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Novel anilinocoumarin derivatives as agents against hepatitis C virus by the induction of IFN-mediated antiviral responses†

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The hepatitis C virus (HCV) is the main cause of progressive liver disease, leading to the development of liver cirrhosis and hepatocellular carcinoma (HCC). Novel anilinocoumarins were synthesized, and their efficacy against HCV replication was evaluated. We demonstrated that 3-(3′,4′,5′-trimethoxyanilin-1′-yl)-methylaminocoumarin (**6**) exhibited strong anti-HCV activity at protein and RNA levels at non-toxic concentrations, with an EC_{50} value of 12 ± 0.3 μM and a selective index (SI) value of 10. Combined treatment of compound **6** and interferon-α (IFN) or telaprevir induced a significant decrease in HCV RNA levels, respectively. We also found that the anti-HCV replication effect of compound **6** was due to the induction of IFN-mediated antiviral responses. This is the first report demonstrating that coumarins inhibit viral replication through an IFN-mediated anti-viral response. Collectively, compound **6** possessed potent activities against HCV replication and could be a new lead compound with higher selectivity and less toxicity.

Introduction

The hepatitis C virus (HCV) is a major cause of liver disease worldwide, including hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma (HCC).[1] It is an enveloped virus belonging to the hepacivirus genus of the *Flaviviridae* family.[2] The positive single-strand RNA genome flanked by unique untranslated regions (UTRs) at 5′ and 3′ ends comprises an open reading frame (ORF).[3] The viral structural and nonstructural proteins generated by post-translational modifications from the viral genome are processed through host and viral proteases.[4] At present, a combination of pegylated interferon-α (PEG-IFN-α) and ribavirin is the standard clinical therapy for HCV-infected patients. However, it is associated with severe side effects and a limited sustained virological response (SVR) of 50–70% in patients infected with HCV genotype 1.[5,6] An effective vaccine against HCV infection has not yet been developed and there is an increasing need for investigating novel agents specially targeted toward anti-HCV replication. Moreover, drug resistance is a serious problem in the development of anti-HCV drugs because of the high rate of viral mutation and replication.[7,8] Because of the side effects and the low cure rate of the current therapy, safer and more effective anti-HCV agents need to be discovered or developed. In the present study, we analyzed the anti-HCV activities of synthesized anilinocoumarin derivatives using the HCV replicon system.

Most compounds and their pharmacophores have been designed from natural products during drug discovery.[9] In particular, the broad biological activities of coumarin derivatives, such as anti-tumor and anti-platelet activities and as oral antithrombotic agents, have been studied extensively.[10,11] Recently, researchers have focused on the anti-viral activities of coumarin derivatives. For example, synthetic analogs from natural products, such as derivatives of novobiocin, clorobiocin,[12] and khellactone, were shown to have potent activities against human immunodeficiency virus (HIV).[13] Similarly, heterobicycle-coumarin conjugated compound **I** with the $-SCH_2-$ linker was synthesized and found to possess significant antiviral activities.[14] Studies of heterobicycle-coumarins have continued, and reports of new anti-HCV agents based on coumarin–purine ribofuranoside conjugate **II** have been published

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(Fig. 1).[15] Studies of anti-HCV inhibitors containing a heterocyclic moiety showed that they exhibited good anti-viral activity. However, reports on the inhibitory effects of anilinocoumarin derivatives on HCV RNA replication are lacking. This dearth of information prompted us to design and synthesize novel lead structures with an aniline moiety conjugated with a natural coumarin pharmacophore to obtain anilinocoumarin derivatives with anti-HCV activity.

Results and discussion

Synthesis of anilinocoumarins

Various syntheses of coumarin derivatives have been developed.[16–20] After careful consideration of the previous work on the syntheses of coumarin derivatives, we used the efficient and chemoselective methodology for the preparation of 3-substituted coumarins.[21,22] The reaction mixture was stirred at room temperature for 14 days with a mixture of 2-hydroxybenzaldehydes (**1**), *t*-butyl acrylate (**2**), and DABCO. The reaction mixture yielded the corresponding Baylis–Hillman adduct **3** (40% yields). The reaction of the adduct with hydrochloric acid and acetic acid at 110 °C then afforded the 3-(chloromethyl)coumarin **4** in an excellent yield (95%). Compound **4** was then reacted with various anilines to generate compounds **5–15** by simple amination. The preparation of anilinocoumarin derivatives is described in Scheme 1. The chemical structure was identified by ^{1}H and ^{13}C nuclear magnetic resonance and confirmed by high-resolution mass spectrometry and elemental analyses.

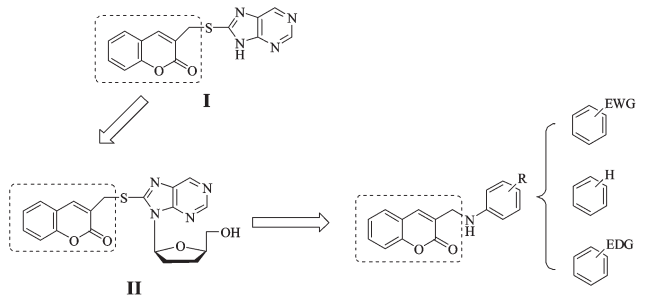
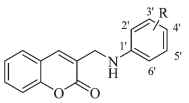


Fig. 1 Structures of newly reported anti-HCV compounds and the design concept of anlinocoumarin derivatives. EWG, electron-withdrawing groups. EDG, electron-donating groups.

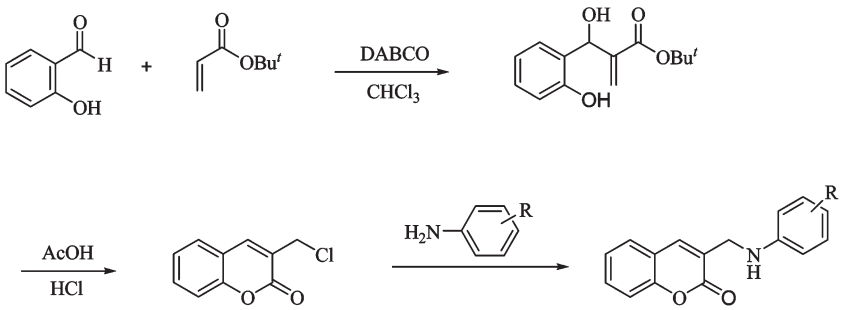
Anilinocoumarins suppressed HCV RNA replication

The new anilinocoumarin derivatives, compounds **5–15**, were evaluated for anti-HCV replication activity against Ava5 cells at 0.5–200 μM. Total cellular RNA of compound-treated cells was subjected to quantitative RT-PCR (qRT-PCR) with specific primers corresponding to HCV RNA after 3 days. All the reported compounds were evaluated for cytotoxicity by the MTS assay. The results for anilinocoumarin derivatives are summarized in Table 1. In the present study of the variety of functional groups of anilinocoumarins, compounds **6**, **7**, **9**, **10**, and **11** exhibited anti-HCV activity at <50 μM. Compounds **5**, **8**, **9**, **10**, **11**, **13**, **14**, and **15** were found to be less cytotoxic (>150 μM). The concentration of the compound at which cell viability was reduced by 50% (CC_{50}), and the concentration of the compound at which HCV RNA replication was reduced by 50% (EC_{50}) were determined. Values of the selective index (SI; CC_{50}/EC_{50}) for compounds **6**, **7**, **9**, and **10** were greater than those for the other compounds. In view of the above-mentioned findings, 3-(3′,4′,5′-trimethoxyanilin-1′-yl)methylaminocoumarin (**6**) showed the highest SI value of 10 in this series of

Table 1 Anti-HCV activity of anilinocoumarin derivatives

Compound		HCV		
		CC_{50}[a] (μM)	EC_{50}[b] (μM)	SI[c]
5	R = H	>200	90	<3
6	R = 3′,4′,5′-triOMe	120	12	10
7	R = 4′-OMe	110	20	5.5
8	R = 2′,4′-diMe	>200	>100	—
9	R = 2′,6′-diMe	152	24	6.5
10	R = 3′-OH	200	50	4
11	R = 4′-Br, 2′-Cl	152	45	3.3
12	R = 4′-$COOC_2H_5$	128	100	<3
13	R = 3′-NO_2	>200	>100	—
14	R = 3′,5′-diNO_2	173	100	<3
15	R = 4′-Cl, 2′-Me	188	85	<3

[a] Concentration of the compound at which cell viability was reduced by 50% when compared to the 0.1% DMSO-treated cells, which served as a mock control. [b] Inhibitory concentration that reduced viral replication by 50%. [c] The selective index was calculated as the ratio of CC_{50} *versus* EC_{50}.



Scheme 1 The synthesis of anilinocoumarin derivatives. DABCO, 1,4-diazabicyclo[2.2.2]octane.

anilinocoumarin derivatives examined for their anti-HCV activity.

Structure–activity relationship (SAR) of anilinocoumarins

Based on EC_{50} and CC_{50} activities, it was intriguing to observe different substituents (OMe, Me, OH, Cl, Br, NO_2, and $COOC_2H_5$) placed at various positions on the phenyl ring. These substituents and their positions affect anti-HCV activity, especially the SI value. Overall, substitution of electron-donating groups such as OMe, Me, and OH on the phenyl ring resulted in a range of SI values of 4–10. Moreover, addition of electron density to the phenyl ring by introducing a strong electron-withdrawing group, such as Cl or NO_2, produces a lower SI value (<3.3). We concluded the brief SAR study by scrutinizing the EC_{50}, CC_{50}, and SI values of anilinocoumarin derivatives. Substituents bearing a strong electron-donating motif on the phenyl ring were crucial for anti-HCV activity. In addition, more substituents on the phenyl ring are important because they resulted in a decrease in potency decreased in the order of compound **10** > compound **7** > compound **9** > compound **6** (Table 1).

Compound 6 reduced HCV protein synthesis and RNA levels

To study the reduction of HCV protein synthesis and RNA levels by compound **6**, quantification of HCV RNA and western blotting were performed. After incubation of compound **6** in Ava5 cells for 4 days, the treated cells were lysed for western blot analyses with anti-HCV NS5B and anti-GAPDH antibodies, which represented the viral protein synthesis and loading control, respectively. Reduced viral protein expression was correlated with increasing concentrations of compound **6** (Fig. 2A). The decrease in HCV RNA replication by compound **6** was dose dependent in both replicon and infectious systems (Fig. 2B and C). The compound exhibited an EC_{50} value of 12 ± 0.3 μM and was not cytotoxic. We concluded that compound **6** was a potent lead compound against HCV. In addition, the inhibition of HCV replication by compound **6** was consistent with that of the reference compounds, heterobicycle-coumarins, which prove the essential role of the pharmacophoric element of the coumarin ring.

Compound 6 enhanced expression of IFN response genes

Anti-HCV drugs can be developed focusing on three specific targets: the internal ribosome entry site (IRES), NS3/4A protease activity, and NS5B polymerase.[23] To evaluate the anti-HCV action of compound **6**, we tested the inhibitory effect of compound **6** on these three specific steps of the HCV replication cycle using several reporter-based assays. No significant inhibition of IRES-mediated translation, NS3/4A protease, or NS5B polymerase activities was observed by compound **6** treatment (data not shown). We further tested whether compound **6** regulated cellular factors to interfere with HCV replication. The IFN-mediated anti-viral pathway is an effective cellular defense against HCV replication.[24] To investigate whether

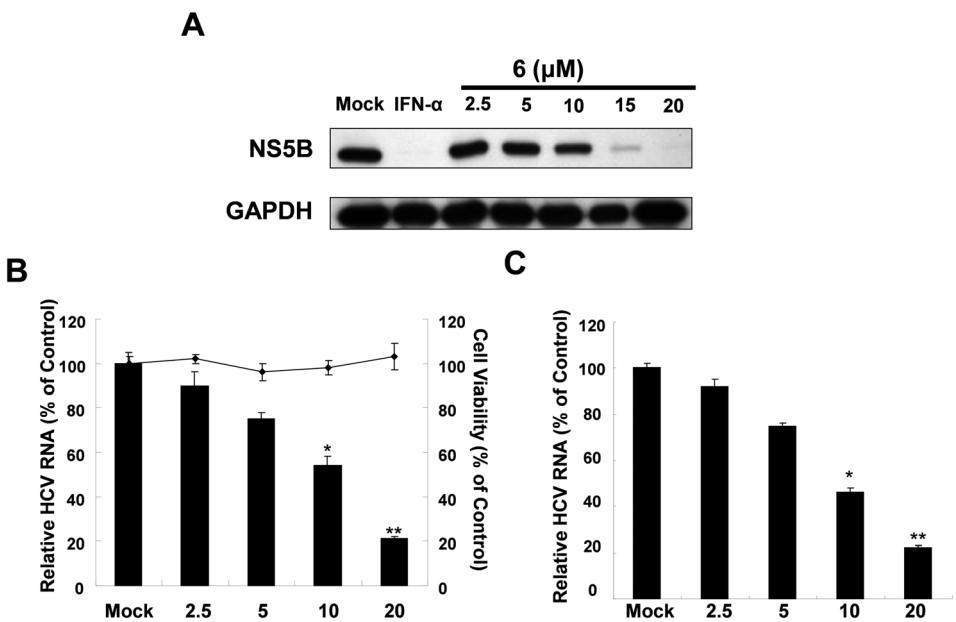


Fig. 2 Inhibition of HCV replication and protein expression in Ava5 cells by compound **6**. Ava5 cells were incubated with compound **6** at the indicated concentration (from 2.5 to 150 μM). (A) After four days of incubation, cell lysates were extracted and analyzed by western blotting with anti-NS5B and anti-GAPDH antibodies (a loading control). (B) After three days of incubation, total RNA was extracted from the Ava5 cells to quantify HCV RNA levels by RT-qPCR. Cell viability was performed simultaneously by the MTS assay. (C) After Huh7 cells were infected with the concentrated virus for 8 hours, the infected cells were treated with compound **6** at the indicated concentrations for 3 days. Total RNA was extracted from the cells to quantify HCV RNA levels by RT-qPCR. The non-linear regression graphs and an effective concentration of 50% inhibition (EC_{50}) were calculated by the GraphPad Prism5.0 program (Version 5.0, GraphPad Software, Inc. La Jolla, CA). Mock indicates the treatment with 0.1% DMSO. Results are expressed as means ± SD (error bar) for triplicate experiments. $^*P < 0.05$; $^{**}P < 0.01$.

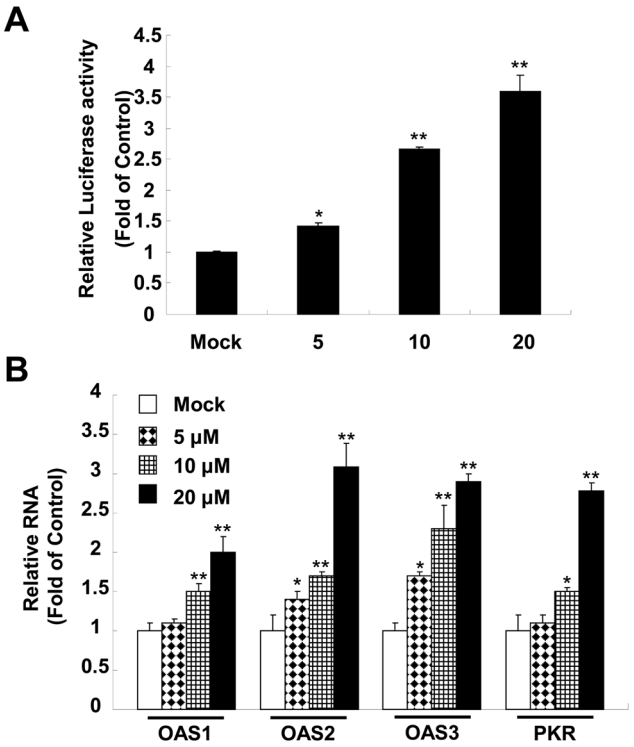


Fig. 3 Induction of IFN response genes expression in Ava5 cells with compound **6**. Ava5 cells were incubated with compound **6** at the indicated concentrations (5, 10 and 20 μM). (A) Ava5 cells were transiently transfected with 1.0 μg of the reporter plasmid containing an interferon-stimulated response element, pISRE-Luc. Subsequently, the transfected cells were treated with the indicated concentrations of compound **6** for 3 days, and total cell lysates were analyzed for luciferase activity. (B) Ava5 cells were treated with the indicated concentrations of compound **6** for 3 days, and total RNA extracts were analyzed by RT-qPCR to quantify the interferon response genes (OAS1, OAS2, OAS3, and PKR) RNA expression normalized by cellular GAPDH mRNA. Mock indicates the treatment with 0.1% DMSO. Results are expressed as means ± SD (error bar) for triplicate experiments. $*P < 0.05$; $**P < 0.01$.

compound **6** induced activation of the IFN pathway, we performed a reporter assay using transient Ava5 cells with a firefly luciferase reporter gene containing the interferon-stimulated response element (ISRE) to verify the level of induction of the IFN pathway by compound **6**. The Ava5 cells transfected with pISRE-Luc and treated with compound **6** at various concentrations were used in the luciferase assay.

Compound **6** significantly induced IFN responses by 3.6-fold at 20 μM compared with the mock control (Fig. 3A). Next, we identified whether the IFN response genes (*e.g.*, 2′-5′-oligoadenylate synthetase (OAS) family and protein kinase R (PKR)) were also induced by compound **6**. The induction level of these genes was analyzed by qRT-PCR in the presence of different concentrations of compound **6**. Compound **6** induced an approximate 3-fold induction of expression of these genes at 20 μM compared with the mock control (Fig. 3B). These results were comparable with the reporter-based assay and indicated that the anti-HCV mechanism of compound **6** may contribute to enhancement of the IFN-mediated anti-viral pathway.

Synergistic inhibitory effects of compound 6 when used in combination with IFN, telaprevir, BMS790052 or PSI7977

A combination of PEG-IFN-α with ribavirin is the current standard therapy against HCV. However, this treatment is associated with a low cure rate and severe side effects.[5] In addition, direct-acting antiviral (DAA) drugs of HCV attenuated the sustained viral response rates by approximately 79% in patients with HCV genotype 1.[25] Based on these findings, we foresaw that compound **6** could be a potent anti-HCV agent in clinical use if it demonstrates enhanced anti-HCV replication activity when combined with IFN-α or telaprevir, a currently used FDA approved protease inhibitor. Besides, we investigated whether the compound **6** enhanced the anti-HCV replication combined the other DAA drugs, such as phase 2 oral anti-viral regiments of HCV polymerase inhibitor, PSI7977 and NS5A replication complex inhibitor, BMS790052.[26,27] To evaluate the effect of combination treatment of compound **6** with IFN-α, telaprevir, BMS790052, or PSI7977 on anti-HCV replication activity, we treated Ava5 cells with various concentrations of compound **6** and IFN-α or telaprevir at fixed ratios as described in the materials and methods section. Combination treatment of compound **6** with IFN-α, telaprevir, BMS790052, or PSI7977 (Fig. 4) increased the reduction of HCV RNA levels (lanes 6–9) compared with the DMSO vehicle control (lane 1) and treatment with each agent alone (lanes 2–5). To determine the effects of combination treatment, we used the isobologram method and the CalcuSyn™ software. CI values of compound **6** combined with IFN, telaprevir, BMS790052 or PSI7977 were 0.8, 0.5, 0.4 and 0.6, respectively. These results strongly supported the notion that compound **6** could be a good adjuvant to treat HCV infection.

Further study to prevent HCC

A strategy for preventing chronic HCC in HCV infection was described by Okamoto *et al.*[28] Previous studies have shown that coumarin compounds, *i.e.*, osthole, imperatorin, and furanocoumarins, which are isolated from plants or chemically synthesized, decrease the levels of plasma alanine aminotransferase (ALT) that correlates with HCC etiology. Oral administration of one of these coumarins, osthole, has been observed to cause decrease in plasma ALT levels in mice with concanavalin A-induced hepatitis and anti-Fas antibody-induced hepatitis. Interestingly, compound **6** is similar to osthole and may prevent HCC by a different mechanism. Osthole was observed to normalize plasma ALT levels, and compound **6** is the first discovered coumarin-like compound known to induce the IFN-mediated anti-viral pathway in HCV replication. The results of the present study will form the basis of further study for prevention of the development of HCC in the future.

Conclusions

In the present study of new potent anti-HCV inhibitors, we synthesized anilinocoumarin derivatives and evaluated their anti-

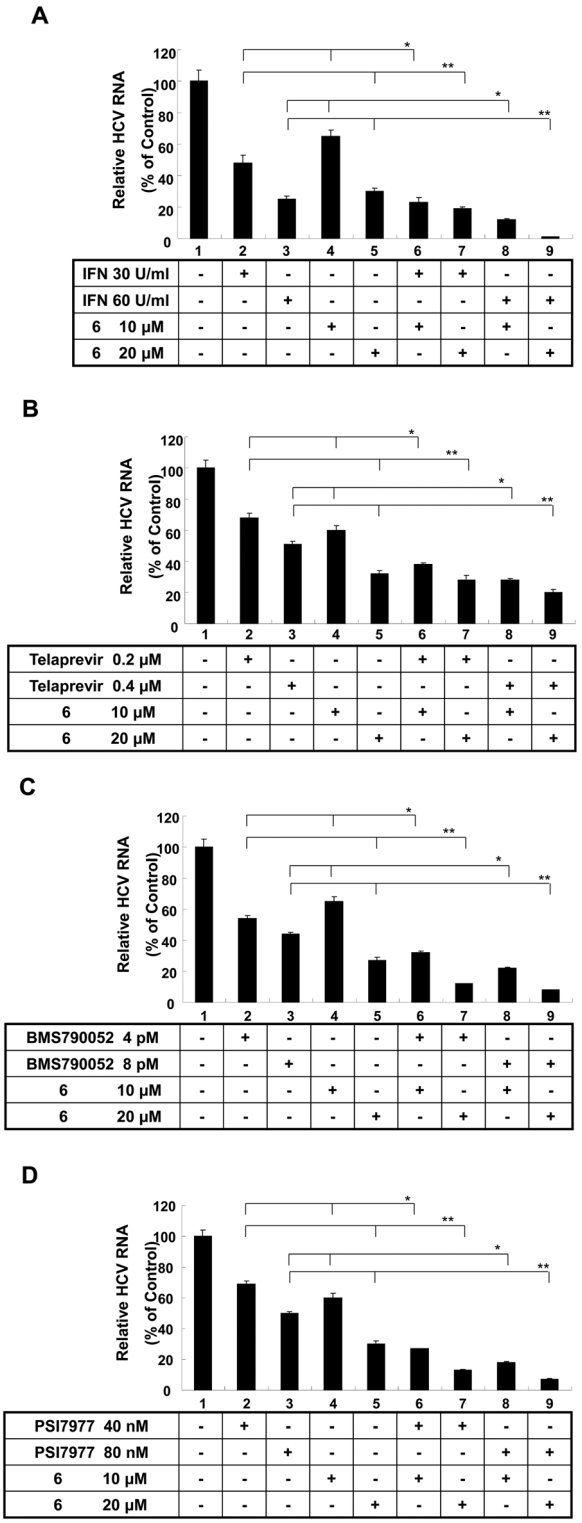


Fig. 4 Enhancement inhibition of the compound **6** combined treatment with IFN, telaprevir, BMS790052, or PSI7977 in HCV replicon cells. Ava5 cells were treated with the indicated concentration of compound **6** and IFN, telaprevir, BMS790052, or PSI7977 for 3 days. The total RNA was extracted and quantified by RT-qPCR analysis. The relative percentage of HCV RNA levels was compared with the cells incubated with 0.1% DMSO (mock control). The errors reflect the SD from triplicate experiments. Results are expressed as means ± SD (error bar) for triplicate experiments. $*P < 0.05$; $**P < 0.01$.

HCV activities. Our results indicated that replacement of a strong electron-donating substituent such as OMe on the 2-anilinocoumarin pharmacophore is a promising characteristic for HCV inhibition. The most potent derivative, 3-(3′,4′,5′-trimethoxyanilin-1′-yl)methylaminocoumarin (**6**), exhibited anti-HCV activity with an EC_{50} value of 12 μM and an SI value of 10. Compound **6** also showed synergistic effects on HCV replication when combined with IFN or telaprevir. This is the first report detailing the mechanism of action of anilinocoumarins, in anti-HCV replication through IFN responses. Compound **6** targeted host factors of the IFN pathway. Hence, it is a promising strategy for circumventing drug resistance and reducing differential responses according to HCV virus mutations and different genotypes. We found a novel molecular mechanism regulated by anilinocoumarin derivatives in liver cells. We suggest that compound **6** could be a lead compound in anti-HCV therapy.

Experimental section

General

All melting points were uncorrected. IR absorption spectra were recorded on a Perkin-Elmer System 2000 FT-IR spectrophotometer. Proton and carbon-13 NMR spectra were measured with a Unity Plus-400 or a Mercury Plus-400 spectrometer. Carbon multiplicities were obtained from DEPT experiments. Chemical shifts (δ) and coupling constants (Hz) were measured with respect to TMS or chloroform-d_1. MS and high-resolution mass spectra (HRMS) were taken on a Thermo-Finnigan TRACE *GC*, *Waters* micromass*ZQ* or JEOL JMS-700 instrument, with a direct inlet system. Elemental analyses were carried out on the elementar vario EL *III* elemental analyzer.

General procedure for the synthesis of 3-(anilin-1′-yl)-methylaminocoumarins 5–15

2-Hydroxybenzaldehyde (**1**) was reacted with *t*-butyl acrylate (**2**) in the presence of DABCO to afford the corresponding Baylis–Hillman adduct **3** in 40% yield. Reaction of the adduct with hydrochloric acid in refluxing acetic acid then afforded the 3-(chloromethyl)coumarin **4** in excellent yield (95%). The compound **4** was then reacted with various anilines by amination for an appropriate time. After the completion of the reaction (TLC monitoring), the reaction mixture was neutralized with saturated $NaHCO_3$ until pH 7 and then partitioned between H_2O (50 mL) and CH_2Cl_2 (50 mL × 3). The organic layer was washed with brine, dried over $MgSO_4$, and evaporated *in vacuo*. The resulting residue was purified by column chromatography (hexane–ethyl acetate) and crystallized from EtOH to give **5–15**.

***tert*-Butyl-3-hydroxy-3-(2-hydroxyphenyl)-2 methylenepropanoate (3).** Yield: 100 mg, 40%. Mp: 108–110 °C (lit.,[22] 108–110 °C). ^1H NMR (400 MHz, $CDCl_3$) δ: 1.51 (s, 9H, CH_3 × 3), 4.38 (d, J = 4.0 Hz, 1H, OH), 5.49 (s, 1H, vinyl H), 5.69 (d, J = 4.0 Hz, 1H, CH), 6.84 (ddd, J = 1.2, 7.2, 7.6 Hz, 1H, ArH), 6.91 (dd, J = 1.2, 8.0 Hz, 1H, ArH), 6.97 (dd, J = 1.6,

7.2 Hz, 1H, ArH), 7.21 (ddd, J = 1.6, 7.6, 8.0 Hz, 1H, ArH), 8.15 (s, 1H, OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 28.0 (CH_3 × 3), 73.7 (CH), 82.6 (C), 117.5 (CH), 119.8 (CH), 124.1 (C), 127.0 (CH_2), 127.8 (CH), 129.5 (CH), 140.8 (C), 156.0 (C), 166.8 (C). EIMS: m/z 250 (M^+), 194, 176, 148, 131, 121, 105, 91, 77, 65, 55, 50, 41.

3-(Chloromethyl)coumarin (4). Yield: 184 mg, 95%. Mp: 113–115 °C (lit.,[22] 108–110 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.56 (d, J = 0.8 Hz, 2H, CH_2), 7.32 (ddd, J = 0.8, 6.4, 8.8 Hz, 1H, ArH), 7.36 (dd, J = 0.8, 8.8 Hz, 1H, ArH), 7.54 (ddd, J = 1.2, 6.4, 7.6 Hz, 1H, ArH), 7.57 (dd, J = 1.2, 7.6 Hz, 1H, ArH), 7.90 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 41.0 (CH_2), 116.6 (CH), 118.8 (C), 124.7 (CH), 125.0 (C), 128.0 (CH), 132.0 (CH), 141.1 (CH), 153.5 (C), 160.1 (C). EIMS: m/z 196 (M^+ + 2), 194 (M^+), 159, 131, 115, 103, 89, 77, 63, 51.

3-(Anilin-1′-yl)methylaminocoumarin (5). Yield: 170 mg, 68%. Mp: 173–175 °C. IR (KBr) ν: 3406, 2671, 2359, 1906, 1700, 1510 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.31 (s, 2H, CH_2), 4.36 (bs, 1H, NH), 6.64 (dd, J = 1.2, 8.8 Hz, 2H, ArH), 6.74 (tt, J = 1.2, 7.6 Hz, 1H, ArH), 7.18 (dd, J = 7.6, 8.8 Hz, 2H, ArH), 7.24 (ddd, J = 1.2, 7.6, 7.6 Hz, 1H, ArH), 7.33 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.49 (ddd, J = 1.2, 7.2, 8.4 Hz, 1H, ArH), 7.68 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 43.7 (CH_2), 113.0 (CH), 116.5 (CH), 118.1 (CH), 119.2 (C), 124.4 (CH), 126.2 (C), 127.7 (CH), 129.4 (CH), 131.1 (CH), 138.6 (CH), 147.2 (C), 153.1 (C), 161.3 (C). ESIMS: m/z 252 (M + H)$^+$, 191, 159, 131, 114, 102, 96. EI-HRMS calcd for $C_{16}H_{13}NO_2$: 251.0946, found: 251.0944. Anal. Calcd for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.21; N, 5.57. Found: C, 76.26; H, 5.27; N, 5.38.

3-(3′,4′,5′-Trimethoxyanilin-1′-yl)methylaminocoumarin (6). Yield: 279 mg, 82%. Mp: 134–136 °C. IR (KBr) ν: 3392, 3054, 2936, 1713, 1605, 1511 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.76 (s, 3H, OCH_3), 3.80 (s, 6H, OCH_3 × 2), 4.20 (bs, 1H, NH), 4.29 (s, 2H, CH_2), 5.89 (s, 2H, ArH), 7.28 (ddd, J = 1.2, 7.2, 7.6 Hz, 1H, ArH), 7.35 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.46 (dd, J = 1.2, 7.6 Hz, 1H, ArH), 7.51 (ddd, J = 1.2, 7.2, 8.4 Hz, 1H, ArH), 7.71 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 44.0 (CH_2), 56.0 (OCH_3 × 2), 61.6 (OCH_3), 90.8 (CH × 2), 116.5 (CH), 119.1 (C), 124.5 (CH), 126.4 (C), 127.7 (CH), 130.6 (C), 131.2 (CH), 138.8 (CH), 144.0 (C), 153.1 (C), 154.1 (C × 2), 161.3 (C). ESIMS: m/z 342 (M + H)$^+$, 310, 279, 183, 168, 159, 140, 115, 109, 97. EI-HRMS calcd for $C_{19}H_{19}NO_5$: 341.1263, found: 341.1265. Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.98; H, 5.37; N, 4.05.

3-(4′-Methoxyanilin-1′-yl)methylaminocoumarin (7). Yield: 219 mg, 78%. Mp: 129–131 °C. IR (KBr) ν: 3400, 2925, 2060, 1710, 1610, 1513 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.73 (s, 3H, OCH_3), 4.12 (bs, 1H, NH), 4.26 (s, 2H, CH_2), 6.60 (d, J = 8.8 Hz, 2H, ArH), 6.77 (d, J = 8.8 Hz, 2H, ArH), 7.25 (ddd, J = 1.2, 7.2, 7.6 Hz, 1H, ArH), 7.33 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.43 (dd, J = 1.6, 7.6 Hz, 1H, ArH), 7.49 (ddd, J = 1.6, 7.2, 8.4 Hz, 1H, ArH), 7.68 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 44.6 (CH_2), 55.7 (OCH_3), 114.4 (CH), 115.0 (CH), 116.5 (CH), 119.2 (C), 124.4 (CH), 126.6 (C), 127.7 (CH), 131.0 (CH), 138.7 (CH), 141.3 (C), 152.5 (C), 135.1 (C), 161.3 (C). ESIMS: m/z 282 (M + H)$^+$, 266, 226, 190, 158, 136, 134, 130, 122, 115, 107, 79. EI-HRMS calcd for $C_{17}H_{15}NO_3$: 281.1052, found: 281.1049. Anal. Calcd for $C_{17}H_{15}NO_3$: C, 72.58; H, 5.37; N, 4.98. Found: C, 72.71; H, 5.50; N, 4.68.

3-(2′,4′-Dimethylanilin-1′-yl)methylaminocoumarin (8). Yield: 206 mg, 74%. Mp: 146–148 °C. IR (KBr) ν: 3422, 2917, 2394, 1711, 1610, 1515 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.22 (s, 6H, CH_3 × 2), 4.13 (bs, 1H, NH), 4.35 (s, 2H, CH_2), 6.43 (d, J = 8.4 Hz, 1H, ArH), 6.88 (d, J = 8.4 Hz, 1H, ArH), 6.93 (s, 1H, ArH), 7.24 (dd, J = 7.2, 7.6 Hz, 1H, ArH), 7.34 (d, J = 8.4 Hz, 1H, ArH), 7.42 (dd, J = 1.6, 7.6 Hz, 1H, ArH), 7.48 (ddd, J = 1.6, 7.2, 8.4 Hz, 1H, ArH), 7.65 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.5 (CH_3), 20.3 (CH_3), 43.9 (CH_2), 110.2 (CH), 116.5 (CH), 119.2 (C), 122.5 (C), 124.4 (CH), 126.5 (C), 126.9 (C), 127.4 (CH), 127.7 (CH), 131.0 (CH), 131.2 (CH), 138.5 (CH), 142.8 (C), 153.1 (C), 161.3 (C). ESIMS: m/z 280 (M + H)$^+$, 262, 190, 158, 132, 121, 115, 106, 103, 117, 103. EI-HRMS calcd for $C_{18}H_{17}NO_2$: 279.1259, found: 279.1258. Anal. Calcd for $C_{18}H_{17}NO_2$: C, 77.40; H, 6.13; N, 5.01. Found: C, 77.08; H, 6.09; N, 4.85.

3-(2′,6′-Dimethylanilin-1′-yl)methylaminocoumarin (9). Yield: 195 mg, 70%. Mp: 106–108 °C. IR (KBr) ν: 3393, 3046, 2930, 1713, 1611 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.31 (s, 6H, CH_3 × 2), 3.60 (bs, 1H, NH), 4.07 (s, 2H, CH_2), 6.84 (t, J = 7.2 Hz, 1H, ArH), 7.00 (d, J = 7.2 Hz, 2H, ArH), 7.27 (ddd, J = 1.2, 7.2, 7.6 Hz, 1H, ArH), 7.34 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.44 (dd, J = 1.6, 7.6 Hz, 1H, ArH), 7.50 (ddd, J = 1.6, 7.2, 8.4 Hz, 1H, ArH), 7.68 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 18.4 (CH_3 × 2), 47.8 (CH_2), 116.6 (CH), 119.2 (C), 122.5 (CH), 124.4 (CH), 127.3 (C), 127.7 (CH), 128.9 (CH), 130.0 (2C), 131.1 (CH), 139.1 (CH), 144.9 (C), 153.3 (C), 161.6 (C). ESIMS: m/z 280 (M + H)$^+$, 190, 158, 132, 121, 115, 106, 104. EI-HRMS calcd for $C_{18}H_{17}NO_2$: 279.1259, found: 279.1258. Anal. Calcd for $C_{18}H_{17}NO_2$: C, 77.40; H, 6.13; N, 5.01. Found: C, 77.36; H, 6.24; N, 4.90.

3-(3′-Hydroxyanilin-1′-yl)methylaminocoumarin (10). Yield: 202 mg, 76%. Mp: 133–135 °C. IR (KBr) ν: 3404, 3061, 2925, 1697, 1604, 1502 cm^{-1}. ^{1}H NMR (400 MHz, acetone-d_6) δ: 4.21 (d, J = 1.6 Hz, 2H, CH_2), 5.50 (bs, 1H, NH), 6.13 (dd, J = 2.0, 8.0 Hz, 1H, ArH), 6.17 (t, J = 2.0 Hz, 1H, ArH), 6.19 (dd, J = 2.0, 8.4 Hz, 1H, ArH), 6.89–6.93 (m, 1H, ArH), 7.30 (ddd, J = 1.2, 7.2, 8.0 Hz, 1H, ArH), 7.34 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.56 (ddd, J = 1.2, 7.2, 8.4 Hz, 1H, ArH), 7.63 (dd, J = 1.2, 8.0 Hz, 1H, ArH), 7.82 (s, 1H, ArH), 7.95 (s, 1H, OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 44.4 (CH_2), 101.2 (CH), 106.2 (CH), 117.6 (CH), 121.0 (C), 125.9 (CH), 128.6 (C), 129.5 (CH), 131.0 (C), 131.3 (CH), 131.4 (CH), 132.5 (CH), 139.4 (CH), 151.2 (C), 154.7 (C), 161.9 (C). ESIMS: m/z 268 (M + H)$^+$, 249, 228, 190, 158, 121, 116, 84, 73. EI-HRMS calcd for $C_{16}H_{13}NO_3$: 267.0895, found: 267.0896. Anal. Calcd for $C_{16}H_{13}NO_3$: C, 71.90; H, 4.90; N, 5.24. Found: C, 71.77; H, 5.00; N, 5.15.

3-(4′-Bromo-2′-methylanilin-1′-yl)methylaminocoumarin (11). Yield: 360 mg, 57%. Mp: 137–139 °C. IR (KBr) ν: 3423, 2069, 1637, 1501 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.37 (d, J = 6.0 Hz, 2H, CH_2), 4.97 (t, J = 6.0 Hz, 1H, NH), 6.43 (d, J = 8.8 Hz, 1H, ArH), 7.17 (dd, J = 2.4, 8.8 Hz, 1H, ArH), 7.27 (ddd, J = 1.2, 7.2, 7.6 Hz, 1H, ArH), 7.35 (dd, J = 1.2, 8.4 Hz, 1H,

ArH), 7.42 (d, J = 2.4 Hz, 1H, ArH), 7.44 (dd, J = 1.6, 7.6 Hz, 1H, ArH), 7.51 (ddd, J = 1.6, 7.2, 8.4 Hz, 1H, ArH), 7.58 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 43.0 (CH_2), 108.6 (C), 112.5 (CH), 116.5 (CH), 118.9 (C), 120.1 (C), 124.6 (CH), 125.2 (C), 127.8 (CH), 130.7 (CH), 131.4 (CH), 131.6 (CH), 138.4 (CH), 142.2 (C), 153.1 (C), 161.0 (C). ESIMS: m/z 369 $(M + H + 4)^+$, 367 $(M + H + 2)^+$, 365 $(M + H)^+$, 335, 330, 284, 267, 245, 120, 195, 181, 151, 120, 96, 81. EI-HRMS calcd for $C_{16}H_{11}BrClNO_2$: 632.9662, found: 362.9663. Anal. Calcd for $C_{16}H_{11}BrClNO_2$: C, 52.70; H, 3.04; N, 3.84. Found: C, 52.67; H, 3.02; N, 3.86.

3-(4′-Ethoxycarbonylanilin-1′-yl)methylaminocoumarin (12). Yield: 203 mg, 63%. Mp: 179–181 °C. IR (KBr) ν: 3375, 2364, 1685, 1560, 1536 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.35 (t, J = 7.2 Hz, 3H, CH_3), 4.31 (q, J = 7.2 Hz, 2H, CH_2CH_3), 4.37 (d, J = 4.4 Hz, 2H, CH_2), 4.82 (bs, 1H, NH), 6.61 (d, J = 9.2 Hz, 2H, ArH), 7.26 (ddd, J = 1.2, 7.2, 7.6 Hz, 1H, ArH), 7.34 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.43 (dd, J = 1.6, 7.6 Hz, 1H, ArH), 7.50 (ddd, J = 1.6, 7.2, 8.4 Hz, 1H, ArH), 7.63 (s, 1H, ArH), 7.88 (d, J = 9.2 Hz, 2H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.4 (CH_3), 43.1 (CH_2), 60.3 (CH_2), 111.9 (CH), 116.6 (CH), 118.9 (C), 119.8 (C), 124.6 (CH), 125.4 (C), 127.8 (CH), 131.4 (CH), 131.6 (CH), 138.8 (CH), 150.9 (C), 153.1 (C), 161.2 (C), 166.2 (C). ESIMS: m/z 324 $(M + H)^+$, 310, 278, 222, 191, 159, 132, 115, 103, 97. EI-HRMS calcd for $C_{19}H_{17}NO_4$: 323.1158, found: 323.1160. Anal. Calcd for $C_{19}H_{17}NO_4$: C, 70.58; H, 5.30; N, 4.33. Found: C, 70.28; H, 5.45; N, 4.23.

3-(3′-Nitroanilin-1′-yl)methylaminocoumarin (13). Yield: 177 mg, 60%. Mp: 147–149 °C. IR (KBr) ν: 3380, 2136, 1662, 1420 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.23 (d, J = 4.8 Hz, 2H, CH_2), 4.65 (bs, 1H, NH), 6.95–6.98 (m, 1H, ArH), 7.03 (dd, J = 2.4, 8.0 Hz, 1H, ArH), 7.32 (dd, J = 7.2, 7.6 Hz, 1H, ArH), 7.35 (d, J = 8.0 Hz, 1H, ArH), 7.38–7.42 (m, 1H, ArH), 7.44 (t, J = 2.4 Hz, 1H, ArH), 7.59 (ddd, J = 1.2, 7.2, 8.0 Hz, 1H, ArH), 7.70 (d, J = 8.0 Hz, 1H, ArH), 7.86 (s, 1H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 42.0 (CH_2), 106.0 (CH), 110.4 (CH), 116.0 (CH), 118.2 (CH), 118.9 (C), 124.5 (CH), 125.5 (C), 128.2 (CH), 130.1 (CH), 131.1 (CH), 138.1 (CH), 148.8 (C), 149.2 (C), 152.6 (C), 160.2 (C). ESIMS: m/z 297 $(M + H)^+$, 279, 191, 159, 132, 116, 97. EI-HRMS calcd for $C_{16}H_{12}N_2O_4$: 296.0797, found: 296.0797. Anal. Calcd for $C_{16}H_{12}N_2O_4$: C, 64.86; H, 4.08; N, 9.46. Found: C, 64.93; H, 4.26; N, 9.07.

3-(3′,5′-Dinitroanilin-1′-yl)methylaminocoumarin (14). Yield: 204 mg, 60%. Mp: 217–219 °C. IR (KBr) ν: 3397, 2919, 2311, 1695, 1536 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.25 (d, J = 6.0 Hz, 2H, CH_2), 6.93 (bs, 1H, NH), 7.31 (ddd, J = 1.2, 7.2, 7.6 Hz, 1H, ArH), 7.36 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.58 (dd, J = 1.6, 7.6 Hz, 1H, ArH), 7.61 (ddd, J = 1.6, 7.2, 8.4 Hz, 1H, ArH), 7.92 (d, J = 2.0 Hz, 2H, ArH), 8.00 (s, 1H, ArH), 8.10 (t, J = 3.0 Hz, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 44.2 (CH_2), 106.7 (CH), 113.1 (CH), 117.7 (CH), 120.8 (C), 126.0 (CH), 126.7 (C), 129.7 (CH), 132.9 (CH), 140.2 (CH), 151.1 (C), 151.9 (C), 154.9 (C), 161.8 (C). ESIMS: m/z 342 $(M + H)^+$, 296, 250, 238, 148, 121, 105. EI-HRMS calcd for $C_{16}H_{11}N_3O_6$: 341.0648, found: 341.0650. Anal. Calcd for $C_{16}H_{11}N_3O_6$: C, 56.31; H, 3.25; N, 12.31. Found: C, 56.30; H, 3.36; N, 12.31.

3-(4′-Chloro-6′-methylanilin-1′-yl)methylaminocoumarin (15). Yield: 182 mg, 61%. Mp: 156–158 °C. IR (KBr) ν: 3429, 2922, 2097, 1506, 1610, 1505 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.21 (s, 3H, CH_3), 4.26 (bs, 1H, NH), 4.35 (s, 2H, CH_2), 6.43 (d, J = 8.4 Hz, 1H, ArH), 7.02 (dd, J = 2.4, 8.4 Hz, 1H, ArH), 7.07 (d, J = 2.4 Hz, 1H, ArH), 7.26 (ddd, J = 1.2, 7.2, 8.0 Hz, 1H, ArH), 7.35 (dd, J = 1.2, 8.4 Hz, 1H, ArH), 7.44 (dd, J = 1.6, 8.0 Hz, 1H, ArH), 7.51 (ddd, J = 1.6, 7.2, 8.4 Hz, 1H, ArH), 7.61 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.4 (CH_3), 43.8 (CH_2), 111.1 (CH), 116.5 (CH), 119.0 (C), 122.2 (C), 124.2 (C), 124.5 (CH), 125.8 (C), 126.8 (CH), 127.8 (CH), 130.1 (CH), 131.3 (CH), 138.7 (CH), 143.7 (C), 153.1 (C), 161.2 (C). ESIMS: m/z 302 $(M + H + 2)^+$, 300 $(M + H)^+$, 264, 191, 158, 132, 61. EI-HRMS calcd for $C_{17}H_{14}ClNO_2$: 299.0713, found: 299.0715. Anal. Calcd for $C_{17}H_{14}ClNO_2$: C, 68.12; H, 4.71; N, 4.67. Found: C, 67.99; H, 4.89; N, 4.43.

Cell culture and reagents

Huh7 cells and Ava5 cells were hapatoma cell lines cultured as described previously.[29] Interferon-α (Roferon©-A) was purchased from Hoffmann-La Roche Inc. (Nutley, NJ, USA). Telaprevir was purchased from Legend Star International Co., Ltd. A series of compounds of anilinocoumarin derivatives were dissolved in DMSO from Sigma-Aldrich. The final concentration of DMSO in all reactions was maintained at 0.1% in all experiments.

Quantification of hepatitis C virus RNAs and western blotting

The standard procedure of western blotting and quantitative real-time RT-PCR (qRT-PCR) was performed as described previously.[28] The total RNA of treated Ava5 cells was extracted and detected using the ABI Step One Real-Time PCR-System (ABI, Warrington, UK) with HCV-specific primers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers; GAPDH was used as an endogenous reference gene. The treated Ava5 cells were lysed in RIPA buffer, and the lysates were analyzed by western blotting. The membranes were probed with an anti-NS5B antibody (1 : 5000 dilution; Abcam, Cambridge, MA, USA) or an anti-GAPDH antibody (1 : 10 000 dilution; GeneTex, Irvine, CA, USA). The signals were detected using an ECL detection kit (PerkinElmer, CT).

Preparation of HCV derived from cell culture (HCVcc) and quantification of hepatitis C virus RNAs

The pcDNA6/TR-Tight/JFH1-FL/AR DNA (kindly provided by Professor Takaji Wakita)[30] was *in vitro* transcripted by a MEGAscript® Kit. The RNA was transfected into Huh7.5 cells by a lipofection method to produce HCVcc. The culture medium was harvested and the virus particle concentrated using polyethylene glycol-8000 (Sigma-Aldrich, USA). The HCVcc infectivity titer was determined by immunostaining the core protein. The Huh7 cells were infected with the concentrated virus for 8 h and treated with different concentrations of compounds for 3 days. The total RNAs were collected and the relative virus RNA quantified by qRT-PCR.

Analyses of combination treatment

Ava5 cells were treated with diluted compound **6** (1.25, 2.5, 5, 10, and 20 μM) in combination with/without diluted IFN-α (7.5, 15, 30 and 60 U mL^{-1}), telaprevir (0.1, 0.2, 0.4 and 0.8 μM), BMS790052 (1, 2, 4 and 8 pM), and PSI7977 (10, 20, 40 and 80 nM) at fixed ratios. After 3 days of incubation, total cellular RNA was collected and analyzed by qRT-PCR, using endogenous cellular GAPDH as a loading control. Values of the combination index (CI) were analyzed using CalcuSyn™ software according to the manufacturer's instructions.[29]

Transfection and luciferase activity assay

To investigate the regulation of IFN by compound **6**, Ava5 cells were transfected with 1 μg of plasmid pISRE-Luc and 0.1 μg of SEAP expression vector (pCMV-SEAP) using the T-Pro™ reagent (Ji-Feng Biotechnology Co. Ltd., Taiwan) to reflect IFN responses and to serve as an internal control, respectively. The transfected cells were incubated with different concentrations of compound **6** for 3 days. They were then lysed to measure luciferase activity using the Steady-Glo™ Luciferase Assay System (Promega, Madison, WI) in accordance with the manufacturer's instructions.

Cytotoxicity assay

Ava5 cells were seeded in 96-well plates overnight. They were then treated with different concentrations of anilinocoumarin derivatives for 4 days. Cell viability was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) assay using the CellTiter 96™ AQueous One Solution Cell Proliferation Assay System (Promega) as described previously.[29]

Statistical analysis

GraphPad Prism ver5.0 (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses and graphical illustrations. Data are presented as the mean ± standard deviation for at least three independent experiments. Statistical significance was analyzed using Student's *t*-test. $P < 0.05$ or $P < 0.01$ was considered significant.

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