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## 4 **Amentoflavone inhibits hepatitis B virus infection via the suppression** 5 **of preS1 binding to host cells**

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#### 54 **Abstract**

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

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

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

75

## 76 **Introduction**

77 Chronic hepatitis B (CHB) infection is caused by the hepatitis B virus (HBV) and  
78 affects more than 250 million people worldwide (1). Chronic infection often leads to the  
79 development of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma  
80 (2, 3).

81 Currently, two types of medications are used for treating CHB, pegylated interferons  
82 (Peg-IFNs) and nucleoside/nucleotide analogs (NAs). The advantage of Peg-IFN therapy  
83 is a higher loss of hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg)  
84 seroconversion within a finite duration of therapy, but the use of Peg-IFNs is limited by  
85 intolerance and side effects. NA drugs, including entecavir, tenofovir disoproxil fumarate,  
86 and tenofovir alafenamide fumarate, can effectively suppress viral loads and are well  
87 tolerated, however, the virological and clinical relapse rates are high after discontinuing  
88 NA drugs. The HBsAg clearance rates are low in patients treated with both NA (1.4%–  
89 5.1%) and Peg-IFN (2.1%–20%) treatment (4). Therefore, the development of anti-HBV

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

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

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

114 proanthocyanidin, betulin derivatives, troglitazone, and epigallocatechin-3-gallate (5)  
115 (10-17); some of these are currently in the clinical trial phase (18). A combination  
116 antiviral drug regiment composed of inhibitors having different mechanisms of actions  
117 may act as an efficient antiviral strategy for treating CHB infections because combination  
118 therapies comprising different direct-acting antivirals have been shown to be highly  
119 effective on chronic hepatitis C treatment (19).

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

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

142

## 143 **Materials and Methods**

### 144 **Cell cultures**

145 HepG2-hNTCP-C4 cells (32) were cultured on type I collagen-coated dish using  
146 media of Dulbecco's Modified Eagle Medium (DMEM)/F-12, GlutaMAX<sup>TM</sup> (Thermo  
147 Fisher Scientific, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS,  
148 Biowest, Nuaille, France), 10 mM HEPES (Nacalai Tesque, Kyoto, Japan), 5 µg/ml  
149 insulin (Wako, Kyoto, Japan), 100 IU/ml penicillin/streptomycin (Nacalai Tesque), and  
150 0.5 mg/ml G418 (Wako). Huh7 cells were grown in DMEM (Wako) supplemented with  
151 10% fetal bovine serum, non-essential amino acid (Wako), and 100 IU/ml  
152 penicillin/streptomycin. Primary human hepatocytes (PXB-cells) were purchased from  
153 PhenixBio (Hiroshima, Japan) and cultured in maintenance medium for PXB-cells  
154 (PhenixBio).

### 155 **Reagents**

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

161 **Virus preparation**

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

173 **HBV infection assay**

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

182 **DNA extraction and quantification of HBV cccDNA**



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

#### 195 **Total RNA extraction, reverse transcription, and quantification of HBV RNA**

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

#### 205 **ELISA**

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

#### 209 **Indirect immunofluorescence analysis**

210 HBV-infected HepG2-hNTCP-C4 cells on day 8 post-infection (p.i.) were fixed with  
211 4% paraformaldehyde (Nacalai Tesque) and permeabilized with 0.5% Triton X-100.  
212 After blocking with 2% block ace solution (DS Pharma Biomedical Co., Osaka, Japan),  
213 the cells were incubated with both anti-HBs polyclonal antibodies (ab9193, Abcam,  
214 Cambridge, MA, USA) and anti-Hep B cAg antibodies (C1-5) (Santa Cruz, Dallas, TX,  
215 USA) as primary antibodies, followed by FITC-conjugated anti-equine (SouthernBiotech,  
216 AL, USA) and Alexa Flour 568 anti-mouse IgG (Abcam) as secondary antibodies,  
217 respectively. Hoechst 33342 (Dojin Chemical Laboratory, Kumamoto, Japan) was used  
218 for nuclear staining and fluorescence signals were observed using a fluorescence  
219 microscope (Axio observer Z1, Carl ZEISS, Germany).

#### 220 **PreS1 binding assay**

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

#### 228 **Virucidal test**

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

#### 234 **HBV adsorption and internalization assay**

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

#### 247 **Transporter assay**

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

#### 250 **Cytotoxicity assay**

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

### 253 **Cell counting**

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

### 259 **Statistical analysis**

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

264

## 265 **Results**

### 266 **Amentoflavone exhibits anti-hepatitis B activity**

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

274 48.6  $\mu\text{g/ml}$  (90.2  $\mu\text{M}$ ) (Fig. 1A). Amentoflavone did not show cellular toxicity in HepG2-  
275 hNTCP-C4 cells at the concentration range tested, except for a slight decrease of cell  
276 viability and viable cell number at the concentration of 80  $\mu\text{g/ml}$  (Fig. 1B). The 50%  
277 cytotoxic concentration ( $\text{CC}_{50}$ ) was 155.2  $\mu\text{g/ml}$ , and the selective index (SI,  $\text{CC}_{50}/\text{EC}_{50}$ )  
278 was 3.2. Next, HBV infection was examined by measuring the expression levels of HBe  
279 and HBc antigens, HBV RNA, and cccDNA. HBV preS1 peptide that mimics N-terminal  
280 aa 2-48 of the HBV large envelope protein, was used as a positive control inhibitor. In  
281 the presence of preS1 peptide, all markers of HBV infection showed remarkable reduction  
282 (Fig. 1C-F). Amentoflavone treatment suppressed the expression levels of extracellular  
283 HBeAg (Fig. 1C), intracellular HBV RNA (Fig. 1D), and cccDNA (Fig. 1E) from HBV-  
284 infected HepG2-hNTCP-C4 cells. Immunofluorescent staining results showed HBs and  
285 HBc protein expression in cells and the number of HBsAg-positive cells are decreased by  
286 amentoflavone treatment as well (Fig. 1F and 1G). We used primary human hepatocytes  
287 PXB-cells to evaluate the impact of amentoflavone in a more physiologically relevant  
288 model. We observed the decrease of HBsAg level from the HBV-infected PXB-cells by  
289 amentoflavone ( $\text{EC}_{50} = 58.6 \mu\text{g/ml}$ ) (Fig. 1H). Amentoflavone did not exhibit cytotoxic  
290 activity against the PXB-cells up to 160  $\mu\text{g/ml}$  (Fig. 1I). We also examined antiviral effect  
291 of amentoflavone against HBV genotype C. Amentoflavone as well as the preS1 peptide  
292 reduced the HBV RNA levels in the cells (Fig. 1J) although the inhibitory effect was  
293 relatively weak. These results suggest that amentoflavone have antiviral activity against  
294 HBV.

295

296 **Amentoflavone blocks preS1-mediated viral attachment 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

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

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



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

375

## 376 **Discussion**

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

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

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

399 NTCP, encoded by the gene *SLC10A1*, is a Na<sup>+</sup>/bile acid cotransporter in hepatocytes.  
400 Recently, NTCP has been identified as the functional entry receptor for HBV and hepatitis  
401 delta virus infection (5). NTCP expression on the cell surface confers HBV susceptibility  
402 among the target cells; thus, a cell line stably expressing human NTCP including HepG2-  
403 hNTCP-C4 cells has been used for the screening of antivirals that target viral entry.  
404 Various entry inhibitors such as attachment inhibitors targeting the preS1 region of  
405 HBsAg, NTCP substrates, direct inhibitors of NTCP, and inhibitors that regulate NTCP  
406 expression have been reported. For instance, a multimeric flavanol, proanthocyanidin,  
407 and its analog, oolonghomobisflavan C, showed anti-HBV activity by directly targeting  
408 the preS1 region of the HBV large surface protein (15). The synthetic myristoylated  
409 PreS1<sub>2-48</sub> lipopeptide has been found to inhibit HBV and HDV infection by blocking viral  
410 binding to the NTCP receptor (41, 42). Bulevirtide, a synthetic PreS1<sub>2-48</sub>-derived  
411 lipopeptide, has been approved as a first-in-class HDV entry inhibitor (43). The  
412 immunosuppressive drug CyA and its derivatives, as well as clinically approved drugs  
413 such as ezetimibe, irbesartan, rosiglitazone, zafirlukast, TRIAC, sulfasalazine, Chicago  
414 Sky Blue 6B, and Evans Blue, have been identified as entry inhibitors that interact with

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

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

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

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

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

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

466

#### 467 **Acknowledgments**

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473

#### 474 **Disclosure**

475 The authors declare no conflicts of interest.

476

#### 477 **Data availability statement**

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

480

#### 481 **List of abbreviations:**

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

485 DNA, HBsAg: hepatitis B surface antigen, HBeAg: hepatitis B e antigen, HBc: HB core,  
486 HSV-1: herpes simplex virus type 1, CyA: cyclosporin A, DMSO: dimethyl sulfoxide,  
487 PXB-cells: primary human hepatocytes, p.i.: post-infection, qPCR: quantitative PCR,  
488 EC<sub>50</sub>: half-maximum effective concentration, CC<sub>50</sub>: 50% cytotoxic concentration, SI:  
489 selectivity index ,TCA: taurocholic acid, ASDs: amorphous solid dispersions

490

#### 491 **References**

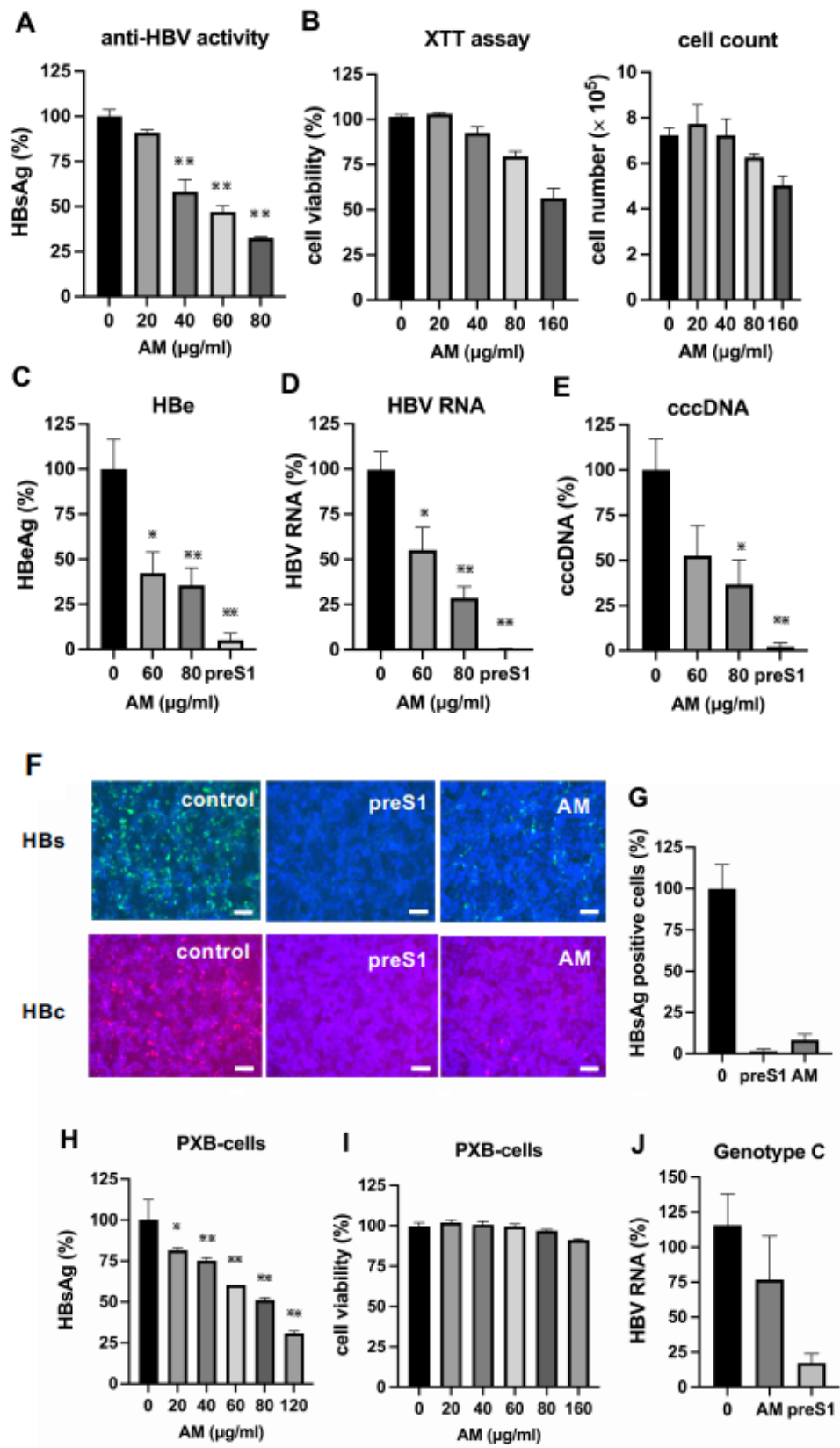
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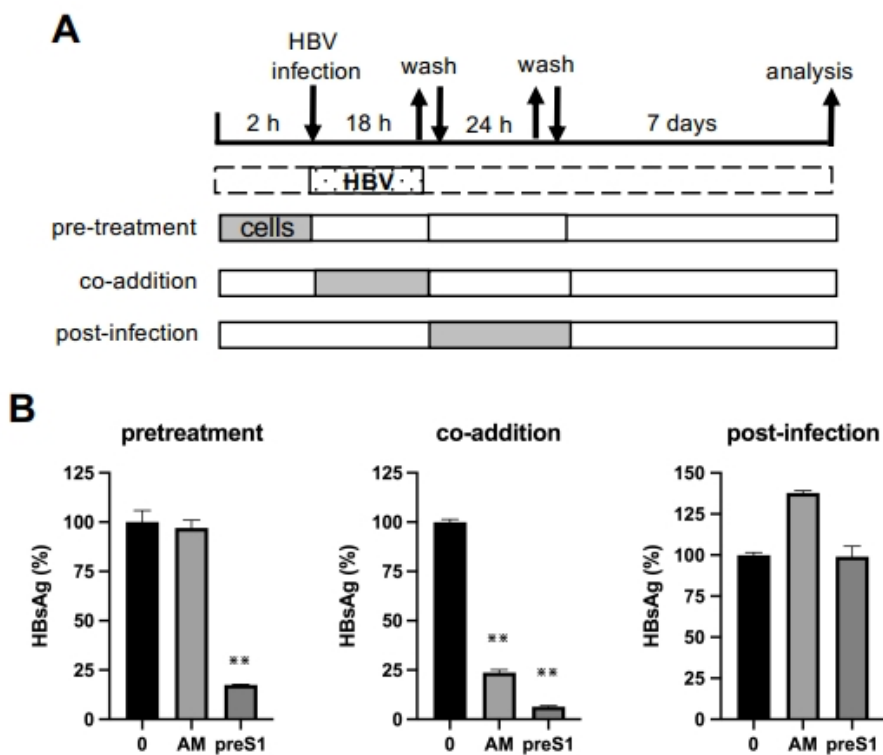
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- 623



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

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

649 HBV RNA levels in the cells were quantified by qPCR. Data show mean  $\pm$  SD from two  
 650 independent experiments. All data except (C) and (F) are expressed as mean  $\pm$  SEM from  
 651 two or three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ . AM: amentoflavone.  
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 656 **Fig. 2. Amentoflavone inhibits HBV entry step.** (A) A schematic of time-of-addition  
 657 experiments. Pre-treatment: cells were pretreated with compounds for 2 h before HBV  
 658 infection. After removing compounds, pretreated cells were challenged with HBV  
 659 infection for 18 h in the absence of compounds. Co-addition: compounds were added to  
 660 cell cultures for 18 h during virus inoculation. Post-infection: cells were infected with  
 661 HBV for 18 h in the absence of compounds. After washing out unbound virus, the

662 infected cells were treated with compounds for 24 h. HBsAg levels in culture  
663 supernatants at day 8 p.i. were determined by ELISA. The gray and white square show  
664 the periods of treatment and nontreatment of compounds. (B) HepG2-hNTCP-C4 cells  
665 were pretreated with amentoflavone (80 µg/ml), preS1 peptide (200 nM), or 0.2%  
666 DMSO as negative control for 2 h at 37°C. After washing out compounds, pretreated  
667 cells were inoculated with HBV without compounds (pretreatment). HBV was  
668 inoculated onto HepG2-hNTCP-C4 cells in the presence of amentoflavone (80 µg/ml),  
669 preS1 peptide (200 nM), or 0.2% DMSO for 18 h at 37°C (co-addition). Cells were  
670 inoculated with HBV without compounds for 18 h at 37°C. After washing out free  
671 virus, cells were treated with amentoflavone (80 µg/ml), preS1 peptide (200 nM), or  
672 0.2% DMSO for 24 h at 37°C (post-infection). Levels of HBsAg in culture supernatants  
673 were determined by ELISA. AM: amentoflavone. Values show mean ± SD from two  
674 independent experiments.

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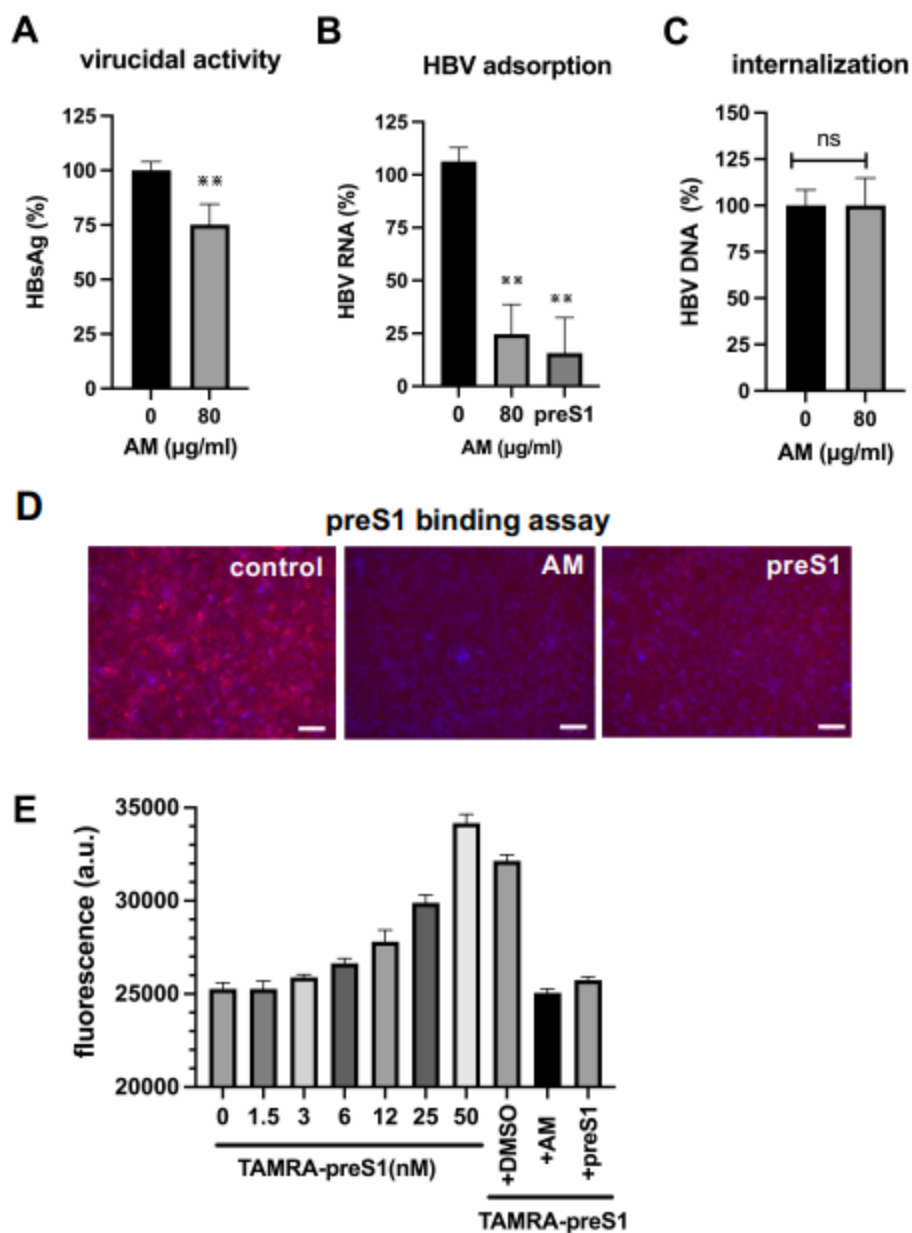
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**Fig. 3. Amentoflavone blocks HBV adsorption via preS1 binding.** (A) Virucidal activity of amentoflavone. A mixture of HBV and amentoflavone was preincubated at 37°C for 2 h. After removal of free amentoflavone with centrifugal filter device, residual 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

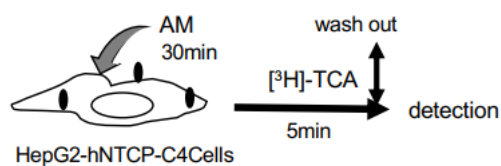
705 cells for 90 min at 4°C to allow HBV adsorption. After extensive washing with cold PBS,  
706 cells were incubated without compounds for 10 days at 37°C. HBV RNA was extracted  
707 and quantified by qPCR analysis. (C) Effect of amentoflavone on virus internalization.  
708 HepG2-hNTCP-C4 cells were exposed with HBV on ice for 3 h in the absence of  
709 compounds, and cultures were then transferred to 37°C in the presence of amentoflavone  
710 for 20 h to allow viral internalization. After trypsinization and extensive washing of the  
711 cells, intracellular HBV DNA were quantified by qPCR. (A-C) Data are expressed as  
712 mean ± SEM from two independent experiments. (D) HepG2-hNTCP-C4 cells were  
713 incubated with 40 nM TAMRA-labeled preS1 peptide in the presence of 80 µg/ml  
714 amentoflavone, 200 nM non-label preS1 peptide, or 0.2% DMSO as a control at 37°C for  
715 45 min. The binding of TAMRA-labeled preS1 to the cell surface was observed by  
716 fluorescence microscopy. Red and blue signals indicate preS1 probe and the nucleus,  
717 respectively. Scale bar, 100 µm. (E) HepG2-hNTCP-C4 cells were incubated with  
718 different concentrations of TAMRA-labeled preS1 peptide (TAMRA-preS1) at 37°C for  
719 45 min. In a competitive binding experiment, cells were exposed with 40 nM TAMRA-  
720 preS1 in the presence or absence of compounds (0.2% DMSO, 200 nM non-label preS1  
721 peptide, or 80 µg/ml amentoflavone). The red fluorescence intensity was measured using  
722 multimode microplate reader. Data are expressed as mean ± SD from triplicate wells. \*\**P*  
723 < 0.01, ns: not significant, AM: amentoflavone, a.u.: arbitrary unit

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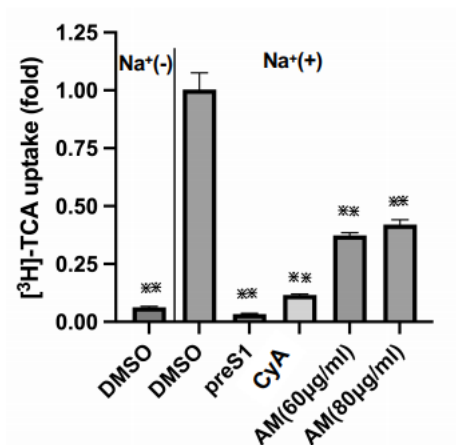
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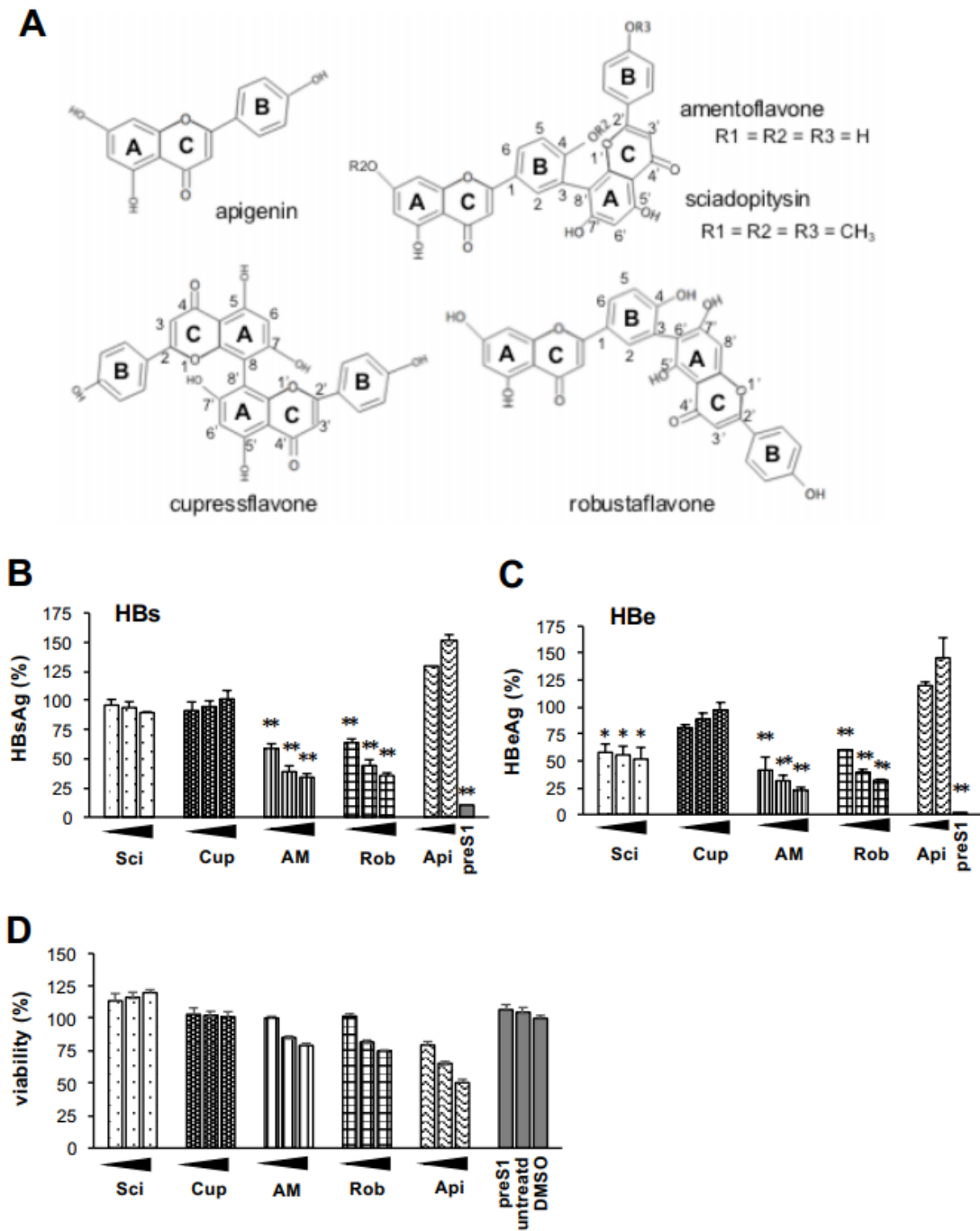
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**Fig. 4. Amentoflavone inhibits NTCP transporter activity.** Cells were treated with compounds (amentoflavone (AM), 200 nM preS1 peptide, 10 µM cyclosporin A (CyA) and, 0.2% DMSO) for 37°C for 30 min followed by the addition of TCA for 5 min. Fold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





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

745 through carbon-carbon. Amentoflavone and sciadopitysin have the same core structure of  
746 C3-8 linkage. Robustaflavone has a structure with C3-6 linkage. Cupressflavone consists  
747 of two apigenin units linking at each A ring. (B) HBV was inoculated to HepG2-hNTCP-  
748 C4 cells in the presence of various concentrations of compounds (sciadopitysin,  
749 cupressflavone, amentoflavone, robustaflavone: 40, 60, and 80 µg/ml, apigenin: 20 and  
750 40 µg/ml, 200 nM preS1 peptide, 0.2% DMSO) for 18 h. Levels of HBsAg (B) and  
751 HBeAg (C) secreted in the culture supernatants on day 7 p.i. were determined by ELISA.  
752 Relative values as compared to the 0.2% DMSO control were calculated. (D) HepG2-  
753 hNTCP-C4 cells were treated with amentoflavone derivatives (sciadopitysin,  
754 cupressflavone, amentoflavone, robustaflavone: 40, 60, and 80 µg/ml, apigenin: 20, 40  
755 and 80 µg/ml), 200 nM preS1 peptide or 0.2% DMSO for 18 h. Cell viability relative to  
756 the 0.2% DMSO control was shown. Data are expressed as mean ± SEM from two  
757 independent experiments. Sci: sciadopitysin, Cup: cupressflavone, AM: amentoflavone,  
758 Rob: robustaflavone, Api: apigenin, \**P* < 0.05, \*\**P* < 0.01.

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