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Amentoflavone inhibits hepatitis B virus infection via the suppression of preS1 binding to host cells

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Abstract

Hepatitis B virus (HBV) is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Current therapeutic drugs for chronic HBV infection use interferon and nucleos(t)ide analogues; however, their efficacy is limited. Thus, there is an urgent need to develop new antivirals for HBV therapy. In this study, we identified a plant-derived polyphenolic bioflavonoid, amentoflavone, as a new anti-HBV compound. Amentoflavone treatment a dose-dependently inhibited HBV infection in HBV susceptible cells with HepG2-hNTCP-C4 and primary human hepatocyte PXB-cells. A mode-of-action study showed amentoflavone inhibits the viral entry step, but not the viral internalization and early replication processes. Attachment of HBV particles as well as HBV preS1 peptide to HepG2-hNTCP-C4 cells were inhibited by amentoflavone. The transporter assay revealed that amentoflavone partly inhibits uptake of sodium taurocholate cotransporting polypeptide (NTCP)-mediated bile acid. Furthermore, effect

of various amentoflavone analogs on HBs and HBe production from HBV-infected HepG2-hNTCP-C4 cells was examined. Robustaflavone exhibited comparable anti-HBV activity to that of amentoflavone and amentoflavone-7,4',4'''-trimethyl ether derivative, sciadopitysin, with moderate anti-HBV activity. Cupressuflavone or the monomeric flavonoid apigenin did not exhibit the antiviral activity. Amentoflavone and its structurally related biflavonoids may provide a potential drug scaffold in the design of a new anti-HBV drug inhibitor targeting NTCP.

Keywords: amentoflavone, hepatitis B virus (HBV), attachment, antiviral, viral entry

Introduction

Chronic hepatitis B (CHB) infection is caused by the hepatitis B virus (HBV) and affects more than 250 million people worldwide (1). Chronic infection often leads to the development of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma (2, 3).

Currently, two types of medications are used for treating CHB, pegylated interferons (Peg-IFNs) and nucleoside/nucleotide analogs (NAs). The advantage of Peg-IFN therapy is a higher loss of hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) seroconversion within a finite duration of therapy, but the use of Peg-IFNs is limited by intolerance and side effects. NA drugs, including entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide fumarate, can effectively suppress viral loads and are well tolerated, however, the virological and clinical relapse rates are high after discontinuing NA drugs. The HBsAg clearance rates are low in patients treated with both NA (1.4%–5.1%) and Peg-IFN (2.1%–20%) treatment (4). Therefore, the development of anti-HBV

drugs with different mechanisms of action is urgently required to improve patient outcomes.

HBV is a small, enveloped virus consisting of approximately 3.2 kb partially double-stranded relaxed circular DNA (rcDNA). HBV enters cells through viral attachment to the low-affinity receptor, heparan sulfate proteoglycan, followed by the binding of HBV particles to a specific receptor, sodium taurocholate cotransporting polypeptide (NTCP) (5). The interaction between the preS1 domain of HBV large surface protein and the NTCP receptor is essential for HBV infectivity (6); accordingly, this interaction triggers HBV internalization (7). Following penetration and uncoating, the viral capsid is transferred and rcDNA is released into the cell nucleus. The rcDNA is converted into covalently closed circular DNA (cccDNA), which resides as a minichromosome inside the nucleus. The cccDNA serves as a template for five mRNAs that encodes the viral proteins: HB core (HBc), HBe, HBV polymerase, HBs, and HBV X. HBV replicates via a pregenomic RNA intermediate that is reversed transcribed into DNA. HBV subviral particles are assembled on the ER membrane, and released through the ER-Golgi secretory pathway (8, 9).

The HBV life cycle is mainly classified into two steps: the viral entry step that includes attachment, penetration, and uncoating and the post-entry step that involves nucleocapsid transportation, cccDNA formation, transcription, translation, reverse transcription, assembly and release (8). Of these, the viral entry step is one of the attractive therapeutic targets since inhibitors that target viral entry may not only prevent the initial viral infection but also have no its immunosuppressive effects. To date, various compounds targeting viral entry have been reported as candidates for HBV treatment, such as Myrcludex B, ezetimibe, irbesartan, cyclosporin A (CyA), vanitaracin A,

proanthocyanidin, betulin derivatives, troglitazone, and epigallocatechin-3-gallate (5) (10-17); some of these are currently in the clinical trial phase (18). A combination antiviral drug regiment composed of inhibitors having different mechanisms of actions may act as an efficient antiviral strategy for treating CHB infections because combination therapies comprising different direct-acting antivirals have been shown to be highly effective on chronic hepatitis C treatment (19).

Plants produce structurally and functionally diverse of secondary metabolites, some of which was used as the molecules for drug production by pharmaceutical industries (20). Flavonoids, including flavones, chalcones, isoflavones, aurones, and xanthones, are low-molecular-weight molecules with variable phenolic structures. Biflavonoids are composed of two monoflavonoids by a direct connection or a linear linker, which have various important bioactivities to human diseases including anti-oxidant, anti-inflammatory, anti-tumor, anti-microbial, anti-diabetes, and neuroprotective activities (21, 22). Amentoflavone is a well-known biflavonoid occurring in numerous medicinal plants from Selaginellaceae, Cupressaceae, and Callaphyllaceae families and the *Garcinia* species (23, 24). Amentoflavone has been reported to exert antiviral activities against some DNA and RNA viruses such as human immunodeficiency virus, influenza A and B viruses, and herpes simplex virus type 1 (HSV-1) (25-27). In our previous research, we screened Indonesian medicinal plants for anti-HBV activity and found that crude extracts from bark and leaf of *Cassia fistula* decreased the production of HBsAg from HBV-infected HepG2-NTCP cells (28). *Cassia* plant species are the major source of bioactive flavonoids such as quercetin, kampferol, catechin, and proanthocyanidin, which were reported to prevent hepatitis B infection (13, 15, 29, 30). Moreover, amentoflavone was isolated from the leaves of *Cassia fistula* (31). Thus, we thought to evaluate the impact

of amentoflavone on the early steps of the HBV life cycle. In this study, we assessed anti-HBV activity of amentoflavone in HBV susceptible cells and determined its antiviral mechanism of action. We also examined antiviral effect of amentoflavone derivatives on HBV infection.

Materials and Methods

Cell cultures

HepG2-hNTCP-C4 cells (32) were cultured on type I collagen-coated dish using media of Dulbecco's Modified Eagle Medium (DMEM)/F-12, GlutaMAX^{TM} (Thermo Fisher Scientific, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaillé, France), 10 mM HEPES (Nacalai Tesque, Kyoto, Japan), 5 μg/ml insulin (Wako, Kyoto, Japan), 100 IU/ml penicillin/streptomycin (Nacalai Tesque), and 0.5 mg/ml G418 (Wako). Huh7 cells were grown in DMEM (Wako) supplemented with 10% fetal bovine serum, non-essential amino acid (Wako), and 100 IU/ml penicillin/streptomycin. Primary human hepatocytes (PXB-cells) were purchased from PhenixBio (Hiroshima, Japan) and cultured in maintenance medium for PXB-cells (PhenixBio).

Reagents

Amentoflavone, cupressuflavone, and sciadopitysin were purchased from EXTRASYNTHESE (Genay, France). Apigenin and dimethyl sulfoxide (DMSO) were obtained from Wako, and robustaflavone was purchased from BioCrick (Sichuan, China). CyA was acquired from Sigma-Aldrich (St. Louis, Missouri, USA). The myristoylated HBV preS1 peptide was purchased from Scram (Tokyo, Japan).

Virus preparation

HBV (genotype D) was prepared from the culture supernatant of Hep38.7-Tet cells (33). Hep38.7-Tet cells were maintained with DMEM/F-12, and GlutaMAX™ supplemented with 10% FBS, 10 mM HEPES, 5 μg/ml insulin, 100 IU/ml penicillin/streptomycin, 0.4 mg/ml G418, and 400 ng/ml tetracycline. The culture supernatants from days 9 to 30 after induction of HBV production by depletion of tetracycline were harvested and concentrated by precipitation with 4% PEG8000 (Sigma-Aldrich) and 2.3% NaCl (Wako). HBV genotype C were prepared from the culture supernatant of Huh7 cells transfected with an HBV expression plasmid (HBV/C-AT, kindly provided by Dr. Masashi Mizokami at National Center for Global Health and Medicine) using Fugene 6 transfection reagent (Promega, Madison, WI, USA). Culture supernatants were harvested at day 3 and 6 post-transfection and concentrated.

HBV infection assay

HepG2-hNTCP-C4 cells and PXB-cells were inoculated with a mixture of compounds and HBV genotype D at 6,000 genome equivalent (GEq)/cell (for HepG2-hNTCP-C4 cells) and 500 GEq/cell (for PXB-cells), respectively, in the presence of 4% PEG8000 for 18 h at 37°C. After removing the virus inoculum by washing, the infected cells were maintained with a culture medium for 7 to 8 days (for HepG2-hNTCP-C4 cells) and 10 days (for PXB-cells and HBV genotype C). Cells and supernatants were then harvested for enzyme-linked immunosorbent assay (ELISA), quantitative PCR (qPCR) analysis, and indirect immunofluorescence analysis, as described below.

DNA extraction and quantification of HBV cccDNA

Total cellular DNA was obtained using QIAamp DNA mini kit (Qiagen, Hilden, Germany) with a slight modification by incubation of Buffer AL at 70°C for 30 min. One microgram of total DNA was treated with 10U of T5 exonuclease (New England Biolabs, MA, USA) to digest all DNA except for cccDNA, followed by 400U of proteinase K treatment (Thermo Fisher Scientific) at 50°C for 30 min and heat inactivation of the enzyme at 95°C for 10 min. The digested samples were used as a template for the quantification of cccDNA using primers and probes as follows: CCC-DNA(F): 5'-CGTCTGTGCCTTCTCATCTGC-3', CCC-DNA(R): 5'-GCACAGCTTGGAGGCTTGAA-3', and CCC-DNA Probe: 5'-CTGTAGGCATAAATTGGT-3' MGB. PCR was carried out at 94°C for 10 min, alongside 50 cycles of 94°C for 15 s and 58°C for 1 min with iTaq Universal Probes Supermix (Bio-Rad, Munich, Germany) using a Bio-Rad CFX Manager.

Total RNA extraction, reverse transcription, and quantification of HBV RNA

Total cellular RNA was isolated using ISOGEN-II (Nippon GENE, Tokyo, Japan) and converted into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). PCR was performed using SYBR Premix Ex Taq II (Takara, Shiga, Japan) using a Bio-Rad CFX Manager as previously described (28). The primers were HBV genotype D-F: 5'-GCTTTCACTTTCTCGCCAAC-3' and HBV genotype D-R: 5'-GAGTTCCGCAGTATGGATCG-3', and HBV genotype C-F: 5'-ACTCACCAACCTCTTGTCCT-3' and HBV genotype C-R: 5'-GACAAACGGGCAACATACCT-3'. The plasmid HBV dilutions from 10^7 to 10^2 copies per reaction were used for generating a standard curve.

ELISA

HBsAg (DIASource Immunoassays S.A, Louvain-la-Neuve, Belgium) and HBeAg (Abnova, Taipei, Taiwan) in the culture supernatants were measured using commercial ELISA kits according to the manufacturer's instructions.

Indirect immunofluorescence analysis

HBV-infected HepG2-hNTCP-C4 cells on day 8 post-infection (p.i.) were fixed with 4% paraformaldehyde (Nacalai Tesque) and permeabilized with 0.5% Triton X-100. After blocking with 2% block ace solution (DS Pharma Biomedical Co., Osaka, Japan), the cells were incubated with both anti-HBs polyclonal antibodies (ab9193, Abcam, Cambridge, MA, USA) and anti-Hep B cAg antibodies (C1-5) (Santa Cruz, Dallas, TX, USA) as primary antibodies, followed by FITC-conjugated anti-equine (SouthernBiotech, AL, USA) and Alexa Flour 568 anti-mouse IgG (Abcam) as secondary antibodies, respectively. Hoechst 33342 (Dojin Chemical Laboratory, Kumamoto, Japan) was used for nuclear staining and fluorescence signals were observed using a fluorescence microscope (Axio observer Z1, Carl ZEISS, Germany).

PreS1 binding assay

HepG2-hNTCP-C4 cells were exposed to 40 nM C-terminally TAMRA-conjugated myristrated preS1 peptide (Scram) with and without the compounds at 37°C for 45 min. The cells were rinsed three times with culture medium and twice with PBS, and then fixed with 4% paraformaldehyde for 20 min at room temperature. Fluorescence-labeled cells were observed using a fluorescence microscope (Axio observer Z1, Carl ZEISS). The red fluorescence intensity was measured at Ex: 530 nm and Em: 590 nm using TriStar 3 multimode microplate reader (Berthold Technologies, Germany)

Virucidal test

The HBV-compound mixture was preincubated at 37°C for 2 h. After applying the mixture onto an Amicon centrifugal filtration device (Millipore, Billerica, MA, USA), the washing-out process was performed three times by centrifugation to remove free amentoflavone. HBV was recovered from the filter and inoculated onto HepG2-hNTCP-C4 cells.

HBV adsorption and internalization assay

For the HBV adsorption assay, the mixture of HBV and amentoflavone was preincubated for 2 h at 37°C prior to inoculation to cells and then added to prechilled HepG2-hNTCP-C4 cells. After incubating at 4°C for 90 min, the cells were extensively rinsed with ice-cold phosphate-buffered saline (PBS) and refed with a fresh medium without amentoflavone. At 10 days p.i., total cellular RNA was extracted using ISOGEN-II. For the internalization assay, pre-chilled cells were inoculated with HBV at 4°C for 3 h. After removing unbound virus, cultures were shifted to 37°C under the media containing amentoflavone for 20 h. Cells were treated with trypsin-EDTA to remove surface-attached virus, and total cellular DNA was extracted using QIAamp DNA mini kit. HBV DNA were quantified with SYBR Premix Ex Taq II using a Bio-Rad CFX Manager. The primers used were Ayw-F:5′-CTCGTGGTGGACTTCTCTC-3′ and Ayw-R: 5′-AAGATGAGGCATAGCCAGCA-3′.

Transporter assay

Bile acid uptake activity in HepG2-hNTCP-C4 cells was measured in the presence and absence of sodium by transporter assay (34).

Cytotoxicity assay

The cytotoxic effects of test compounds on cultured cells were determined using Cell Proliferation Kit II (XTT, Sigma-Aldrich) as previously described (28).

Cell counting

A total of 2.4×10^5 HepG2-hNTCP-C4 cells per well were seeded in a collagen-coated 24-well plate. Next day, cells were treated with different concentrations of amentoflavones at 37°C for 24 h. After removing the culture medium, the cells were washed by PBS and further incubated in medium without amentoflavone with changing every 2 or 3 days for 7 days. Living cells were counted using manual hemocytometer.

Statistical analysis

Data were analyzed with GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Differences between control and treated groups were evaluated by Student's *t*-test or one-way analysis of variance (ANOVA). A *P*-value less than 0.05 was considered as statistically significant.

Results

Amentoflavone exhibits anti-hepatitis B activity

We evaluated anti-HBV activity of amentoflavone using HepG2-hNTCP-C4 cells, a HepG2 cell line overexpressing the human NTCP gene and supporting HBV infection. HepG2-hNTCP-C4 cells were inoculated with genotype D of HBV derived from Hep38.7-Tet cells for 18 h at 37°C in the presence or absence of amentoflavone, and the levels of HBsAg released in the culture supernatants on day 7 p.i. were analyzed by ELISA. Amentoflavone treatment reduced HBsAg production from infected cells in a dose-dependent manner with the half-maximum effective concentration (EC_{50}) value of

48.6 μg/ml (90.2 μM) (Fig. 1A). Amentoflavone did not show cellular toxicity in HepG2-hNTCP-C4 cells at the concentration range tested, except for a slight decrease of cell viability and viable cell number at the concentration of 80 μg/ml (Fig. 1B). The 50% cytotoxic concentration (CC_{50}) was 155.2 μg/ml, and the selective index (SI, CC_{50}/EC_{50}) was 3.2. Next, HBV infection was examined by measuring the expression levels of HBe and HBc antigens, HBV RNA, and cccDNA. HBV preS1 peptide that mimics N-terminal aa 2-48 of the HBV large envelope protein, was used as a positive control inhibitor. In the presence of preS1 peptide, all markers of HBV infection showed remarkable reduction (Fig. 1C-F). Amentoflavone treatment suppressed the expression levels of extracellular HBeAg (Fig. 1C), intracellular HBV RNA (Fig. 1D), and cccDNA (Fig. 1E) from HBV-infected HepG2-hNTCP-C4 cells. Immunofluorescent staining results showed HBs and HBc protein expression in cells and the number of HBsAg-positive cells are decreased by amentoflavone treatment as well (Fig. 1F and 1G). We used primary human hepatocytes PXB-cells to evaluate the impact of amentoflavone in a more physiologically relevant model. We observed the decrease of HBsAg level from the HBV-infected PXB-cells by amentoflavone (EC_{50} = 58.6 μg/ml) (Fig. 1H). Amentoflavone did not exhibit cytotoxic activity against the PXB-cells up to 160 μg/ml (Fig. 1I). We also examined antiviral effect of amentoflavone against HBV genotype C. Amentoflavone as well as the preS1 peptide reduced the HBV RNA levels in the cells (Fig. 1J) although the inhibitory effect was relatively weak. These results suggest that amentoflavone have antiviral activity against HBV.

Amentoflavone blocks preS1-mediated viral attachement to the cells

297 To determine at which step of the HBV life cycle amentoflavone is acting, time-of-
298 addition studies were performed. Three sets of experiments were conducted in parallel
299 (Fig. 2A): (i) Pre-treatment: To examine the possible effect of amentoflavone on the
300 expression of host factors associated with virus entry, HepG2-hNTCP-C4 cells were
301 pretreated with amentoflavone for 2 h before HBV infection. After removing
302 amentoflavone, pretreated cells were challenged with HBV infection for 18 h in the
303 absence of amentoflavone. After removing the unbound virus, infected cells were further
304 cultured until HBsAg expression-level analysis. This experiment determines the antiviral
305 effect on pre-virus inoculation, especially against the host factor. (ii) Co-addition:
306 amentoflavone was added to cell cultures during virus inoculation for 18 h. After washing
307 out free amentoflavone and virus, infected cells were further incubated in the absence of
308 amentoflavone until HBsAg expression-level analysis. This experiment determines the
309 antiviral effect during the viral entry. (iii) Post-infection: cells were infected with HBV
310 in the absence of amentoflavone for 18 h. After washing out unbound virus, the infected
311 cells were treated with amentoflavone for 24 h. After removing free amentoflavone,
312 infected cells were further incubated until HBsAg expression-level analysis. This
313 experiment determines the antiviral effect at the early post-viral entry step. HBV preS1
314 peptide was used as a positive control. Amentoflavone treatment effectively inhibited
315 HBV infection at co-addition treatment, but not at pre-virus inoculation or early post-
316 infection (Fig. 2B). These results suggest that amentoflavone inhibits the virus entry step.
317 We further examined whether every stage of virus entry is suppressed by
318 amentoflavone. Firstly, virucidal activity test to examine a direct inactivation of
319 amentoflavone on the virus particles was conducted. HBV was premixed with
320 amentoflavone and the mixture was incubated for 2 h at 37°C. The mixture was applied

onto a centrifugal filtration device and the process of washing-out process was repeated by centrifugation to remove free amentoflavone. As shown in Fig. 3A, amentoflavone significantly reduced HBV infectivity to 75% of untreated control ($P < 0.01$). We then tested the effect of amentoflavone on virus attachment. After pretreatment of virus and amentoflavone mixture for 2 h at 37°C, the mixture was inoculated onto the pre-chilled HepG2-hNTCP-C4 cells and incubated for 90 min at 4°C to allow HBV particles to bind to cell surface. After removing unbound virus particles by extensive washing with cold PBS, cells were incubated in culture medium without amentoflavone for 10 days. The expression levels of HBV RNA in the cells were measured by qRT-PCR analysis. The result showed that amentoflavone markedly inhibited HBV RNA levels by 25% of untreated control (Fig. 3B). Next, we examined the effect of amentoflavone on viral penetration. Pre-chilled cells were inoculated with HBV at 4°C for 3 h and then cultures were shifted to 37°C in the presence of amentoflavone for 20 h to allow the internalization of the virus particles into the cells. After non-internalized HBV was removed with trypsinization, HBV DNA levels were measured by qRT-PCR analysis. The result showed that amentoflavone did not decrease intracellular HBV DNA levels (Fig. 3C).

HBV entry is mediated by the binding of the HBV preS1 domain to the NTCP molecule (5). To evaluate whether amentoflavone can block the preS1 binding to the cell surface of HepG2-hNTCP-C4 cells, a preS1 binding assay was conducted. The addition of amentoflavone or non-label preS1 peptide as a positive control strongly inhibited the binding of TAMRA-labeled preS1 peptide to the surface of HepG2-hNTCP-C4 cells as shown in the decrease of red fluorescence signals (Fig. 3D and 3E). Collectively, these results suggest that amentoflavone inhibits preS1-mediated HBV attachment to the cell surface.

345

346 **Amentoflavone inhibits NTCP transporter activity**

347 The viral receptor function of NTCP has been reported to share common
348 determinants with bile acid transporter activity (5, 35). Since amentoflavone blocked the
349 binding of a HBV preS1 peptide to HepG2-hNTCP-C4 cell surface, we tested the ability
350 of amentoflavone to affect the functional activity of NTCP. NTCP transporter activity
351 was evaluated by treating HepG2-hNTCP-C4 cells using [^{3}H]-labeled taurocholic acid
352 (TCA) as a NTCP substrate in the presence or absence of amentoflavone. As shown in
353 Fig. 4, amentoflavone and CyA, a known compound to impair the NTCP-mediated bile
354 acid uptake (11, 36), suppressed TCA uptake by 40% and 12%, respectively, of the
355 untreated control.

356

357 **Comparison of amentoflavone derivatives for anti-HBV activity**

358 Naturally occurring biflavonoids are dimers of flavonoid moieties linked by either
359 C-C or C-O-C bonds. Amentoflavone is a C-C type compound with apigenin residues
360 coupled at C-8- and C-3- positions. We then examined the structure essential for anti-
361 HBV activity. Commercially available C-C type bioflavonoids, cupressuflavone,
362 robustaflavone, and sciadopitysin and the monomeric flavonoid apigenin were used for
363 antiviral examination. Chemical structures of these compounds are shown in Fig. 5A.
364 HBV was inoculated onto HepG2-NTCP-C4 cells in the presence of compounds for 16 h.
365 After removing unbound virus and compounds, cells were further incubated for 7 days
366 and HBsAg and HBeAg levels in the culture supernatants from infected cells were
367 determined by ELISA assay. Among the compounds, robustaflavone most strongly

reduced the production of both HBsAg and HBeAg from infected cells. Anti-HBV efficiency and cytotoxic effects by robustaflavone were almost the same as those of amentoflavone (HBsAg, EC_{50}: 56.4 μg/ml, CC_{50}: >80 μg/ml) (Fig. 5C and 5D). Sciadopitysin, the trimethyl ether derivative of amentoflavone, decreased the production of HBeAg, but not HBsAg. A symmetrical C-8, C-8" biapigenin, cupressuflavone, did not show any notable antiviral effect (Fig. 5C and 5D). Contrarily, the monomeric flavonoid, apigenin, enhanced HBsAg and HBeAg production from infected cells.

Discussion

In this study, we identified amentoflavone as a new entry inhibitor of HBV infection. Until recently, amentoflavone has shown antiviral effects against other viruses. For example, early infections with HSV-1 and acyclovir-resistant strains were inhibited by amentoflavone through affecting cofilin-mediated F-actin reorganization and reducing viral nuclear transportation to the nucleus (27). Amentoflavone suppressed coxsackievirus B3 replication through the inhibition of fatty acid synthesis (37). The proteolytic activity of SARS-CoV 3-chymotrypsin-like protease ($3CL^{pro}$) was inhibited by amentoflavone isolated from *Torreya nucifera* (38). Amentoflavone also inhibited viral entry, replication, and translation steps of the hepatitis C virus life cycle (39). Antiviral effect of amentoflavone against HBV were not reported so far, however an amentoflavone derivative, robustaflavone, showed inhibition of HBV replication in Hep2.2.15 cells (40).

We found that amentoflavone inhibits HBV infection to HBV susceptible cells, HepG2-hNTCP-C4, and primary human hepatocyte PXB-cells (Fig. 1A and 1H). Notably,

amentoflavone treatment decreased levels of all HBV biomarkers (extracellular HBsAg and HBeAg, intracellular HBsAg and HBcAg, cccDNA, and HBV RNA) in HBV-infected cells (Fig. 1). Time-of-addition studies showed that amentoflavone exerts anti-HBV activity at the viral entry step, not the early post-viral infection step including viral penetration (Fig. 2, 3B and 3C). Amentoflavone not only interrupted the HBV preS1 binding to HepG2-hNTCP-C4 cell surface (Fig. 3D and 3E), but also inhibited NTCP transporter activity (Fig. 4). These results suggest that amentoflavone exerts anti-HBV activity by suppression of HBV attachment via the HBV preS1 binding to the host cells.

NTCP, encoded by the gene *SLC10A1*, is a Na+/bile acid cotransporter in hepatocytes. Recently, NTCP has been identified as the functional entry receptor for HBV and hepatitis delta virus infection (5). NTCP expression on the cell surface confers HBV susceptibility among the target cells; thus, a cell line stably expressing human NTCP including HepG2-hNTCP-C4 cells has been used for the screening of antivirals that target viral entry. Various entry inhibitors such as attachment inhibitors targeting the preS1 region of HBsAg, NTCP substrates, direct inhibitors of NTCP, and inhibitors that regulate NTCP expression have been reported. For instance, a multimeric flavanol, proanthocyanidin, and its analog, oolonghomobisflavan C, showed anti-HBV activity by directly targeting the preS1 region of the HBV large surface protein (15). The synthetic myristoylated $PreS1_{2\text{-}48}$ lipopeptide has been found to inhibit HBV and HDV infection by blocking viral binding to the NTCP receptor (41, 42). Bulevirtide, a synthetic $PreS1_{2\text{-}48}$-derived lipopeptide, has been approved as a first-in-class HDV entry inhibitor (43). The immunosuppressive drug CyA and its derivatives, as well as clinically approved drugs such as ezetimibe, irbesartan, rosiglitazone, zafirlukast, TRIAC, sulfasalazine, Chicago Sky Blue 6B, and Evans Blue, have been identified as entry inhibitors that interact with

NTCP (10-12, 14, 36, 44, 45). Ro41-5253, a selective antagonist of retinoic acid receptor antagonist inhibited HBV infection through suppression of NTCP expression (46). A flavonoid, epigallocatechin gallate, blocked HBV infection into immortalized primary human hepatocytes by blocking the virus endocytosis/cell fusion step (13). Curcumin inhibited HBV infection by suppressing viral attachment and internalization (47).

As an apigenin dimer with C3-8 linkage, amentoflavone is a small molecule that is chemically distinct from compounds identified thus far as potential entry inhibitors. Interestingly, the amentoflavone-derivative robustaflavone (C3-6 linkage) also reduced production of HBsAg and HBeAg from infected cells and its antiviral potency was comparable efficacy to those of amentoflavone (Fig. 5C and 5D). On the other hand, amentoflavone-7,4',4'"-trimethyl ether derivative, sciadopitysin, another C-C type of biflavonoid, cupressuflavone, or monomeric flavonoid apigenin were none or moderate inhibitory effect (Fig. 5C and 5D). These suggested that two apigenin motifs with C3-8 or C3-6 linkage are important for anti-HBV activity. Meanwhile, amentoflavone also showed low selectively against HepG2-hNTCP-C4 cells (SI = 3.2) (Fig. 1B). Chemical modifications of amentoflavone to increase the anti-HBV property should be considered in the future study.

Antiviral mechanism of action demonstrated that amentoflavone inhibited viral adsorption to host cells as the preS1 peptide does (Fig. 3B), but pretreatment of cells with amentoflavone before the viral inoculation failed to block HBV infection (Fig. 2B). In this connection, receptor antagonists can be classified as either reversible or irreversible, depending on the longevity of the antagonist-receptor complex. Amentoflavone might dissociate readily from the binding site of the NTCP receptor. Further research is needed to confirm type of interaction between amentoflavone and the NTCP molecule.

The inhibition of TCA uptake activity is associated with anti-HBV activity on HBV infection (10, 35). In the present study, we observed that amentoflavone impairs function of NTCP-mediated bile acid transport activity (Fig. 4) in agreement with most of HBV entry inhibitors targeting NTCP reported by other groups (e.g., CyA, betulin derivatives and, curcumin) (17, 36, 47). Although amentoflavone showed a dose-dependent inhibition in HBV infection, TCA inhibition activity of amentoflavone reached a plateau at an inhibition of 60%. The difference in degree of inhibition between infection assay and transporter assay might result from the difference in conditions of experimental temperature. Virus adsorption assay was carried out at 4°C to allow HBV particles to bind to cell surface, while transporter assay was conducted at 37°C. HBV entry inhibitor is expected to inhibit the viral entry without impairing hepatic bile acid uptake by NTCP. Thus, amentoflavone may offer the less adverse effect on interference with bile acid transport.

Amentoflavone has shown to possess numerous biological activities to human diseases such as anti-cancer, anti-inflammatory, anti-oxidative, antidiabetic, and antibacterial activity. Our result also revealed that amentoflavone is a good candidate for development of a new anti-HBV drug. Therefore, amentoflavone is considered as a promising therapeutic agent for clinical research. However, its low aqueous solubility and poor oral bioavailability hamper the clinical application of amentoflavone. In recent years, amorphous solid dispersions (ASDs) technique has been developed to improve the solubility and bioavailability of the poorly-soluble drugs (48, 49) and the Food and Drug Administration has approved 19 commercial ASD products from 2007 to 2017 (50). ASDs for oral drug delivery could be useful for pharmacokinetics and antiviral studies of the amentoflavone *in vivo*.

In conclusion, amentoflavone inhibits HBV infection by the suppression of the HBV attachment. Amentoflavone and its structurally related biflavonoids can provide new insights into the design of a novel anti-HBV entry inhibitor.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

List of abbreviations:

CHB: chronic hepatitis B, HBV: hepatitis B virus, Peg-IFNs: pegylated interferons, NAs: nucleoside/nucleotide analogs, NTCP, sodium taurocholate cotransporting polypeptide**,** rcDNA: double-stranded relaxed circular DNA, cccDNA: covalently closed circular

DNA, HBsAg: hepatitis B surface antigen, HBeAg: hepatitis B e antigen, HBc: HB core, HSV-1: herpes simplex virus type 1, CyA: cyclosporin A, DMSO: dimethyl sulfoxide, PXB-cells: primary human hepatocytes, p.i.: post-infection, qPCR: quantitative PCR, EC_{50}: half-maximum effective concentration, CC_{50}: 50% cytotoxic concentration, SI: selectivity index ,TCA: taurocholic acid, ASDs: amorphous solid dispersions

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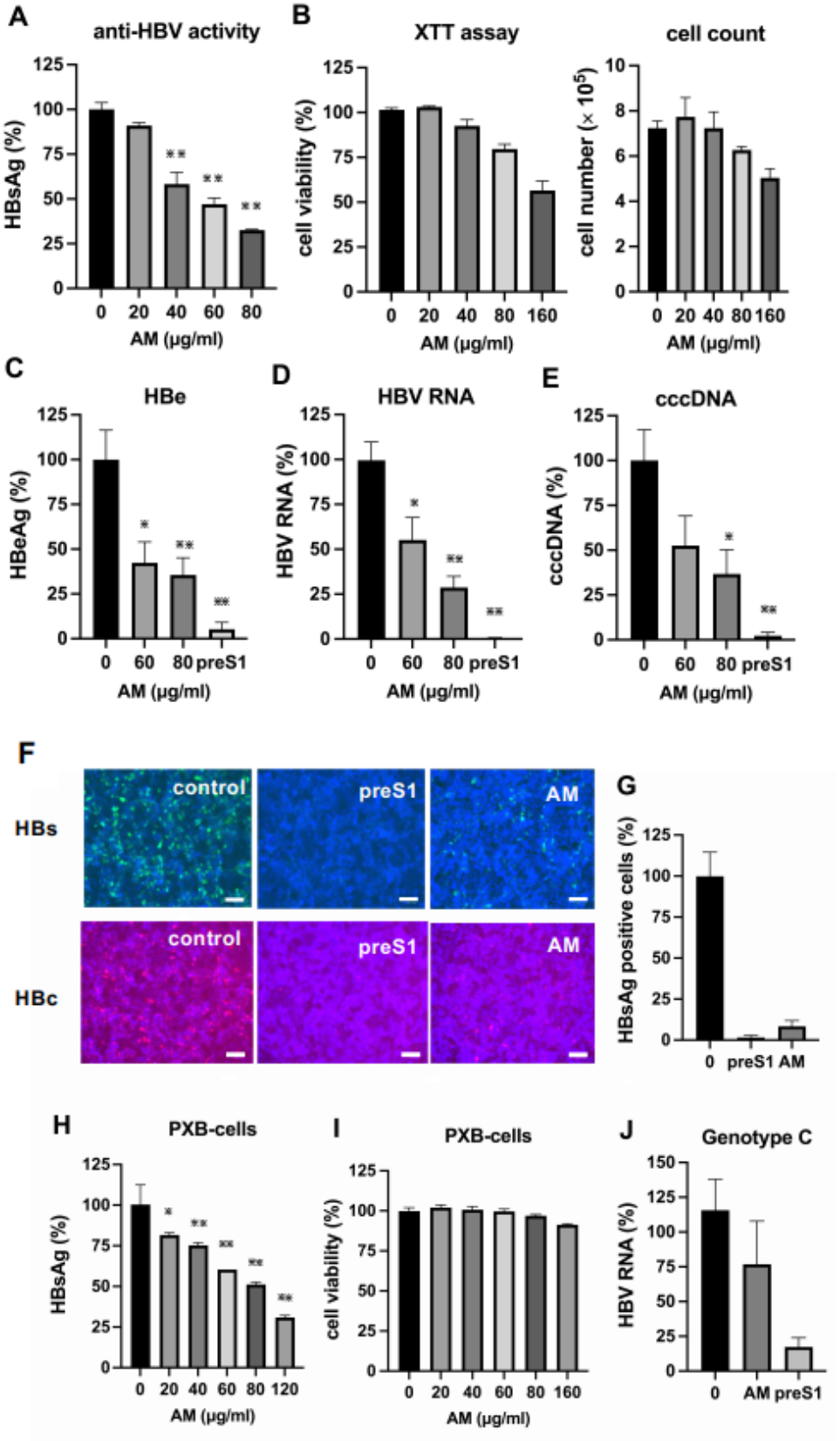

624 **Fig. 1. Amentoflavone inhibits HBV infection.** Anti-HBV activity and cytotoxicity of

amentoflavone on HepG2-hNTCP-C4. (A) Levels of HBsAg production in culture supernatants of HepG2-hNTCP-C4 cells were determined by ELISA. (B) To monitor cytotoxic levels of amentoflavone, viability assessment (left) and cell count (right) were performed. Cell viability was determined by XTT assay. HepG2-hNTCP-C4 cells in a 24-well plate were treated with different concentrations of amentoflavones at 37°C for 24 h. After the removal of amentoflavone, cells were incubated in medium without amentoflavone for 7 days. Living cells were manually counted. Data are expressed as mean ± SD from triplicate wells. (C to I) HBV was inoculated onto HepG2-hNTCP-C4 cells in the presence of amentoflavone or 200 nM preS1 peptide at 37°C for 18 h. Secretion of HBeAg (C) in culture supernatants and HBV RNA (D) and cccDNA (E) levels in infected cells were determined by ELISA and qPCR, respectively. (F) HepG2-hNTCP-C4 cells were infected with HBV in the presence of amentoflavone (80 μg/ml), preS1 peptide (200 nM), or 0.2% DMSO as the untreated control. Infected cells were stained with anti-HBs (green) and anti-HBc (red) antibodies. Nuclear was stained with Hoechst 33342 (blue). Scale bar, 100 μM. (G) HBV-infected HepG2-hNTCP-C4 cells were stained with anti-HBs antibody, as shown in (F). The number of HBsAg-positive cells from seven randomly selected areas was counted. The percentages of HBsAg-positive cells by the compounds compared to the untreated control are shown. Data show mean ± SD from two independent experiments. (H, I) Anti-HBV activity and cytotoxicity of amentoflavone on PXB-cells. The HBsAg levels in culture supernatants of PXB-cells were determined by ELISA (H) and cell viability was determined by the XTT assay (I). (J) HepG2-hNTCP-C4 cells were infected with HBV genotype C in the presence of amentoflavone (80 μg/ml), preS1 peptide (200 nM), or 0.2% DMSO. After the removal of amentoflavone, cells were incubated in medium without amentoflavone for 10 days.

HBV RNA levels in the cells were quantified by qPCR. Data show mean ± SD from two independent experiments. All data except (C) and (F) are expressed as mean ± SEM from two or three independent experiments. *$P < 0.05$, **$P < 0.01$. AM: amentoflavone.

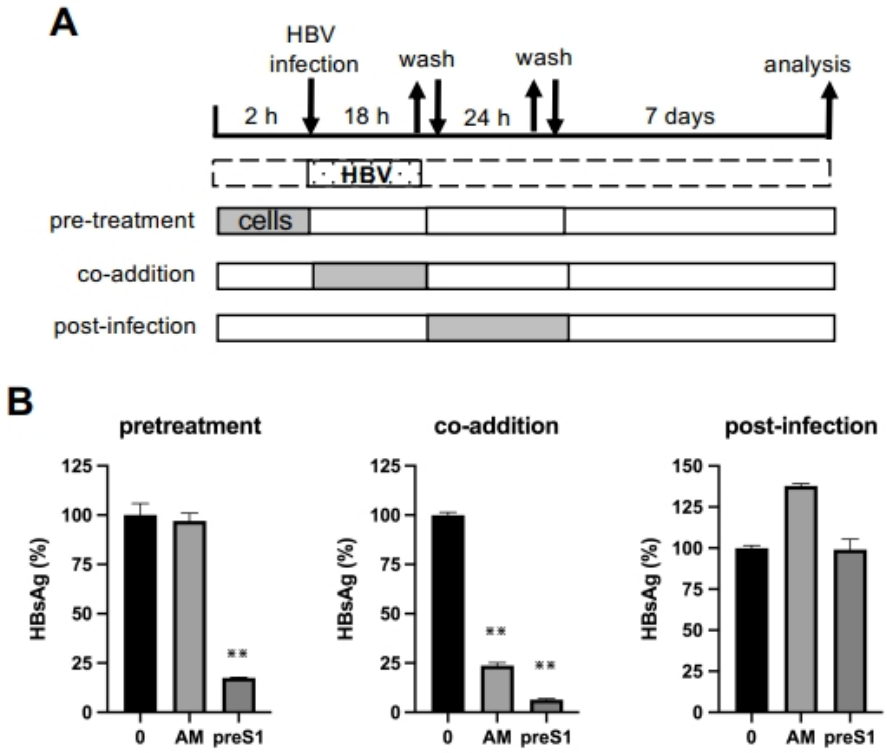

Fig. 2. Amentoflavone inhibits HBV entry step. (A) A schematic of time-of-addition experiments. Pre-treatment: cells were pretreated with compounds for 2 h before HBV infection. After removing compounds, pretreated cells were challenged with HBV infection for 18 h in the absence of compounds. Co-addition: compounds were added to cell cultures for 18 h during virus inoculation. Post-infection: cells were infected with HBV for 18 h in the absence of compounds. After washing out unbound virus, the

infected cells were treated with compounds for 24 h. HBsAg levels in culture supernatants at day 8 p.i. were determined by ELISA. The gray and white square show the periods of treatment and nontreatment of compounds. (B) HepG2-hNTCP-C4 cells were pretreated with amentoflavone (80 μg/ml), preS1 peptide (200 nM), or 0.2% DMSO as negative control for 2 h at 37°C. After washing out compounds, pretreated cells were inoculated with HBV without compounds (pretreatment). HBV was inoculated onto HepG2-hNTCP-C4 cells in the presence of amentoflavone (80 μg/ml), preS1 peptide (200 nM), or 0.2% DMSO for 18 h at 37°C (co-addition). Cells were inoculated with HBV without compounds for 18 h at 37°C. After washing out free virus, cells were treated with amentoflavone (80 μg/ml), preS1 peptide (200 nM), or 0.2% DMSO for 24 h at 37°C (post-infection). Levels of HBsAg in culture supernatants were determined by ELISA. AM: amentoflavone. Values show mean ± SD from two independent experiments.

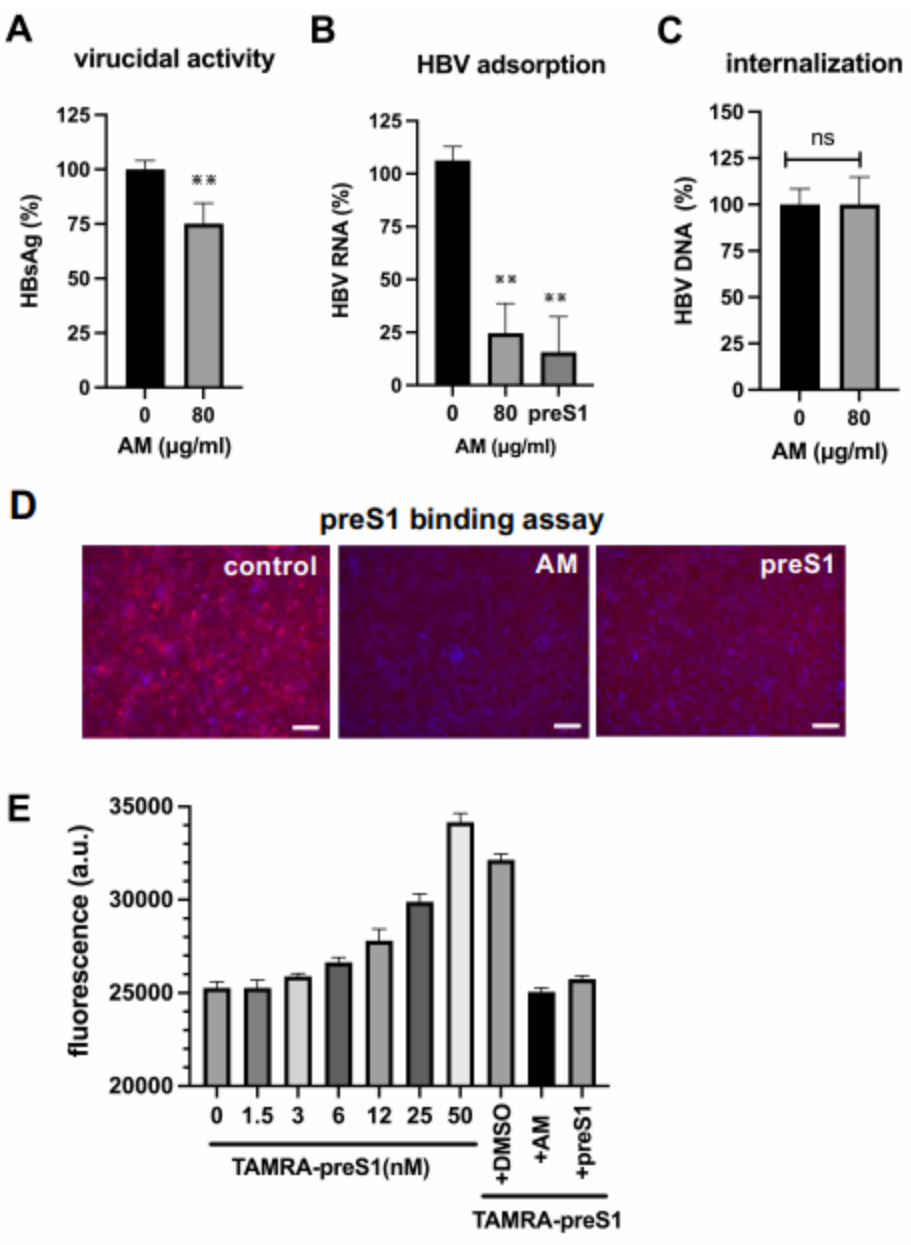

Fig. 3. Amentoflavone blocks HBV adsorption via preS1 binding. (A) Virucidal activity of amentoflavone. A mixture of HBV and amentoflavone was preincubated at 37°C for 2 h. After removal of free amentoflavone with centrifugal filter device, residual virus infectivity was titrated and expressed as a percentage relative to the untreated control. (B) Effect of amentoflavone on HBV adsorption. HBV- amentoflavone mixture was preincubated for 2 h at 37°C, then inoculated onto pre-chilled HepG2-hNTCP-C4

cells for 90 min at 4°C to allow HBV adsorption. After extensive washing with cold PBS, cells were incubated without compounds for 10 days at 37°C. HBV RNA was extracted and quantified by qPCR analysis. (C) Effect of amentoflavone on virus internalization. HepG2-hNTCP-C4 cells were exposed with HBV on ice for 3 h in the absence of compounds, and cultures were then transferred to 37°C in the presence of amentoflavone for 20 h to allow viral internalization. After trypsinization and extensive washing of the cells, intracellular HBV DNA were quantified by qPCR. (A-C) Data are expressed as mean ± SEM from two independent experiments. (D) HepG2-hNTCP-C4 cells were incubated with 40 nM TAMRA-labeled preS1 peptide in the presence of 80 μg/ml amentoflavone, 200 nM non-label preS1 peptide, or 0.2% DMSO as a control at 37°C for 45 min. The binding of TAMRA-labeled preS1 to the cell surface was observed by fluorescence microscopy. Red and blue signals indicate preS1 probe and the nucleus, respectively. Scale bar, 100 μm. (E) HepG2-hNTCP-C4 cells were incubated with different concentrations of TAMRA-labeled preS1 peptide (TAMRA-preS1) at 37°C for 45 min. In a competitive binding experiment, cells were exposed with 40 nM TAMRA-preS1 in the presence or absence of compounds (0.2% DMSO, 200 nM non-label preS1 peptide, or 80 μg/ml amentoflavone). The red fluorescence intensity was measured using multimode microplate reader. Data are expressed as mean ± SD from triplicate wells. $^{**}P < 0.01$, ns: not significant, AM: amentoflavone, a.u.: arbitrary unit

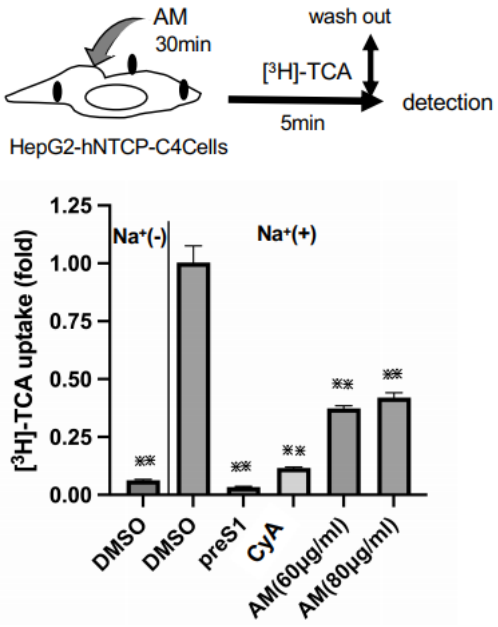

Fig. 4. Amentoflavone inhibits NTCP transporter activity. Cells were treated with compounds (amentoflavone (AM), 200 nM preS1 peptide, 10 μM cyclosporin A (CyA) and, 0.2% DMSO) for 37°C for 30 min followed by the addition of TCA for 5 min. Fold reduction of cellular TCA uptake compared to the 0.2% DMSO (Na+) control was calculated. Data are expressed as mean ± SD from triplicate wells. $^{**}P < 0.01$.

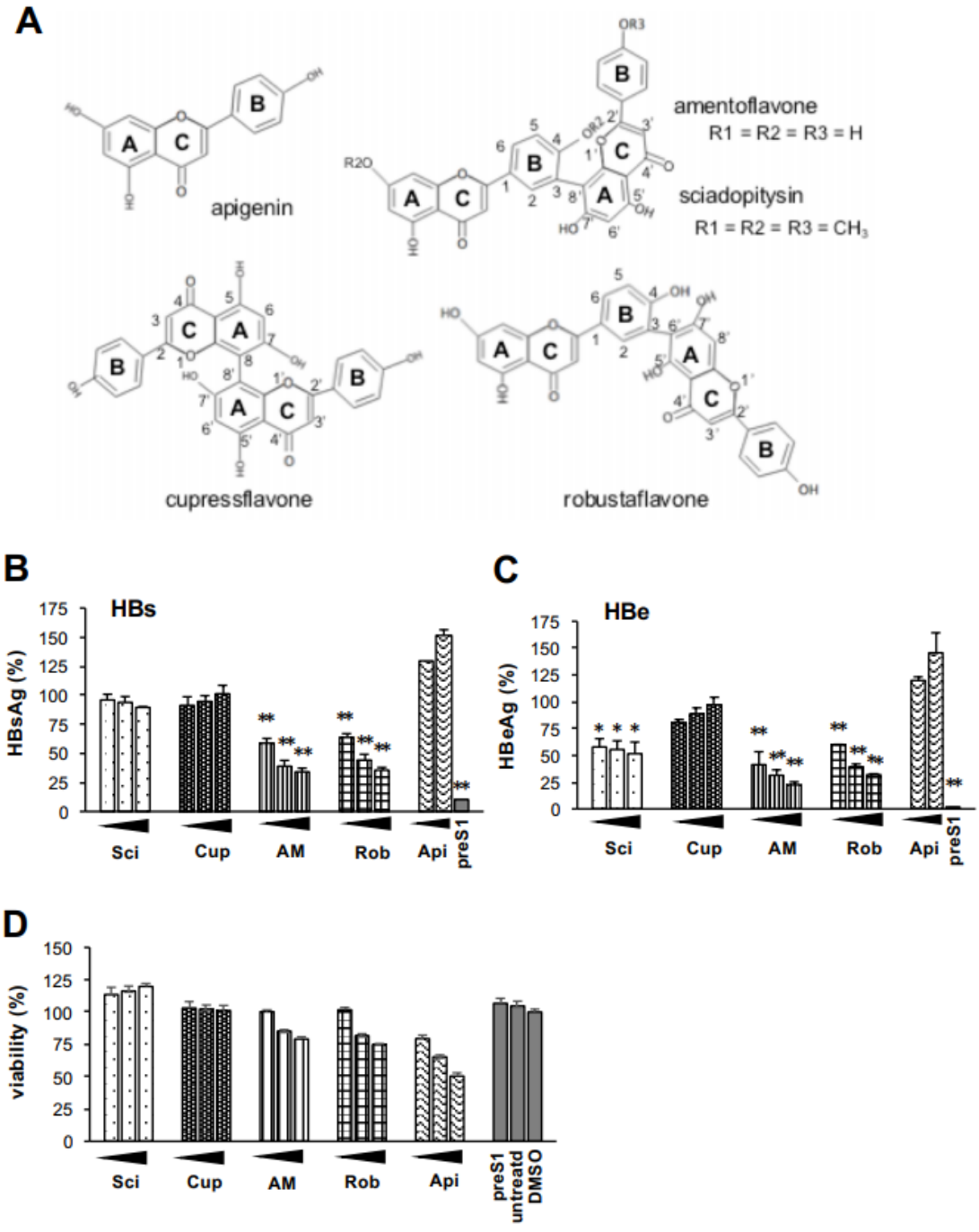

Fig. 5. Anti-HBV and cytotoxic activities of amentoflavone derivatives. (A) Chemical structure of amentoflavone and amentoflavone derivatives. Sciadopitysin, cupressflavone, amentoflavone, and robustaflavone consists of two apigenin moieties (rings A-C) linked

through carbon-carbon. Amentoflavone and sciadopitysin have the same core structure of C3-8 linkage. Robustaflavone has a structure with C3-6 linkage. Cupressflavone consists of two apigenin units linking at each A ring. (B) HBV was inoculated to HepG2-hNTCP-C4 cells in the presence of various concentrations of compounds (sciadopitysin, cupressflavone, amentoflavone, robustaflavone: 40, 60, and 80 μg/ml, apigenin: 20 and 40 μg/ml, 200 nM preS1 peptide, 0.2% DMSO) for 18 h. Levels of HBsAg (B) and HBeAg (C) secreted in the culture supernatants on day 7 p.i. were determined by ELISA. Relative values as compared to the 0.2% DMSO control were calculated. (D) HepG2-hNTCP-C4 cells were treated with amentoflavone derivatives (sciadopitysin, cupressflavone, amentoflavone, robustaflavone: 40, 60, and 80 μg/ml, apigenin: 20, 40 and 80 μg/ml), 200 nM preS1 peptide or 0.2% DMSO for 18 h. Cell viability relative to the 0.2% DMSO control was shown. Data are expressed as mean ± SEM from two independent experiments. Sci: sciadopitysin, Cup: cupressflavone, AM: amentoflavone, Rob: robustaflavone, Api: apigenin, $*P < 0.05$, $**P < 0.01$.