenoside library compounds was performed by the addition of each compound (50 μM final concentration) from DMSO stock solution to cultured HepG2.2.15 cells, which were then further incubated at 37 °C under 5% CO_2. At day 4 post-administration of each ginsenoside library compound to cells, percentage cell viabilities were determined by cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer's instructions. Thereafter, more extensive cytotoxicity studies were performed with appropriate quantities of selected ginsenosides (Rg6, Rh4, and Rb3) added to cultured HepG2.2.15 cells at final concentrations of 100, 50, 25, 12.5, 6.25 or 3.12 μM, following which cells were incubated at 37 °C under 5% CO_2. At day 2, 4, and 6 post-administration of the selected ginsenosides to cells, percentage cell viabilities were determined by CCK-8 assay as above. Additional cytotoxicity studies were also performed with combinations of ginsenoside compounds (Rg6, Rh4 and Rb3) (at 80, 40, 20, 10, 5 or 2.5 μM) and LMV (at 0.08, 0.04, 0.02, 0.01, 0.005 or 0.0025 μM) respectively added to cultured HepG2.2.15 cells, following which cells were incubated at 37 °C under 5% CO_2. At day 5 post-administration of ginsenoside/LMV combinations to cells, percentage cell viabilities were determined by CCK-8 assay once again.

2.4. HepG2.2.15 cell assays for HBV mRNA expression, HBV particle, and HBsAg secretion

HepG2.2.15 cells were prepared as above. Once cells had reached confluence, initial screening of ginsenoside library compounds was performed with each compound (50 μM final concentration) added individually to culture media, then cells were further incubated at 37 °C under 5% CO_2. At day 4 post-administration of compounds, cells were collected to determine the percentage inhibition of HBV mRNA expression levels relative to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels (as described below).

Thereafter, more extensive studies were performed with HepG2.2.15 cells as follows. After cells were grown to confluence, as above, culture media were replaced with media containing one of the selected ginsenosides as appropriate, or else with LMV or ginsenoside/LMV combinations at the indicated concentrations, after which cells were incubated at 37 °C under 5% CO_2. At the indicated times (at day 2, 4, or 6, post-administration; or just at day 5 post-administration), cells were collected for subsequent detection of HBV mRNA expression levels whilst cell culture media samples were also collected for the detection of cell secreted HBV particles (measured by HBV DNA detection) and HBsAg (as described below).

RNA extraction from cells was performed using the QIAamp® Viral RNA Mini kit (Qiagen, Germany), according to manufacturer's instructions. RNA integrity was evaluated by electrophoresis on 1% agarose gels, and quantified using an ND1000 spectrophotometer (NanoDrop Technologies, Germany). Following this, extracted RNA (1 μg) was used to synthesize cDNA using a SensiFAST™ cDNA Synthesis Kit (Meridian Bioscience Inc, Cincinnati, OH, USA) according to manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) reactions were then performed with qPCR 2 × SYBR master mix (Top-Bio, Czech Republic) on a LightCycler® real-time PCR system (Roche, Switzerland), using the indicated primers specific for amplification of HBV mRNA and GAPDH control mRNAs (Table 1). PCR amplification was performed on multiple RT-qPCR reaction mixtures (each 20 μl) located in individual wells of a 96-well optical-grade PCR plate, sealed with optical sealing tape (Bio-Rad Laboratories, Hercules, CA, USA). Each RT-qPCR reaction mixture comprised synthesized cDNA (2 μl) (diluted 1:1), appropriate primers (each 0.4 μl, 10 mM), 2xSYBR green PCR master mix (10 μl) and ddH_2O (7.2 μl). PCR amplification involved one cycle at 94 °C (5 min), followed by 40 cycles of denaturation at 94 °C (20 s), then annealing and extension at 57 °C for 20 s, plus 72 °C for 30 s. Finally, melting curve analyses were performed by slowly heating the PCR reaction mixtures from 65 to 95 °C, in increments of 0.5 °C every 5 s, with simultaneous measurements of the SYBR green signal intensities. HBV mRNA expression levels were determined and normalized relative to GAPDH mRNA levels using calculated $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen, 2001), then plotted in terms of mRNA expression (percentage) or conversely inhibition (percentage) of expression. Three biological replicates were performed for each data point where fold change (FC) ≥ 2 is considered statistically significant.

Percentage inhibitions of HBV particle secretion were determined using RT-qPCR to quantitate HBV-DNA levels in cell culture media collected from HepG2.2.15 cells previously treated with LMV, ginsenoside

Table 1. Primers for RT-qPCR detection of mRNAs.

mRNA	Primers	Accession Number
HBs-Fr	5′-CCTAGGACCCCTGCTCGTGT-3′	M38454.1
HBs-Rv	5′-AACGCCGCAGACACATCCAA-3′	M38454.1
GADPH-Fr	5′-CGGATTTGGTCGTATTGGG-3′	J02642.1
GADPH-Rv	5′-TCTCGCTCCTGGAAGATGG-3′	J02642.1

or ginsenoside/LMV combinations, relative to levels in cell culture media collected from control HepG2.2.15 cells [treated with DMSO 0.5 % (v/v) alone] (100%). HBV-DNA was isolated from culture media samples by means of the QIAGEN QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. RT-qPCR reactions were then performed with the Luna® Universal Probe qPCR master mix (New England Biolabs, Ipswich, MA, USA) on a LightCycler® real-time PCR system (Roche, Switzerland), using the indicated primers (Table 2). PCR amplification involved initial denaturation at 95 °C (60 s), followed by 45 cycles at 95 °C (15 s), then a single cycle at 60 °C (30 s).

Percentage inhibitions of HBsAg secretion were determined using ELISA to quantitate HBsAg levels in cell culture media samples collected from HepG2.2.15 cells previously treated with LMV, ginsenoside or ginsenoside/LMV combinations, relative to levels observed in cell culture media collected from control HepG2.2.15 cells [treated with DMSO 0.5 % (v/v) alone] (100%). ELISA reactions were performed with a commercial ELISA assay kit (Cell Biolabs, Inc., San Diego, CA) according to manufacturer's instructions, wherein quantifications of HBsAg levels (above a threshold of 1 ng/ml) were performed by measuring optical density (OD) values at 450/630nm using a microtiter plate spectrophotometer.

2.5. HepG2-NTCPsec+ cytotoxicity and cell assays for HBV particle and HBsAg secretion

HBV infection was performed as described previously (König et al., 2019). Briefly, HepG2-NTCPsec+ cells were seeded at a density of 8×10^3 cells per well in 384-well plates and inoculated with cell culture-derived HBV genotype D at a final genome copy equivalent (GEq) of 5000 per cell in the presence of 4% polyethylene glycol (PEG) (w/v). Typically, HBV-infected HepG2-NTCPsec+ cells were treated with ginsenosides (Rb3, Rh4, or Rg6) and/or LMV, at the indicated concentrations, 2 h before HBV infection. Next day, cells were washed out 3 times with Dulbecco's phosphate-buffered saline (DPBS), replenished with fresh media, then further incubated at 37 °C under 5% CO_2. At 1 and 2 weeks post-administration of ginsenosides and/or LMV, cell viabilities were measured by automated cell counting. Briefly, cells were incubated with a nuclear dye, multiple images taken per compound concentration, analyzed by image-mining software determining cell nuclei, and data were then normalized with respect to data obtained using control HBV-infected HepG2-NTCPsec+ cells [treated with DMSO 0.5 % (v/v) alone] (100%). Cell culture media samples were collected for analysis of secretion of either HBV particles (via analysis of HBV DNA levels) or HBsAg, as stated above with HepG2.2.15 cells. All experiments were performed in quadruplicate for accurate statistical analysis (of HBV DNA levels), or samples were pooled prior to unicate analysis (HBsAg).

2.6. Determination of ginsenoside/LMV combination indices

The effects of the combined treatments were analyzed using the Chou-Talalay method. The combination index (CI) values of drug combinations were calculated using CompuSyn software (CompuSyn Inc., Paramus, NJ). CI values of >1.10, 0.9 to 1.10, and <0.9 to 0.3 indicated if drug combinations were antagonistic, additive, or synergistic, respectively (Chou, 2006, 2010).

Table 2. Primers for RT-qPCR detection of HBV DNA.

DNA	Primers	Accession Number
HBV-Fr	5′-ACTCACCAACCTCCTGTCCT-3′	X02763.1
HBV-Rv	5′-GACAAACGGGCAACATACCT-3′	X02763.1
DNA probe	5′- FAM-TATCGCTGGATGTGTCTGCGGCGT-3′-TAMRA	X02763.1

2.7. Statistical analysis

All experiments were performed in triplicate, unless otherwise indicated in the figures, and data analyzed by GraphPad Prism 7 (Graph Pad Software, Inc., USA). Results are expressed as mean +/− standard deviation (SD). Statistical comparisons were made using the Holm-Sidak multiple t-test adjusted for multiplicity.

3. Results

3.1. Screening a ginsenoside compound library for anti-HBV activity

A critical aim of the studies described here was to identify new compounds capable of interfering with the HBV replication cycle. Accordingly, a ginsenoside library was initially screened against HepG2.2.15 cells for cytotoxic effects at a high but not excessive concentration. At day 4 post-administration of each member of the ginsenoside library (at 50 μM), HepG2.2.15 cell viabilities were measured by CCK-8 assay. The ginsenoside library compounds (prepared as stock solutions in DMSO) did not cause significant cytotoxicity compared to the situation with control cells [treated with DMSO 0.5 % (v/v) alone] (Figure 2). Thereafter, the ginsenoside library compounds were screened for their ability to inhibit HBV mRNA expression levels in HepG2.2.15 cells. At day 4 post-administration of each individual member of the ginsenoside library (at 50 μM) to cells, percentage inhibitions of HBV mRNA expression levels were determined by RT-qPCR. All members of the ginsenoside library appeared able to inhibit HBV mRNA expression levels in HepG2.2.15 cells >50% in comparison to GAPDH control mRNA expression levels. However, ginsenosides Rg6, Rh4, Rb3, and Rg3 were shown to be especially effective (Figure 2). Given that the activity of Rg3 against HBV had been described previously (Kang et al., 2013) then only ginsenosides Rg6, Rh4, and Rb3 were selected for further investigation here. Our particular approach here and throughout was to determine if HBV mRNA expression levels could be inhibited specifically relative to GAPDH mRNA expression levels, in analogy to the way in which RNA interference (RNAi) effects are studied. GAPDH is a "housekeeping" protein central to cell metabolism so, is a well-established specificity control in studying RNAi effects. In determining HBV mRNA expression levels relative to GAPDH mRNA expression levels, the particular HBV RT-qPCR probes used in detection of HBV mRNA expression levels were hepatitis B surface (HBs) mRNA probes (Table 1). However, these probes do not actually discriminate in detection between levels of the viral pre-S2/pre-S mRNA transcript, that translates into the medium and small HBs (M-HBs and S-HBs) proteins respectively, and levels of the pre-S1/pre-S2/pre-S mRNA transcript that translates into the large HBs (L-HBs) protein (Duraisamy et al., 2020). Moreover, these HBs probes also overlap with the pgRNA viral transcript around which immature nucleocapsids are assembled in hepatocytes. Accordingly, our mRNA inhibition data should only be seen as a reflection of the inhibition of HBV mRNA expression levels *in toto* relative to GAPDH mRNA control expression levels. The data do not allow for the unambiguous detection of any ginsenoside-mediated differential inhibition of certain HBV mRNA transcripts relative to others.

3.2. Evaluation of HepG2.2.15 cytotoxicity effects of selected ginsenosides

Assessment of cytotoxicity is an important part of evaluating any potential antiviral agent since a useful compound should show neither acute nor long-term toxicity against host cells. Such a compound should be selective for virus-specific processes with few or no effects on cellular metabolism (Simões et al., 1999). Accordingly, the HepG2.2.15 cytotoxicities of all three selected ginsenosides (Rg6, Rh4, and Rb3) were analyzed in a dose-dependent manner, at day 2, 4, and 6 post-administration of the ginsenosides, using the CCK-8 assay once again. All three selected ginsenosides showed no significant HepG2.2.15 cytotoxicities at day 2 and 4 post-administration. Slightly reduced cell viabilities were observed in

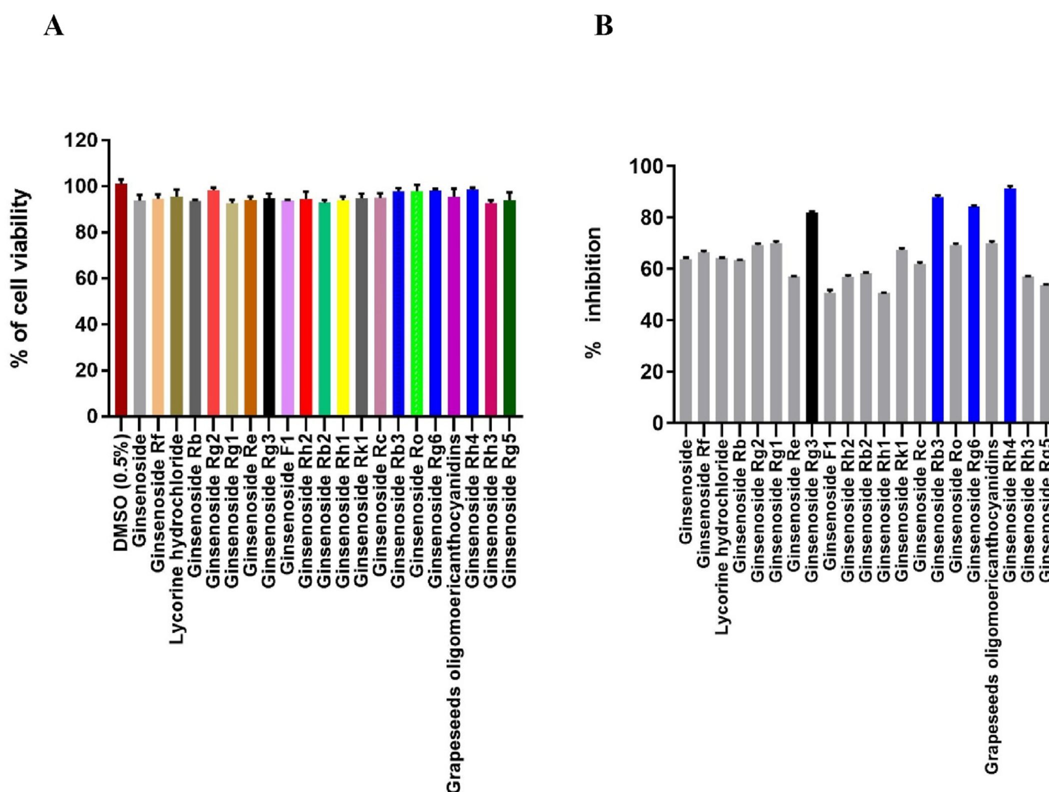


Figure 2. Screening of ginsenoside library compounds. A) Ginsenoside library compounds were screened for their impact on cell viability. In this instance, HepG2.2.15 cells were treated with the indicated ginsenosides or other natural products (added from DMSO stock solutions to give 50 μM/well final concentration), then incubated until end day 4 post-administration of natural products. Cell viabilities were assessed by CCK-8 assay relative to control HepG2.2.15 cells [treated with DMSO 0.5 % (v/v) alone] (100%), then plotted in terms of percentage cell viability; **B**) ginsenoside library compounds were then analyzed for inhibition of HBV mRNA expression levels. In this case, HepG2.2.15 cells were treated with the indicated ginsenosides or other natural products (at 50 μM/well) then incubated until end day 4 post-administration of natural products. Thereafter, HBV mRNA expression levels in cells were determined by means of RT-qPCR and normalized relative to GAPDH mRNA levels, then plotted in terms of percentage inhibition. All data are represented as mean ± SD from experiments performed in duplicate ($p < 0.05$).

general at day 6 post-administration in a manner unrelated to ginsenoside doses, so indicative of a non-specific decline in HepG2.2.15 cell viability at day 6 post-administration, compared to control HepG2.2.15 cells [treated with DMSO 0.5% (v/v) alone] (Figure 3). Others have reported how ginsenosides (at 100 μM), such as Rg3, Rh2, Rb1, Rb2, Rc, Rd, and Rp1, are only weakly cytotoxic or not at all (Kang et al., 2013; Song et al., 2014; Kim et al., 2017). Hence, we were confident that Rg6, Rh4, and Rb3 could be further evaluated to determine anti-HBV effects without complications from cell cytotoxicities.

3.3. Efficient inhibition of HBV mRNA expression by selected ginsenosides

Thereafter, HepG2.2.15 cells were treated with each of the selected ginsenosides at the indicated concentrations causing efficient, dose-dependent inhibition of HBV mRNA expression levels relative to control GAPDH mRNA levels (Figure 4). Efficient reductions of intracellular HBV mRNA expression levels were observed at days 2 and 4, post-administration of ginsenosides Rg6, Rh4, and Rb3. However, these inhibition effects were observed to be transient and to wear off completely by day 6, post-administration (data not shown). Estimated IC_{50} values for ginsenosides Rg6, Rh4, and Rb3 were 5.1, 4.3, and 4.1 at day 2, and 3.2, 5.6, and 3.2 μM at day 4, respectively, post-administration to HepG2.2.15 cells. All three of the selected ginsenosides behaved similarly in mediating efficient dose-dependent inhibition of HBV mRNA expression levels in HepG2.2.15 cells. Ginsenoside/LMV combination studies were then performed using HepG2.2.15 cells wherein efficient inhibitory effects were observed at day 5 post-administration at the indicated concentrations (Figure 5). Effects were studied 5 days post-administration on the basis that this was the most extended time period of ginsenoside treatment of HepG2.2.15 cells at which ginsenoside-mediated inhibition of HBV mRNA expression levels could still be observed, although less effective than observed at shorter treatment periods (Figure 4). Unsurprisingly, LMV was found to have no noticeable impact upon HBV mRNA expression levels in stark contrast to the selected ginsenosides. Accompanying CCK-8 assay cytotoxicity data also demonstrated that HepG2.2.15 cells were largely refractory to the presence of up to 80 μM of Rg6, Rh4, or Rb3 in combination with up to 0.08 μM of LMV.

3.4. Ginsenosides inhibit HBV particle secretion in HepG2.2.15 and HepG2-NTCPsec+ cells

The impact of the selected ginsenoside compounds on levels of HBV particle secretion from HepG2.2.15 and HBV-infected HepG2-NTCPsec+ cells was then studied in the presence and absence of LMV. Prior to these studies, cell cytotoxicity studies all indicated that both cell lines were largely refractory to the presence of up to 80 μM of Rg6, Rh4, or Rb3 in combination with up to 0.08 μM of LMV (Figure 6). Following this, the respective impacts of LMV, ginsenosides, or ginsenoside/LMV combinations on percentage inhibitions of HBV particle secretion were then evaluated in both cell lines by the quantitation of HBV-DNA levels in cell culture media, removed from cells previously treated with LMV, ginsenoside or ginsenoside/LMV combinations, relative to those levels observed in cell culture media removed from control cells. As anticipated LMV alone significantly inhibited the levels of HBV particle secretion, the selected ginsenosides much less so (Figure 6). Importantly, ginsenoside/LMV combinations acted in different ways, and based upon the available

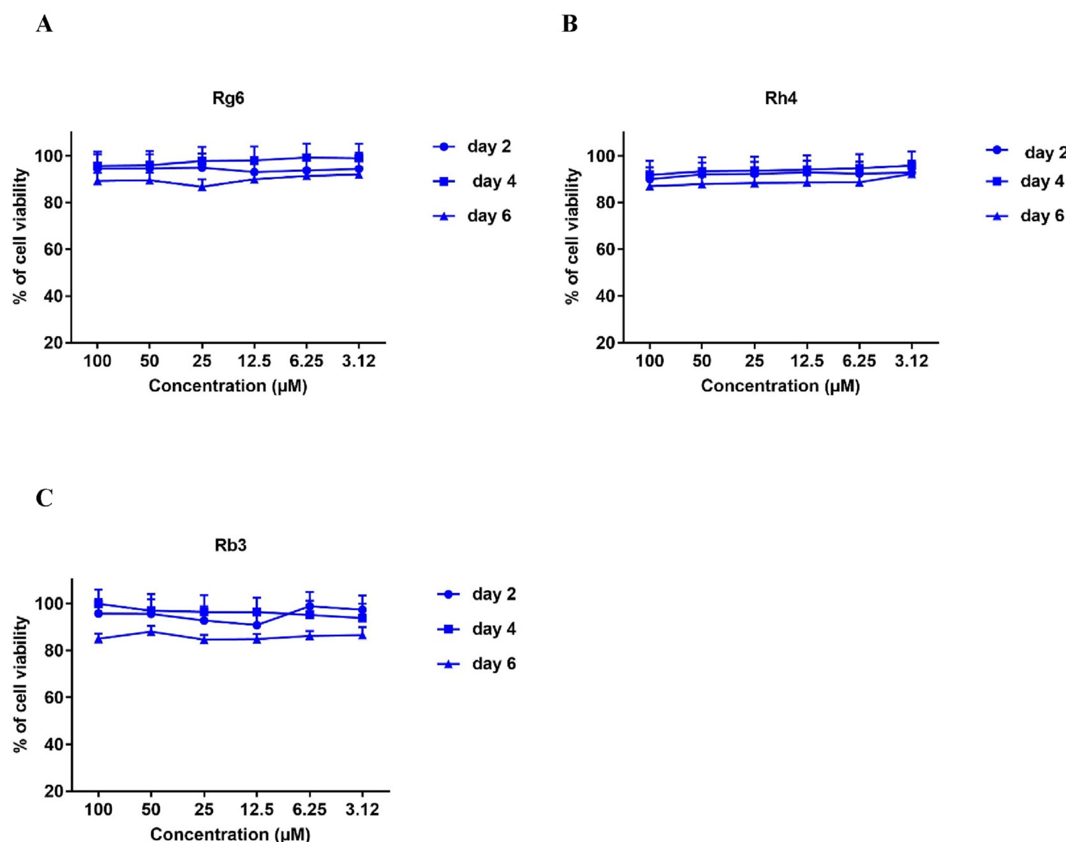


Figure 3. Ginsenoside-mediated cytotoxicity effects. Selected ginsenosides (**A**-Rg6, **B**-Rh4, and **C**-Rb3) were studied with HepG2.2.15 cells. Cells were treated with the selected ginsenosides (added from DMSO stock solutions) at the indicated concentrations, then incubated until end day 2, 4 or 6 post-administration of ginsenosides. Cell viabilities were then assessed by CCK-8 assay relative to control HepG2.2.15 cells [treated with DMSO 0.5 % (v/v) alone] (100%), then plotted in terms of percentage cell viability. All data are represented as mean ± SD from experiments performed in triplicate ($p < 0.05$ or $p < 0.01$).

data, CI values were then computed by the Chou-Talalay method (Chou, 2006, 2010). These values indicated that only Rh4/LMV combinations acted synergistically in both cell lines to inhibit HBV particle secretion, whilst Rg6/LMV combinations acted antagonistically, and Rb3/LMV combinations exhibited either additive behavior in HepG2.2.15 cells or moderate synergism in HBV-infected HepG2-NTCPsec+ cells (Table 3A and Table 3B).

Generally speaking, in terms of the graphical data (Figure 6), ginsenoside/LMV combinations in HBV-infected HepG2-NTCPsec+ cells appeared to be generally more effective than LMV alone over the complete concentration range used. In HepG2.2.15 cells, ginsenoside/LMV combinations were apparently more effective than LMV alone at ginsenoside concentrations of 10 μM or below, with the opposite appearing true at higher ginsenoside concentrations.

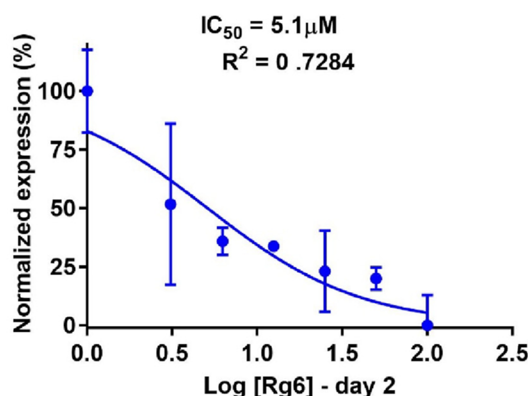
3.5. Ginsenosides inhibit HBsAg secretion in HepG2.2.15 and HepG2-NTCPsec+ cells

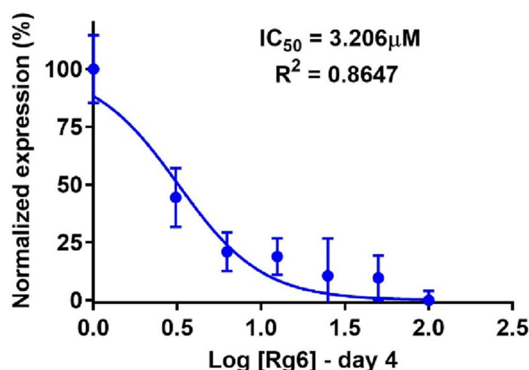
Turning to the effects of the selected ginsenosides on HBsAg secretion, HepG2.2.15, and HBV-infected HepG2-NTCPsec+ cells were treated with LMV alone, ginsenosides, or various ginsenoside/LMV combinations, as above, and the impacts on inhibition of HBsAg secretion were also evaluated. In HepG2.2.15 cells, significant reductions in HBsAg secretion were observed with the selected ginsenosides alone and generally more so with LMV alone. However, ginsenoside/LMV combinations were the most effective (Figure 7). Indeed, a maximum reduction of 52 % in secreted HBsAg levels was reached when HepG2.2.15 cells were treated with the Rh4/LMV combination (at respective drug concentrations of 80 μM and 0.08 μM) compared to the situation with control cells. Furthermore, Rh4/LMV combinations appeared to be more effective overall than either Rg6/LMV or Rb3/LMV combinations. Once again, CI values were then computed by the Chou-Talalay method (Chou, 2006, 2010). These values indicated that Rh4/LMV combinations acted slightly synergistically to inhibit HBsAg secretion, whilst Rg6/LMV combinations acted antagonistically, and Rb3/LMV combinations exhibited near additive behavior (Table 4A).

In the case of studies with HBV-infected HepG2-NTCPsec+ cells, there were no observable effects measured on HBsAg secretion 1 week post-administration (data not shown). However, at 2 weeks post-administration, secreted HBsAg levels were found to decrease (Figure 7). In the case of Rb3 experiments, LMV alone (up to 0.04 μM) was more effective than both Rb3 (up to 40 μM) and Rb3/LMV combinations (up to 40 μM and 0.04 μM, respectively), thereby suggesting that Rb3 is only a weak inhibitor of HBsAg secretion (below 40 μM), and Rb3/LMV combinations are antagonistic. On the other hand, although LMV alone (up to 0.02 μM) was more effective than Rh4 (up to 20 μM), >60% inhibition of HBsAg secretion was observed with Rh4/LMV combinations (up to 40 μM and 0.04 μM, respectively). Nonetheless, although LMV alone (up to 0.01 μM) was more effective than Rg6 (up to 10 μM), Rg6/LMV combinations (up to 40 μM and 0.04 μM, respectively) were able to mediate >90% inhibition of HBsAg secretion.

In calculating CI values by the Chou-Talalay method (Chou, 2006, 2010), values for Rb3 were not determined given the fact that the graphical data clearly indicated that Rb3/LMV combinations were acting with antagonism throughout. In the cases of Rh4 and Rg6, before calculation, inhibition data obtained at ginsenoside and LMV concentrations of 5 μM and 0.005 μM, respectively, were disregarded for data fitting reasons. In addition, inhibition data collected at the highest ginsenoside and

A





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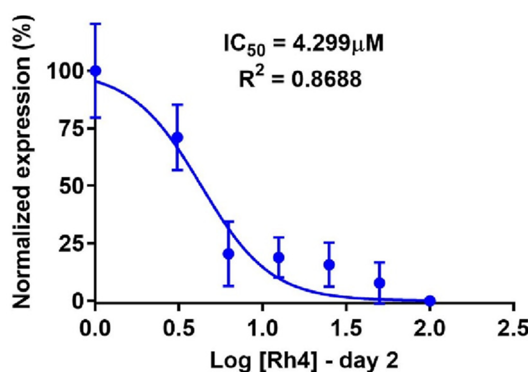

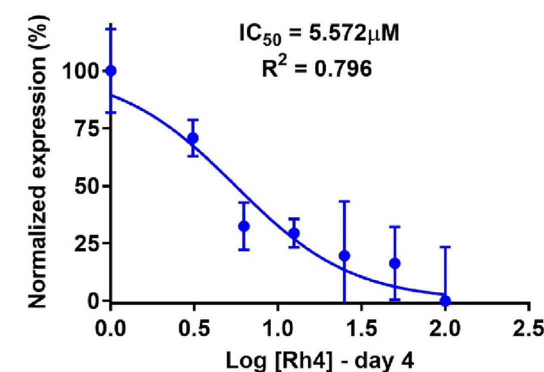

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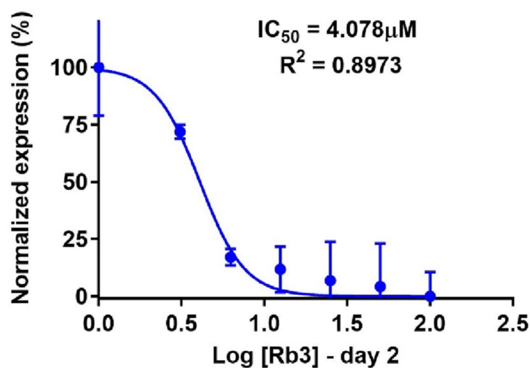


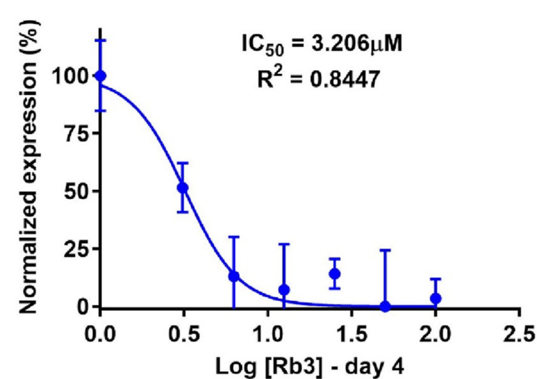


Figure 4. Ginsenoside-mediated efficient inhibition of HBV mRNA expression levels. Selected ginsenosides (**A**-Rg6, **B**-Rh4, and **C**-Rb3) were studied with HepG2.2.15 cells. Cells were treated separately with selected ginsenosides (added from DMSO stock solutions) at the indicated concentrations (3.1, 6.25, 12.5, 25, 50, or 100 μM), or with DMSO 0.5 % (v/v) alone, then incubated until end day 2, 4 or 6 post-administration of added compounds. Thereafter, HBV mRNA expression levels in cells were determined by means of RT-qPCR, normalized relative to GAPDH mRNA levels, then plotted in terms of percentage (normalized) expression. Only day 2 and 4 data are shown. IC_{50} is the concentration of ginsenoside causing 50% normalized expression; R^2 is the corresponding coefficient of determination which needs to be in the range 0.5–0.9, indicating a "good" fit.

LMV concentrations (80 μM and 0.08 μM, respectively) were also disregarded, given the clear HepG2-NTCPsec+ cell cytotoxicities observed at these concentrations. Thereafter, resulting CI values revealed that both Rh4/LMV or Rg6/LMV combinations acted with general synergism to inhibit HBsAg secretion (Table 4B).

4. Discussion

CHB infection remains a worldwide public health concern given its impact on the formation of severe liver diseases such as HCC. Currently, NUC therapies, including treatment with telbivudine (LdT), entecavir

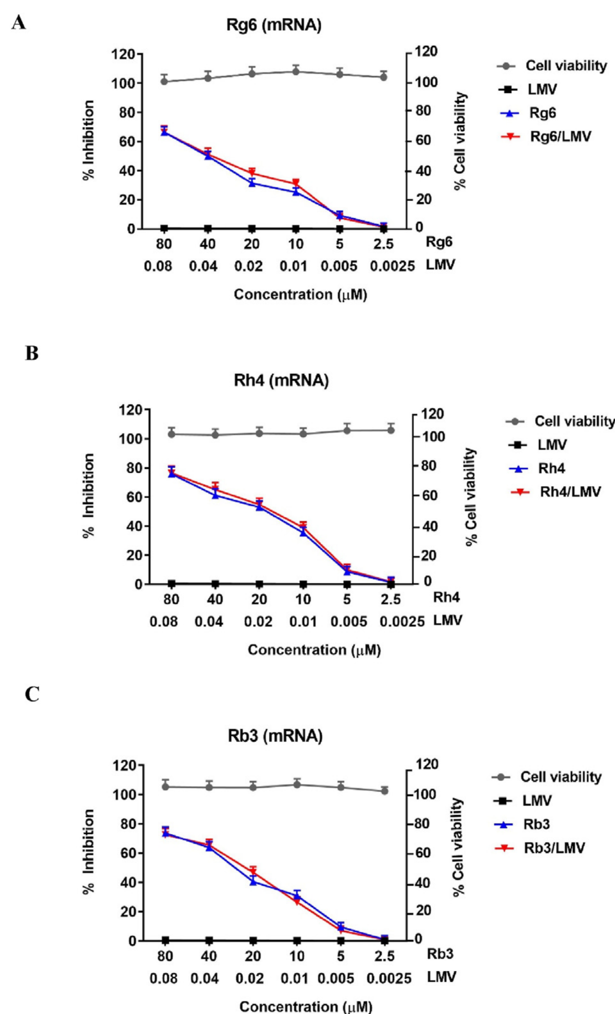


Figure 5. Ginsenoside/LMV-mediated inhibition of HBV mRNA expression levels. Selected ginsenosides (**A**-Rg6, **B**-Rh4, and **C**-Rb3) were studied in HepG2.2.15 cells. Cells were initially treated with ginsenoside/LMV combinations (added from DMSO stock solutions) to the indicated concentrations, then incubated until end day 5 post-administration of ginsenoside/LMV combinations. Cell viabilities were then assessed by CCK-8 assay relative to control HepG2.2.15 cells [treated with DMSO 0.5 % (v/v) alone] (100%), then plotted in terms of percentage cell viability. All data are represented as mean ± SD from experiments performed in duplicate ($p < 0.05$ or $p < 0.01$). Thereafter, HepG2.2.15 cells were treated separately with LMV, ginsenosides, or ginsenoside/LMV combinations (added from DMSO stock solutions) at the indicated concentrations, then incubated until end day 5 post-administration of added compounds. HBV mRNA expression levels in cells were determined by means of RT-qPCR and normalized relative to GAPDH mRNA levels, then plotted in terms of percentage inhibition. All data are represented as mean ± SD from experiments performed in triplicate ($p < 0.05$ or $p < 0.01$).

(ETV), adefovir dipivoxil (ADV), or tenofovir disoproxil fumarate (TDF) (Duraisamy et al., 2020; Tavakolpour et al., 2018), are approved for the treatment of CHB infection. However, NUC treatments are not sufficient to achieve functional cure (Duraisamy et al., 2020; Kim et al., 2018). Accordingly, new APIs are required (Lok et al., 2017). Here we demonstrate for the first time the anti-HBV activities of ginsenosides Rg6, Rh4, and Rb3 in two *in vitro* cell models of HBV infection, although this is not the first time that ginsenosides family members have been reported to have antiviral properties, for example, against hepatitis A virus (Lee et al., 2013), HSV (Liang et al., 2012), and also against HBV using Rg3 (Kang et al., 2013). Critically, here is the first time that ginsenosides Rg6, Rh4, and Rb3 have been shown to modulate the HBV replication cycle by a mechanism that also involves dose-dependent inhibition of HBV mRNA expression (Figures 4 and 5). As stated above, these RT-qPCR data can and should be seen at this stage as a reflection of HBV mRNA expression levels *in toto* relative to a well-established GAPDH mRNA control expression levels. On the other hand, what can be said with certainty is that ginsenosides Rg6, Rh4, and Rb3 do inhibit HBV mRNA expression levels specifically in a dose dependent manner relative to GAPDH mRNA expression levels. Obviously, this does not rule out the possibility that ginsenosides Rg6, Rh4, and Rb3 are also interfering with the expression of other cellular genes and pathways in order to impact on HBV mRNA expression levels. Indeed, this seems probable. For example, Rg3 has been reported to exhibit anti-HBV effects by stimulating TNF Receptor Associated Factor 6/transforming growth factor-β-activated kinase 1 (TRAF6/TAK1) degradation and inhibiting the cJun NH_2-terminal kinase/activator protein 1 (JNK/AP-1) signalling pathway (Kang et al., 2013). Furthermore, Rg3 has been shown to have clear anti-lipid accumulation properties in HepG2 cells too by interference in the expression of sterol regulatory element binding protein-2 (SREBP-2) plus 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR), and AMP-activated protein kinase (AMPK) activation (Lee et al., 2012). Also, where transcriptome analyses have been carried out previously to study the impact of ginsenoside Rh2 administration on HepG2 cells, then a comparison of RNA-transcriptome profiles from control and Rh2-treated groups revealed that the expression levels of 2116 genes were most significantly affected, comprising 971 up-regulated genes and 1145 down-regulated genes. Differential expression of genes from the p53 signaling pathway and from DNA replication pathways were suggested to account for the ability of Rh2 to cause apoptosis in HepG2 cells (Zhang et al., 2019). Accordingly, the clear implication is that ginsenosides Rg6, Rh4, and Rb3 are unlikely to interfere with just HBV mRNA expression levels alone. Hence follow up mechanistic studies will be essential to determine possible Rg6, Rh4, and Rb3 targets that help mediate dose-dependent inhibition of HBV mRNA expression levels at the molecular level in hepatocytes.

A critical aspect of our studies, as reported here, has been the use of two *in vitro* cell models for HBV infections and propagation based on HepG2.2.15 and HBV-infected HepG2-NTCPsec+ cell lines. These two models were selected on the basis that any observable anti-HBV effects would be observed in the context of sustained HBV cellular replication (HepG2.2.15 cell model) and in the context of actual HBV cellular infection and replication (HBV-infected HepG2-NTCPsec+ cell model). In this way, there can be some confidence that any observed anti-HBV effects mediated by LMV, selected ginsenosides, or ginsenoside/LMV combinations, should be reasonable predictive of data that might be obtained using *in vivo* animal models of HBV infection. Importantly, preliminary studies were performed with the HBV-infected HepG2-NTCPsec+ cell model to show that all three ginsenosides Rg6, Rh4, and Rb3 were unable to inhibit HBV entry into HepG2-NTCPsec+ cells (results not shown). This is important since the primary function of the NTCP receptor is receptor-mediated uptake of sodium taurocholate and other bile acids from the blood into liver cells. Since bile acids and ginenosides are similarly bio-active derivatives of cholesterol then the possibility that ginsenosides Rg6, Rh4, and Rb3 might be inhibitors of NTCP receptor-mediated HBV cell entry needed to be ruled out for the sake of completeness.

In terms of observed results, our data clearly indicate that administration of LMV does not result in significant inhibition of HBV mRNA levels (Figure 5) but does cause significant inhibition of HBV particle and HBsAg secretion, in keeping with its primary function as a reverse transcriptase (RT) inhibitor of the viral DNA polymerase (Figures 6 and 7) (Wu, 2016; Kang et al., 2013). Similarly, our data show clearly that administration of selected ginsenosides Rg6, Rh4, and Rb3 can cause significant inhibition of HBV particle and HBsAg secretion (Figures 6 and 7), in keeping with the fact that all three ginsenosides are mediating a dose-dependent inhibition of HBV mRNA expression levels (Figures 4 and 5), hence resulting in the inhibition of HBV particle and HBsAg secretion in due course. Obviously as noted above, we cannot rule out the possibility that ginsenoside-mediated interference of other cellular genes

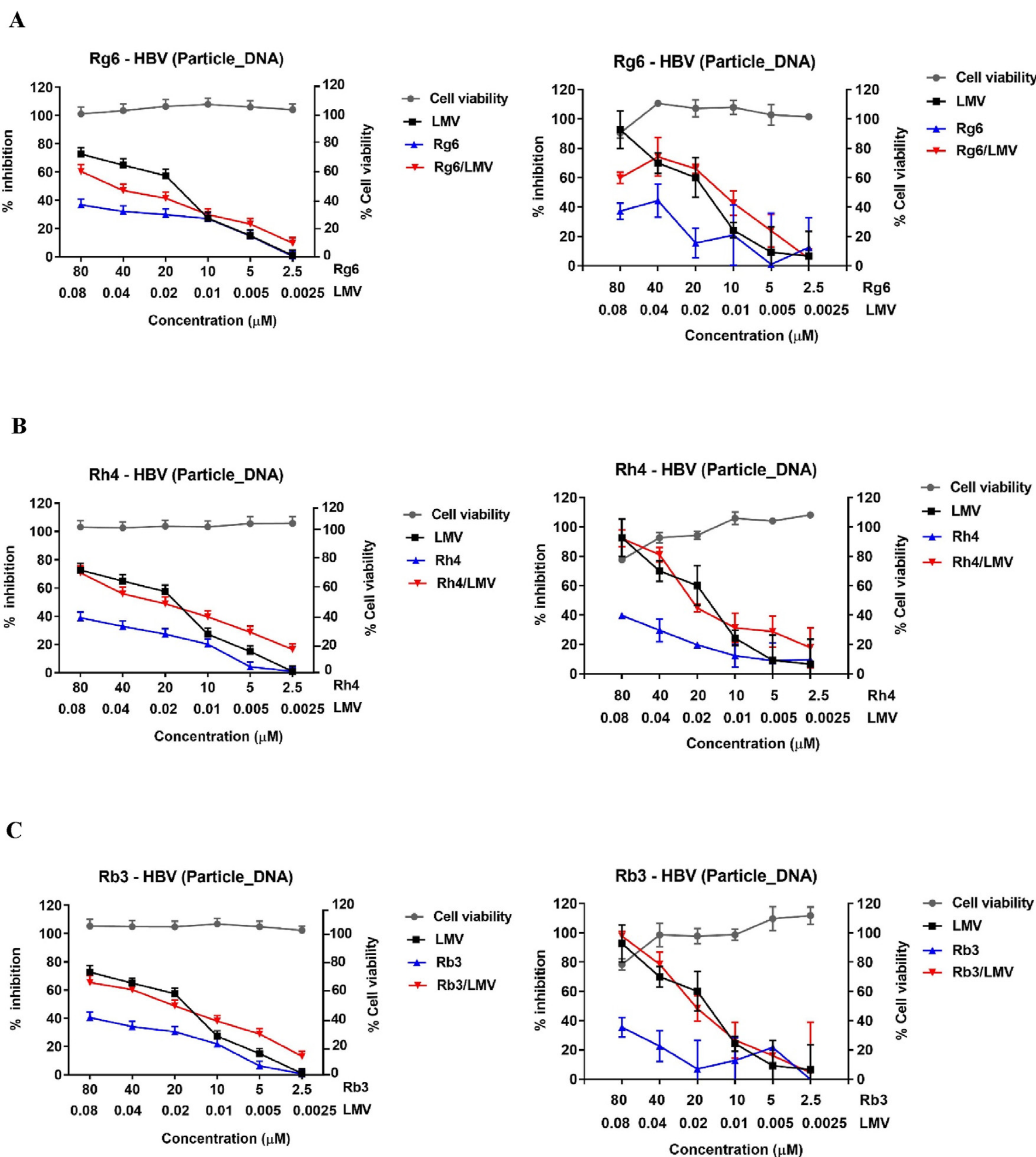


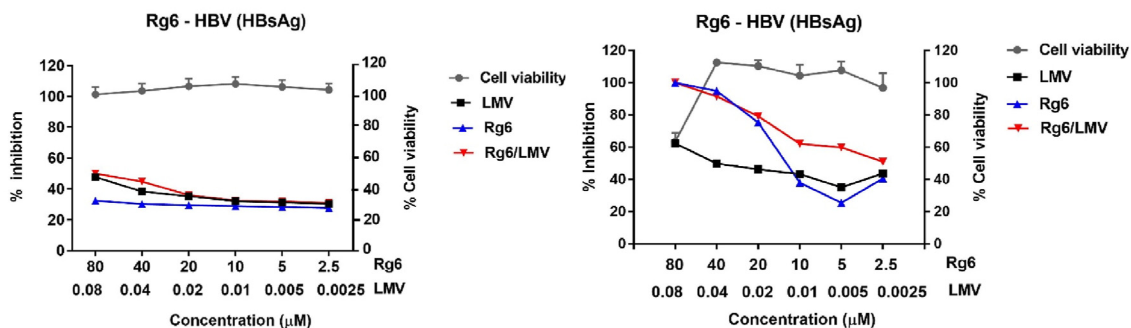
Figure 6. Ginsenoside/LMV-mediated inhibition of HBV particle secretion. Selected ginsenosides (**A**-Rg6, **B**-Rh4, and **C**-Rb3) were studied with HepG2.2.15 cells (left), and HBV-infected HepG2-NTCPsec+ cells (right). In the case of HepG2.2.15, percentage cell viabilities were assessed by CCK-8 assay at end day 5 post-administration of ginsenoside/LMV combinations, as in Figure 5. All data are represented as mean ± SD from experiments performed in duplicate ($p < 0.05$ or $p < 0.01$). Thereafter, HepG2.2.15 cells were treated with LMV, ginsenosides, or ginsenoside/LMV combinations (added from DMSO stock solutions) at the indicated concentrations, then incubated until end day 5 post-administration of added compounds. Percentage inhibitions of HBV particle secretion were determined using RT-qPCR to quantitate HBV-DNA levels in HepG2.2.15 cell culture media, relative to levels in cell culture media collected from control HepG2.2.15 cells [treated with DMSO 0.5 % (v/v) alone] (100%). All data are represented as mean ± SD from experiments performed in triplicate ($p < 0.05$ or $p < 0.01$). In the case of HBV genotype D-infected HepG2-NTCPsec+, cells were initially treated with ginsenoside/LMV combinations (added from DMSO stock solutions) at the indicated concentrations, then incubated for a week post-administration of ginsenoside/LMV combinations. Cell viabilities were assessed relative to control HBV-infected HepG2-NTCPsec+ cells [treated with DMSO 0.5 % (v/v) alone] (100%) by cell nuclei counting assay, then plotted in terms of percentage cell viability; all data are represented as mean ± SD (n = 4). Thereafter, HBV-infected HepG2-NTCPsec+ cells were treated separately with LMV, ginsenosides, or ginsenoside/LMV combinations (added from DMSO stock solutions) at the indicated concentrations, then incubated for a week post-administration of added compounds. Percentage inhibitions of HBV particle secretion were determined by RT-qPCR as described above; all data are represented as mean ± SD (n = 4).

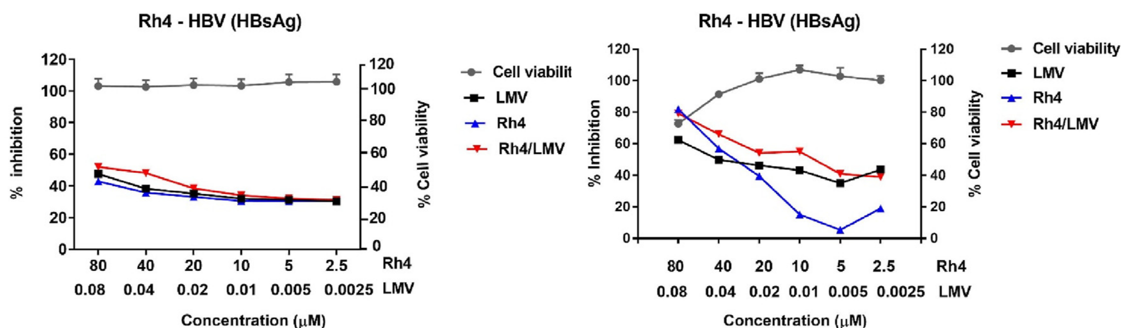
Table 3A. CI values for inhibition of HBV particle secretion from HepG2.2.15 cells.

Drug combination	Ratio	CI values for modulation of HBV DNA levels				Weighted average CI values	Assigned symbol	Description
		50%	75%	90%	95%			
Rg6/LMV	1000:1	1.2045	1.1976	1.1868	1.0827	1.18	-	Slight antagonism
Rh4/LMV	1000:1	1.0617	0.9231	0.8316	0.8219	0.88	+	Slight synergism
Rb3/LMV	1000:1	1.0865	0.9636	0.9032	0.8334	0.98	±	Near additive

Table 3B. CI values for inhibition of HBV particle secretion from HBV-infected HepG2-NTCPsec+ cells.

Drug combination	Ratio	CI values for modulation of HBV DNA levels				Weighted average CI values	Assigned symbol	Description
		50%	75%	90%	95%			
Rg6/LMV	1000:1	1.9778	1.5846	1.2697	1.0922	1.33	–	Moderate antagonism
Rh4/LMV	1000:1	1.2131	0.8329	0.5777	0.4524	0.64	+++	Synergism
Rb3/LMV	1000:1	1.5968	0.9912	0.7387	0.6779	0.85	++	Moderate synergism





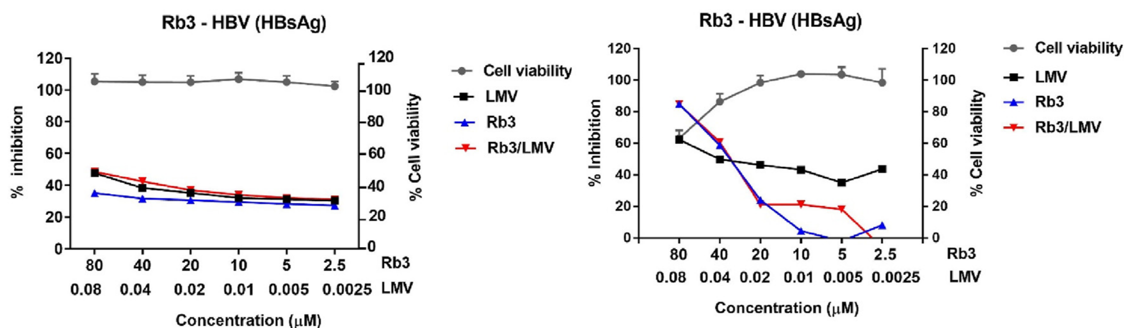


Figure 7. Ginsenoside/LMV-mediated inhibition of HBsAg secretion. Selected ginsenosides (**A**-Rg6, **B**-Rh4, and **C**-Rb3) were studied with HepG2.2.15 cells (left), and HBV-infected HepG2-NTCPsec+ cells (right). In the case of HepG2.2.15, percentage cell viabilities were assessed by CCK-8 assay at end day 5 post-administraion of ginsenoside/LMV combinations, as in Figure 5. All data are represented as mean ± SD from experiments performed in duplicate ($p < 0.05$ or $p < 0.01$). Thereafter, HepG2.2.15 cells were treated with LMV, ginsenosides, or ginsenoside/LMV combinations (added from DMSO stock solutions) at the indicated concentrations, then incubated until end day 5 post-administration of added compounds. Percentage inhibitions of HBsAg secretion levels were determined by ELISA relative to HBsAg secretion levels from control HepG2.2.15 cells [treated with DMSO 0.5 % (v/v) alone] (100%). All data are represented as mean ± SD from experiments performed in triplicate ($p < 0.05$ or $p < 0.01$). In the case of HBV genotype D-infected HepG2-NTCPsec+, percentage cell viabilities were assessed by cell nuclei counting assay 2 weeks post-administration of ginsenoside/LMV combinations, as in Figure 6; all data are represented as mean ± SD (n = 4). Thereafter, HBV-infected HepG2-NTCPsec+ cells were treated separately with LMV, ginsenosides, or ginsenoside/LMV combinations (added from DMSO stock solutions) at the indicated concentrations, then incubated for 2 weeks post-administration of added compounds. Percentage inhibitions of HBV particle secretion were determined by ELISA as described above; all data are from experiments performed in quadruplicate and analysed as pooled samples.

Table 4A. CI values for inhibition of HBsAg secretion from HepG2.2.15 cells.

Drug combination	Ratio	CI values at inhibition of HBsAg secretion				Weighted average CI values	Assigned symbol	Description
		50%	75%	90%	95%			
Rg6/LMV	1000:1	1.6218	1.3533	1.2869	0.7847	1.39	–	Moderate antagonism
Rh4/LMV	1000:1	1.6511	0.9757	0.7208	0.5200	0.90	+	Slight synergism
Rb3/LMV	1000:1	1.1234	1.0565	0.9229	0.7594	1.07	±	Near additive

Table 4B. CI values for inhibition of HBsAg secretion from HBV-infected HepG2-NTCPsec+ cells.

Drug combination	Ratio	CI values at inhibition of HBsAg secretion				Weighted average CI values	Assigned symbol	Description
		50%	75%	90%	95%			
Rg6/LMV	1000:1	1.0161	0.5259	0.3336	0.2457	0.40	+++	Synergism
Rh4/LMV	1000:1	0.5292	0.4208	0.5113	0.7097	0.55	+++	Synergism

and pathways (Lee et al., 2012; Zhang et al., 2019) may also account for the observed inhibitions of HBV particle and HBsAg secretion (Figures 6 and 7), at least in part. Nevertheless, a mechanistic link between the inhibition of HBV mRNA expression levels and the inhibition of HBV particle and HBsAg secretion would be logical in mammalian cells given their rapid protein turnover, even though we cannot be completely categoric at this stage without further detailed mechanistic studies.

Regarding the administration of ginsenoside/LMV combinations, there appear to be two key outcomes. Firstly, Rh4/LMV combinations exceptionally inhibit HBV particle (Figure 6, Table 3A and Table 3B)] and HBsAg secretion (Figure 7, Table 4A and Table 4B) with synergism in both cell lines. Secondly, Rg6/LMV combinations act to inhibit HBsAg secretion from HBV-infected HepG2-NTCPsec+ cells (Figure 7, Table 4B) with strong synergism too. Such observed differences in inhibition behavior between cell lines may be put down simply to the fact that HepG2.2.15 cells have multiple HBV genome integration sites and only stably express and replicate HBV (Sells et al., 1987; Watanabe et al., 2015), whereas *de novo* HBV-infected HepG2-NTCPsec+ cells actually support authentic viral covalently closed circular DNA (cccDNA) formation and regulation (König et al., 2019). However, secretion data obtained with the HBV-infected HepG2-NTCPsec+ cell model might well be considered to have extra weight given that this cell model is a genuine HBV cell infection model and therefore more representative of the HBV infection process *in vivo* than the recombinant HepG2.2.15 cell model. Interestingly, from the perspective of potential structure-activity relationships, we also note that Rh4 and Rg6 are related closely in structure compared to Rb3 and Rg3 (Figure 1).

The main caveat to these observed ginsenoside/LMV combination effects is the problem of relative doses. In the case of all three selected ginsenosides, these are administered at concentrations 10^3 times higher than corresponding LMV concentrations in combinations. Given this, there might be real practical implications in proceeding to combination studies using *in vivo* animal models of HBV infection. Both LMV and all selected ginsenosides are poorly water soluble, hence why stock solutions of these compounds were prepared using the solvent DMSO. This is acceptable for *in vitro* but not for *in vivo* experiments. Critically, where ginsenoside pharmacokinetic studies have been performed with Rb3 and Rg5, both were found very liver cell tropic, consistent with their structural similarities to bile acids (as noted above), and prone to liver metabolic processing including deglycosylation and glucuronidation. In the case of Rb3, the mean plasma half-lives were found to be 13.77 ± 1.23 min and 2045.70 ± 156.20 min for the bio-distribution and elimination phases, $t_{1/2\alpha}$ and $t_{1/2\beta}$ respectively, indicating that Rb3 biodistribution after administration *in vivo* is very rapid to liver cells while the subsequent elimination phase is slow. Furthermore, several different metabolic products were identified, some or all of which could be active drug candidates (Zhao et al., 2018). In the case of Rg5, feasible metabolic pathways were determined comprising a total of 17 different metabolic products some or all of which could also be active drug candidates (Hong et al., 2018). Accordingly, if we make the reasonable assumption that Rg6 and Rh4 have similar chemical and biophysical properties to Rb3 then rapid entry to the liver after administration *in vivo* seems probable. Nevertheless, given our own experiences *in vitro*, we would propose that follow up studies *in vivo* should make use of drug delivery nanoparticles which are also liver tropic and designed to enhance the functional delivery of poorly water-soluble drugs to liver hepatocytes following systemic or per oral administration. In so doing, not only would combination studies be rendered much more practical but drug bioavailability to HBV-infected hepatocytes *in vivo* could be enhanced too, so potentially reducing total required drug doses and potentially enabling reductions in the current concentration difference of 10^3 between ginsenosides and LMV, as used here to realize effective ginsenoside/LMV combination studies.

Overall, our combination study data presented here suggest that at least Rh4 can act in synergy with LMV to inhibit HBV particle and HBsAg secretion from two *in vitro* cell models designed to mimic sustained HBV cellular replication (HepG2.2.15 cell model) and actual HBV cellular infection and replication (HBV-infected HepG2-NTCPsec+ cell model). Given this, we would propose that Rh4 should be tested further *in vivo* in combination with well-established NUCs such as LMV. Clearly, in the event that such studies might be realized successfully, then Rh4/NUC combinations could represent an opportunity for new, cost-effective, NUC-related therapeutic approaches for the treatment of CHB infections. It remains to be seen if co-administration of Rh4 with LMV might enable the future repurposing of LMV itself as an effective new treatment for CHB infection going forward.

5. Conclusion

All our *in vitro* data interlock to suggest that ginsenoside Rh4 could be a possible agent for the treatment of CHB infections when used in combination with anti-HBV drugs such as NUCs. *In vivo* studies are now essential to test this promise further.

Declarations

Author contribution statement

Ganesh Selvaraj Duraisamy; Eunji Jo; Ivana Huvarová; Kyu-Ho P. Park: Performed the experiments; Analyzed and interpreted the data.

Zbyněk Heger; Vojtěch Adam; Daniel Růžek: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Marc P. Windisch: Conceived and designed the experiments; Wrote the paper.

Andrew D. Miller: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare the following conflict of interests: G.S.D, Z.H., V.A., D.R. and A.D.M. have filed a patent together related to this work. A.D.M. is a shareholder in KP Therapeutics (Europe) s.r.o.

Additional information

No additional information is available for this paper.

References

Arbab, A.H., Parvez, M.K., Al-Dosari, M.S., Al-Rehaily, A.J., 2017. *In vitro* evaluation of novel antiviral activities of 60 medicinal plants extract against hepatitis B virus. Exp. Ther. Med. 14, 626–634.

Attele, A.S., Wu, J.A., Yuan, C.S., 1999. Ginseng pharmacology: multiple constituents and multiple actions. Biochem. Pharmacol. 58, 1685–1693.

Bouredja, N., Bouthiba, M., Kebir, M., 2020. Ethnobotanical study of medicinal plants used by herbalists for the treatment of respiratory diseases in the region of Oran, Algeria. Br. J. Med. Health Sci. 2, 92–97.

Caly, L., Druce, J.D., Catton, M.G., Jans, D.A., Wagstaff, K.M., 2020. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 *in vitro*. Antivir. Res. 178, 104787.

Chen, Y., Zhu, J., 2013. Anti-HBV effect of individual traditional Chinese herbal medicine *in vitro* and *in vivo*: an analytic review. J. Viral Hepat. 20, 445–452.

Cheng, Z., Sun, G., Guo, W., Huang, Y., Sun, W., Zhao, F., Hu, K., 2015. Inhibition of hepatitis B virus replication by quercetin in human hepatoma cell lines. Virol. Sin. 30, 261–268.

Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621–681.

Chou, T.C., 2010. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70, 440–446.

Chou, S.C., Huang, T.J., Lin, E.H., Huang, C.H., Chou, C.H., 2012. Anti-hepatitis B virus constituents of *Solanum erianthum*. Nat. Prod. Commun. 7, 153–156.

Duraisamy, G.S., Bhosale, D., Lipenská, I., Huvarova, I., Růžek, D., Windisch, M.P., Miller, A.D., 2020. Advanced therapeutics, vaccinations, and precision medicine in the treatment and management of chronic hepatitis B viral infections; where are we and where are we going? Viruses 12, 998.

Farrell, G.C., Teoh, N.C., 2006. Management of chronic hepatitis B virus infection: a new era of disease control. Intern. Med. J. 36, 100–113.

Glatthaar-Saalmüller, B., Rauchhaus, U., Rode, S., Haunschild, J., Saalmüller, A., 2011. Antiviral activity *in vitro* of two preparations of the herbal medicinal product Sinupret® against viruses causing respiratory infections. Phytomedicine 19, 1–7.

Goh, V.S.L., Mok, C.K., Chu, J.J.H., 2020. Antiviral natural products for arbovirus infections. Molecules 25, 2796.

Helfer, M., Koppensteiner, H., Schneider, M., Rebensburg, S., Forcisi, S., Müller, C., Schmitt-Kopplin, P., Schindler, M., Brack-Werner, R., 2014. The root extract of the medicinal plant *Pelargonium sidoides* is a potent HIV-1 attachment inhibitor. PLoS One 9, e87487.

Hong, C., Yang, P., Li, S., Guo, Y., Wang, D., Wang, J., 2018. *In vitro/in vivo* metabolism of ginsenoside Rg5 in rat using ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry. Molecules 23, 2113.

Huang, Q., Zhang, S., Huang, R., Wei, L., Chen, Y., Lv, S., Liang, C., et al., 2013. Isolation and identification of an anti-hepatitis B virus compound from *Hydrocotyle sibthorpioides* Lam. J. Ethnopharmacol. 150, 568–575.

Huang, J., Su, D., Feng, Y., Liu, K., Song, Y., 2014. Antiviral herbs–present and future. Infect. Disord.: Drug Targets 14, 61–73.

Huang, S., Liu, Y., Zhang, Y., Zhang, R., Zhu, C., Fan, L., Pei, G., Zhang, B., Shi, Y., 2020. Baicalein inhibits SARS-CoV-2/VSV replication with interfering mitochondrial oxidative phosphorylation in a mPTP dependent manner. Signal Transduct. Targeted Ther. 5, 266.

Kang, L.J., Choi, Y.J., Lee, S.G., 2013. Stimulation of TRAF6/TAK1 degradation and inhibition of JNK/AP-1 signalling by ginsenoside Rg3 attenuates hepatitis B virus replication. Int. J. Biochem. Cell Biol. 45, 2612–2621.

Kim, S.J., Jang, J.Y., Kim, E.J., Cho, E.K., Ahn, D.G., Kim, C., Park, H.S., et al., 2017. Ginsenoside Rg3 restores hepatitis C virus-induced aberrant mitochondrial dynamics and inhibits virus propagation. Hepatology 66, 758–771.

Kim, S.S., Ahn, E.K., Cho, S.Y., Park, R.W., Cho, H.J., Kim, J.H., Kim, H.G., et al., 2018. Impact of nucleos(t)ide analog treatment on the development of malignancy in patients with chronic hepatitis B. Medicine (Baltim.) 97, e11087.

König, A., Yang, J., Jo, E., Park, K.H.P., Kim, H., Than, T.T., Song, X., Qi, X., Dai, X., et al., 2019. Efficient long-term amplification of hepatitis B virus isolates after infection of slow proliferating HepG2-NTCP cells. J. Hepatol. 71, 289–300.

Lee, S., Lee, M.S., Kim, C.T., Kim, I.H., Kim, Y., 2012. Ginsenoside Rg3 reduces lipid accumulation with AMP-activated protein kinase (AMPK) activation in HepG2 cells. Int. J. Mol. Sci. 13, 5729–5739.

Lee, M.H., Lee, B.H., Lee, S., Choi, C., 2013. Reduction of hepatitis A virus on FRhK-4 cells treated with Korean red ginseng extract and ginsenosides. J. Food Sci. 78, M1412–M1415.

León, P., Pozo, F., Echevarría, J.M., 2004. Detection of hepatitis B virus variants resistant to lamivudine and famciclovir among randomly selected chronic carriers from Spain. Enferm. Infecc. Microbiol. Clín. 22, 133–137.

Li, J., Meng, A.P., Guan, X.L., Li, J., Wu, Q., Deng, S.P., Su, X.J., et al., 2013. Anti-hepatitis B virus lignans from the root of *Streblus asper*. Bioorg. Med. Chem. Lett 23, 2238–2244.

Liang, Y.Y., Wang, B., Qian, D.M., Li, L., Wang, Z.H., Hu, M., Song, X.X., 2012. Inhibitory effects of Ginsenoside Rb1 on apoptosis caused by HSV-1 in human glioma cells. Virol. Sin. 27, 19–25.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Lok, A.S., Zoulim, F., Dusheiko, G., Ghany, M.G., 2017. Hepatitis B cure: from discovery to regulatory approval. Hepatology 66, 1296–1313.

Lü, J.M., Yao, Q., Chen, C., 2009. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr. Vasc. Pharmacol. 7, 293–302.

Musarra-Pizzo, M., Pennisi, R., Ben-Amor, I., Mandalari, G., Sciortino, M.T., 2021. Antiviral activity exerted by natural products against human viruses. Viruses 13, 828.

Parvez, M.K., Arab, A.H., Al-Dosari, M.S., Al-Rehaily, A.J., 2016. Antiviral natural products against chronic hepatitis B: recent developments. Curr. Pharmaceut. Des. 22, 286–293.

Qiu, L.P., Chen, K.P., 2013. Anti-HBV agents derived from botanical origin. Fitoterapia 84, 140–157.

Ryu, Y.B., Jeong, H.J., Kim, J.H., Kim, Y.M., Park, J.Y., Kim, D., Nguyen, T.T., et al., 2010. Biflavonoids from *Torreya nucifera* displaying SARS-CoV 3CL(pro) inhibition. Bioorg. Med. Chem. 18, 7940–7947.

Sagar, S., Kaur, M., Minneman, K.P., 2010. Antiviral lead compounds from marine sponges. Mar. Drugs 8, 2619–2638.

Sanna, G., Farci, P., Busonera, B., Murgia, G., La Colla, P., Giliberti, G., 2015. Antiviral properties from plants of the Mediterranean flora. Nat. Prod. Res. 29, 2065–2070.

Serkedjieva, J., Nikolova, E., Kirilov, N., 2010. Synergistic inhibition of influenza A virus replication by a plant polyphenol-rich extract and epsilon-aminocaproic acid *in vitro* and *in vivo*. Acta Virol 54, 137–145.

Shaikh, T., Cooper, C., 2012. Reassessing the role for lamivudine in chronic hepatitis B infection: a four-year cohort analysis. Can. J. Gastroenterol. 26, 148–150.

Simões, C.M., Falkenberg, M., Schenkel, Mentz L.A., Amoros, E.P., Girre, M., 1999. Antiviral activity of south Brazilian medicinal plant extracts. Phytomedicine 6, 205–214.

Song, M.Y., Kim, B.S., Kim, H., 2014. Influence of Panax ginseng on obesity and gut microbiota in obese middle-aged Korean women. J. Ginseng Res. 38, 106–115.

Sells, M.A., Chen, M.L., Acs, G., 1987. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. U.S.A. 84, 1005–1009.

Tavakolpour, S., Darvishi, M., Mirsafaei, H.S., Ghasemiadl, M., 2018. Nucleoside/nucleotide analogues in the treatment of chronic hepatitis B infection during pregnancy: a systematic review. Inf. Disp. 50, 95–106.

Tian, Y., Sun, L.M., Liu, X.Q., Li, B., Wang, Q., Dong, J.X., 2010. Anti-HBV active flavone glucosides from *Euphorbia humifusa* Willd. Fitoterapia 81, 799–802.

Watanabe, Y., Yamamoto, H., Oikawa, R., Toyota, M., Yamamoto, M., Kokudo, N., Tanaka, S., et al., 2015. DNA methylation at hepatitis B viral integrants is associated with methylation at flanking human genomic sequences. Genome Res 25, 328–337.

Wu, Y.H., 2016. Naturally derived anti-hepatitis B virus agents and their mechanism of action. World J. Gastroenterol. 22, 188–204.

Xiao, Y., Liu, C., Tang, W., Zhang, H., Chen, X., 2019. Evans blue inhibits HBV replication through a dual antiviral mechanism by targeting virus binding and capsid assembly. Front. Microbiol. 10, 2638.

Xu, R., Hu, P., Li, Y., et al., 2021. Advances in HBV infection and replication systems *in vitro*. Virol. J. 18, 105.

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., et al., 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 1, e00049.

Yu, J.S., Roh, H.S., Baek, K.H., Lee, S., Kim, S., So, H.M., Moon, E., et al., 2018. Bioactivity-guided isolation of ginsenosides from Korean Red Ginseng with cytotoxic activity against human lung adenocarcinoma cells. J. Ginseng Res. 42, 562–570.

Zandi, K., Musall, K., Oo, A., Cao, D., Liang, B., Hassandarvish, P., Lan, S., Slack, R.L., Kirby, K.A., Bassit, L., Amblard, F., Kim, B., AbuBakar, S., Sarafianos, S.G., Schinazi, R.F., 2021. Baicalein and baicalin inhibit SARS-CoV-2 RNA-dependent-RNA polymerase. Microorganisms 9, 893.

Zhang, J., Li, W., Yuan, Q., Zhou, J., Zhang, J., Cao, Y., Fu, G., Hu, W., 2019. Transcriptome analyses of the anti-proliferative effects of 20(S)-ginsenoside Rh2 on HepG2 cells. Front. Pharmacol. 10, 1331.

Zhang, Y.B., Zhang, X.L., Chen, N.H., Wu, Z.N., Ye, W.C., Li, Y.L., Wang, G.C., 2017. Four matrine-based alkaloids with antiviral activities against HBV from the seeds of *Sophora alopecuroides*. Org. Lett. 19, 424–427.

Zhao, L., Ma, Y., Chen, C., Liu, S., Wu, W., 2018. Pharmacokinetic and metabolic studies of ginsenoside Rb3 in rats using RRLC-Q-TOF-MS. J. Chromatogr. Sci. 56, 480–487.

Zhou, X., Liu, J., Yang, B., Lin, X., Yang, X.W., Liu, Y., 2013. Marine natural products with anti-HIV activities in the last decade. Curr. Med. Chem. 20, 953–973.

Zhou, N.J., Geng, C.A., Huang, X.Y., Ma, Y.B., Zhang, X.M., Wang, J.L., Chen, J.J., 2015. Anti-hepatitis B virus active constituents from *Swertia chirayita*. Fitoterapia 100, 27–34.