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Discovery of 3-Amino-2-Hydroxypropoxyisoflavone Derivatives as Potential Anti-HCV Agents

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Abstract: Synthesis and anti-hepatitis C virus (anti-HCV) effects of certain 3-amino-2-hydroxy-propoxy isoflavone derivatives, **6a–i**, were described. The known 3-(3,4-dimethoxyphenyl)- 7-(oxiran-2-ylmethoxy)-4*H*-chromen-4-one (**5**) was reacted with substituted amines to give the desired isoflavone derivatives, **6a–i**. Among them, 7-{3-[(3,4-dimethoxy-phenethyl)amino]-2-hydroxypropoxy}-3-(3,4-dimethoxyphenyl)-4*H*-chromen-4-one (**6b**) was the most active, exhibiting approximately 2-fold higher anti-HCV effects than standard antiviral drug ribavirin (EC_{50} of 6.53 vs. 13.16 μM). In addition, compound **6b** was less cytotoxic than ribavirin. The selectivity index (SI) of **6b** is approximately 2.6-fold higher than ribavirin. The compounds **6e**, **6h**, and **6i** were also found to possess higher anti-HCV effects than ribavirin. Compound **6b** was found to inhibit the HCV RNA expression in Ava5 cells in a dose-dependent manner; furthermore, we found that the antiviral mechanism of compounds **6b**, **6e**, **6h**, and **6i** gave rise to induction of HO-1 expression. With the HO-1 promoter-based analysis, we found compounds **6b**, **6e**, **6h**, and **6i** induced HO-1 expression through increasing Nrf-2 binding activity. Taken together, compound **6b** may serve as a potential lead compound for developing novel anti-HCV agents.

Keywords: 3-amino-2-hydroxypropoxyisoflavones; ribavirin; hepatitis C virus; cytotoxicity

1. Introduction

Hepatitis C virus (HCV) infection has significantly increased in the past decades and becomes a severe problem in liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Globally, an estimated 200 million people are infected with hepatitis C virus and more than 350,000 people die every year from HCV-related liver diseases [1–3]. In clinical therapies, there are still no approved vaccines for the treatment of HCV infection [4]. The therapeutic agents for HCV patients still present a drug-resistant problem, so the development of supplemental agents or more effective and safer agents is required for such therapy [5–10]. Recently, Andreev et al. [11] identified 1-benzyl-2-phenyl-4,5,6,7-tetrahydro-1*H*-indole

(Compound **1**) as a potent anti-HCV agent which displayed EC_{50} values of 7.9 and 2.6 μM in genotype 1b and 2a, respectively. Kaushik-Basu et al. [12] reported that (3a*S*,8a*S*,*E*)-ethyl-4-(2-phenylhydrazono)-1-tosyldecahydro-cyclohepta[*b*]pyrrole-2-carboxylate (Compound **2**) exhibited EC_{50} values of 1.8 and 4.5 μM in genotype 1b and 2a, respectively. Zhong et al. [13] prepared certain quercetin analogues for anti-HCV evaluations and found 7-[(3-chlorobenzyl)oxy]-2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-4*H*-chromen-4-one (Compound **3**) was the most potent, exhibiting an EC_{50} value of 3.8 μM. We have also synthesized certain naphtho [1,2-*d*]oxazole derivatives for anti-HCV evaluations and discovered 2-(furan-2-yl)-*N*-(4-methoxyphenyl)naphtho[1,2-*d*]oxazol-5-amine (Compound **4**) [14] to be the most active, exhibiting an EC_{50} value of 0.63 μM.

A number of natural isoflavonoids along with their synthetic analogues have been found to possess extensive biological activities including antiparasitic, anti-cancer, antiviral, anti-inflammatory, antioxidant, and anti-osteoporosis effects [15–19]. In order to further explore antiviral effects of isoflavonoids, we describe herein the synthesis of 3-amino-2-hydroxy-propoxyisoflavone derivatives (target compounds, Figure 1) and their evaluations related to the inhibition of HCV replication by inducing HO-1 expression.

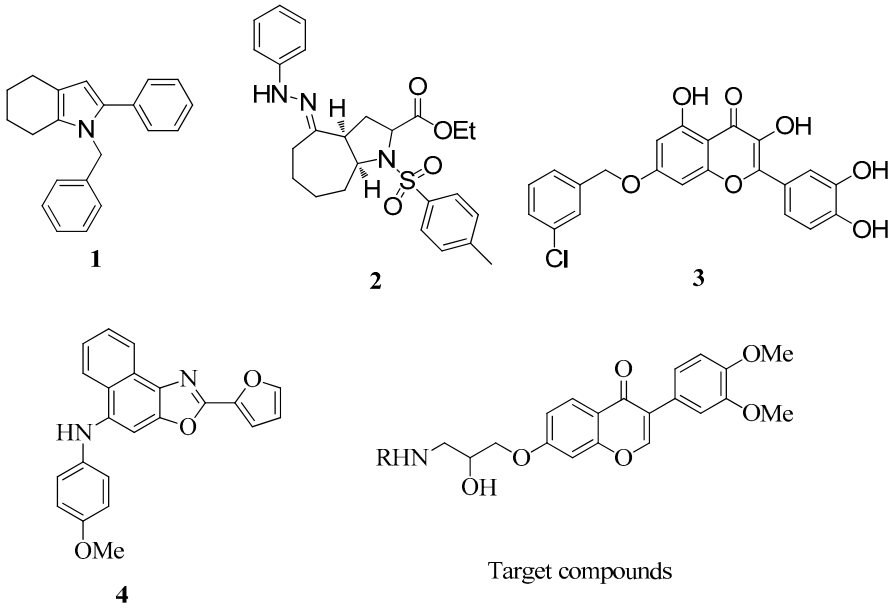


Figure 1. Structures of compounds **1**–**4**, and target compounds.

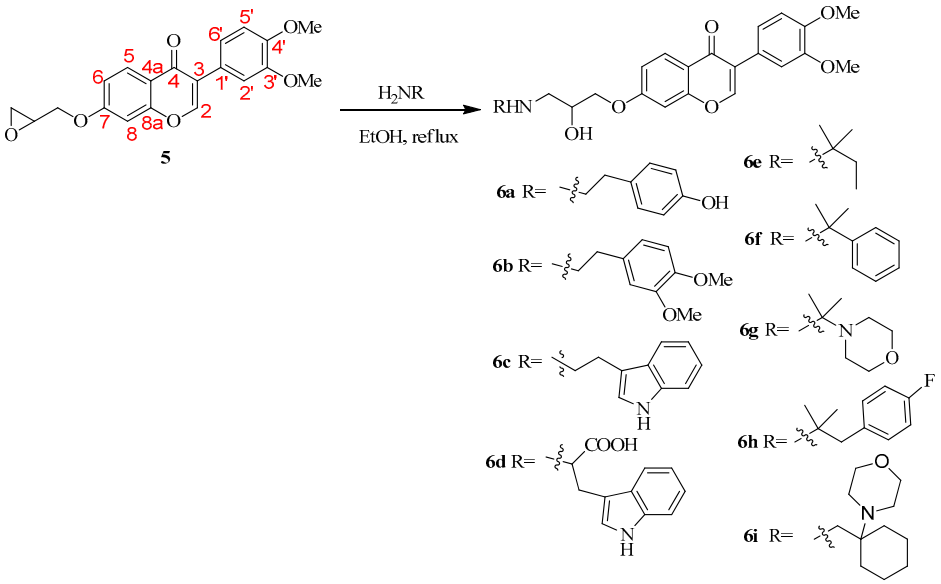
2. Results and Discussion

2.1. Chemistry

Preparation of 3-Amino-2-Hydroxypropoxyisoflavone Derivatives

The desired 3-amino-2-hydroxypropoxyisoflavone derivatives, **6a**–**i**, have been prepared as described in Scheme 1. Reaction of 3-(3,4-dimethoxyphenyl)-7-(oxiran-2-ylmethoxy)-4*H*-chromen-4-one (**5**) [19] with 4-hydroxyphenylethylamine in ethanol gave 3-(3,4-dimethoxyphenyl)-7-{2-hydroxy-3- [(4-hydroxyphenethyl) amino]propoxy}-4*H*-chromen-4-one (**6a**) in 76% yield. 7-{3-[(3,4-Dimethoxyphenethyl)amino]-2-hydroxypropoxy}-3-(3,4-dimethoxyphenyl)-4*H*-chromen- 4-one (**6b**) was obtained by the treatment of **5** with 3,4-dimethoxyphenylethylamine. The structure of **6b** was determined by ^{13}C (100 MHz), ^{1}H (400 MHz), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser enhancement spectroscopy (NOESY) nuclear magnetic resonance (NMR) (Table 1). The spectra of **6b** (Table 1) revealed the presence of two sets of

3,4-dimethoxyphenyl-aromatic rings [δ_C 124.52 (C-1′) and 132.05 (C-3′″); δ_C 112.42 (C-2′)/δ_H 7.20 (2′-CH, d, *J* = 2.0 Hz) and δ_C 111.89 (C-4′″)/δ_H 6.74 (4′″-CH, m); δ_C 148.71 (C-3′) and 147.52 (C-5′″); δ_C 148.94 (C-4′) and 149.05 (C-6′″); δ_C 111.10 (C-5′)/δ_H 6.92 (5′-CH, d, *J* = 8.4 Hz) and δ_C 111.27 (C-7′″)/δ_H 6.80 (7′″-CH, d, *J* = 8.0 Hz); δ_C 120.98 (C-6′)/δ_H 7.04 (6′-CH, dd, *J* = 8.4, 2.0 Hz) and δ_C 20.55 (C-8′″)/δ_H 6.75 (m)], four methoxy groups [δ_C 55.89/δ_H 3.84 (s) for 3′-OMe, δ_C 55.91/δ_H 3.88 (s) for 4′-OMe, δ_C 55.82/δ_H 3.91 (s) for 5′″-OMe, and δ_C 55.87/δ_H 3.93 (s) for 6′″-OMe], *4H*-chromen-4-one moiety [δ_C 152.26 (C-2)/δ_H 7.94 (2-CH, s), δ_C 124.89 (C-3), δ_C 75.81 (C-4), δ_C 118.55 (C-4a), δ_C 127.75 (C-5)/δ_H 8.20 (5-CH, d, *J* = 8.8 Hz), δ_C 114.74 (C-6)/δ_H 6.99 (6-CH, d, *J* = 8.8, 2.4 Hz), δ_C 162.95 (C-7), δ_C 157.73 (C-8)/δ_H 6.86 (8-CH, d, *J* = 2.4 Hz), and δ_C 157.73 (C-8a)], and the 3-amino-2-hydroxypropoxy-spacer [δ_C 67.72 (C-1″)/δ_H 4.07 (1″-CH_2, m), δ_C 70.95 (C-2″)/δ_H 4.07 (2″-CH, m), δ_C 51.32 (C-3″)/δ_H 2.91 (3″-CH_2, m), δ_C 50.98 (C-1′″)/δ_H 2.91 and 2.78 (1′″-CH_2, m), δ_C 35.85 (C-2′″)/δ_H 2.78 (2′″-CH_2, m), and δ_H 2.23 (2″-OH and 3″-NH, br s)]. Its HMBC spectrum provided key correlations: (1) from H-2′ to C-3, 4′, 6′, and H-6′ to C-3, 2′, 4′ suggested the 3,4-dimethoxyphenyl group was attached to C-3 of the *4H*-chromen-4-one moiety; (2) from H-1″ to C-7, 3″, H-1′″ to C-3″, 3′″ indicated the other 3,4-dimethoxyphenyl group was attached to C-2′″ of the 3-amino-2-hydroxypropoxy-spacer and the spacer was attached to C-7 of the *4H*-chromen-4-one moiety (Figure 2A). The relative connection was established according to nuclear Overhauser effect (NOE) correlations between H-2/H-2′, 6′; H-1″/H-6, 8; H-1′″/H-2″, 8′″; and H-2′″/H-8′″ in the NOESY experiment (Figure 2B). Accordingly, compounds **6c–6i** have been prepared by amination of **5** in a yield of 65–83%. The structure of **6a–i** was determined by NMR (^{1}H and ^{13}C) (spectra data can be found in Supplementary Materials) and further confirmed by elemental analysis.



Scheme 1. Synthesis of 3-amino-2-hydroxypropoxyisoflavone derivatives **6a–i**.

Table 1. ^{13}C (100 MHz), ^{1}H (400 MHz), *HC* HMBC and NOESY nuclear magnetic resonance (NMR) Data for 7-{3-[(3,4-dimethoxy phenethyl)amino]-2-hydroxypropoxy}-3-(3,4-dimethoxyphenyl)-*4H*-chromen-4-one (**6b**) in $CDCl_3$. [a]

	^{13}C	^{1}H [b]		
Position	**δ_C (mult.)**	**δ_H (mult., *J*, Hz)**	***HC* HMBC**	**NOESY**
2	152.26 (CH)	7.94 (s)	4, 8a, 1′	2′, 6′
3	124.89 (C)			
4	175.81 (C)			
4a	118.55 (C)			
5	127.75 (CH)	8.20 (d, 8.8)	4, 7, 8a	6
6	114.74 (CH)	6.99 (dd, 8.8, 2.4)	4a, 8	5, 1″
7	162.95 (C)			
8	100.84 (CH)	6.86 (d, 2.4)	4a, 6, 7, 8a	1″
8a	157.73 (C)			
1′	124.52 (C)			
2′	112.42 (CH)	7.20 (d, 2.0)	3, 4′, 6′	2, 3′-OMe
3′	148.71 (C)			
3′-OMe	55.89 (CH_3)	3.84 (s)		2′
4′	148.94 (C)			
4′-OMe	55.91 (CH_3)	3.88 (s)		5′
5′	111.10 (CH)	6.92 (d, 8.4)	1′, 3′	6′, 4′-OMe
6′	120.98 (CH)	7.04 (dd, 8.4, 2.0)	3, 2′, 4′	2, 5′
1″	67.72 (CH_2)	4.07 (m), overlapped	7, 3″	6, 8
2″	70.95 (CH)	4.07 (m), overlapped		3″, 1‴
2″-OH		2.23 (br s), overlapped		
3″	51.32 (CH_2)	2.91 (m), overlapped		2″
3″-NH		2.23 (br s), overlapped		
1‴	50.98 (CH_2)	2.91 and 2.78 (m), overlapped	3″, 3‴	2″, 8‴
2‴	35.85 (CH_2)	2.78 (m), overlapped	4‴, 8‴	8‴
3‴	132.05 (C)			
4‴	111.89 (CH)	6.74 (m), overlapped	2‴, 6‴, 8‴	5‴-OMe
5‴	147.52 (C)			
5‴-OMe	55.82 (CH_3)	3.91 (s)		4‴
6‴	149.05 (C)			
6‴-OMe	55.87 (CH_3)	3.93 (s)		7‴
7‴	111.27 (CH)	6.80 (d, 8.0)	3‴	6‴-OMe
8‴	120.55 (CH)	6.75 (m), overlapped	2‴, 4‴, 6‴	1‴, 2‴

[a] s: Singlet, d: Doublet, dd: Doublet of doublets, br s: Broad singlet, m: Multiple, ov: Overlapped; [b] The ^{13}C and ^{1}H correlations were confirmed by the heteronuclear multiple quantum coherence (HMQC) experiment.

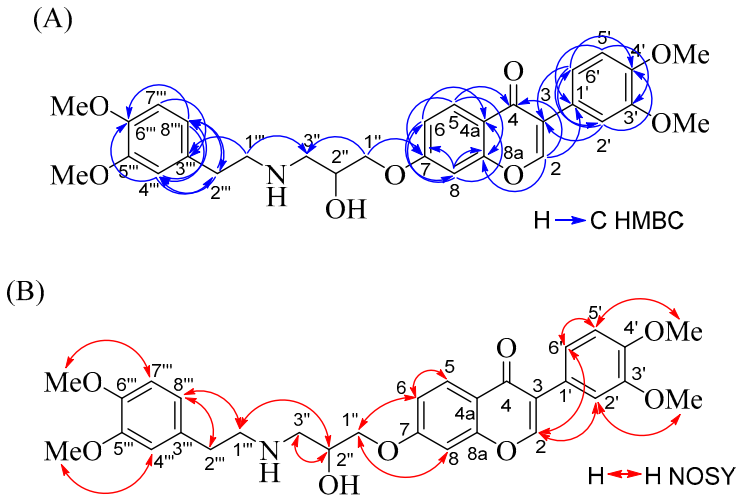


Figure 2. HMBC (**A**) and NOESY (**B**) correlations for 7-{3-[(3,4-dimethoxyphenethyl)amino]-2-hydroxypropoxy}-3-(3,4-dimethoxyphenyl)-*4H*-chromen-4-one (**6b**).

2.2. Biological Activities

2.2.1. Anti-HCV Activities and Cytotoxicities

The anti-HCV and cytotoxicities of 3-amino-2-hydroxypropoxyisoflavone derivatives are summarized in Table 2. Ava-5 cells were treated with compounds **6a–i** or the positive ribavirin for three days, and then analyzed through firefly luciferase assay. The concentration that inhibited 50% HCV replication (EC_{50}), the concentration that inhibited 50% cell growth (CC_{50}), and the selectivity index (SI: CC_{50}/EC_{50}) of compounds were determined with ribavirin as a positive control. Results indicated that compounds **6b**, **6e**, **6h** and **6i** were more active than ribavirin. Among them, compound **6b** was the most active, exhibiting approximately 2-fold more anti-HCV activity (EC_{50} of 6.53 μM) than that of ribavirin (EC_{50} = 13.16 μM). In addition, compound **6b** was less cytotoxic than ribavirin. The selectivity index (SI) of **6b** is approximately 2.6-fold higher than that of ribavirin (21.08 vs. 8.08).

Table 2. Antiviral activities and cytotoxicities of isoflavone derivatives.

Compound	EC_{50} (μM) [a]	CC_{50} (μM) [b]	SI [c]
6a	>20	71.65 ± 4.44	<3.58
6b	6.53 ± 0.57	137.68 ± 6.91	21.08
6c	16.32 ± 0.95	36.93 ± 0.46	2.26
6d	>20	98.84 ± 3.67	<4.94
6e	8.14 ± 1.74	87.91 ± 2.13	10.80
6f	14.31 ± 0.84	47.19 ± 2.74	3.30
6g	>20	143.57 ± 3.82	<7.17
6h	9.35 ± 0.97	110.98 ± 4.39	11.87
6i	10.71 ± 0.87	155.87 ± 1.58	14.55
ribavirin	13.16 ± 1.63	106.27 ± 3.69	8.08

[a] The EC_{50} is the concentration of the compound resulting in a 50% inhibition in virus production; [b] The CC_{50} is the concentration of the compound causing a 50% growth inhibition of uninfected ava-5 cells; [c] SI: Selectivity index. SI = CC_{50}/IC_{50}.

2.2.2. Compound **6b** Reduced HCV Replication in HCV-Infected Ava-5 Cells

To further confirm the anti-HCV effect of compound **6b**, we treated compound **6b** at indicated concentrations in Ava-5 cells for 3 days. Both western blotting and RT-qPCR were performed to determine the resultant activity of compound **6b** against HCV replication showing that compound **6b** dose-dependently reduced HCV protein synthesis and RNA replication without cell cytotoxicity in Ava5 cells. Treatment of 0.1% dimethyl sulfoxide (DMSO) served as a mock control on inhibition of HCV replication (Figures 3 and 4).

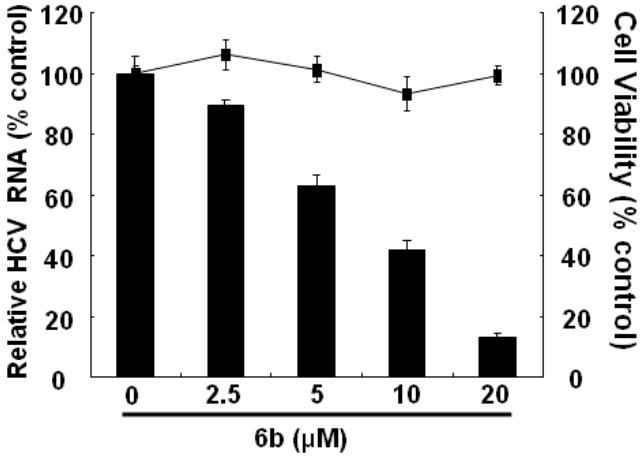


Figure 3. Inhibition of HCV RNA expression in HCV-infected Ava-5 cells by **6b**. Ava-5 cells were treated with 2.5, 5, 10 and 20 μM of **6b** for 3 days. Total RNA was extracted and quantified HCV RNA levels by RT-qPCR. HCV RNA expression was normalized by cellular GAPDH mRNA. Treatment with 0.1% DMSO served as a mock control. The results are expressed as the means ± standard deviations (SD) of triplicate experiments.

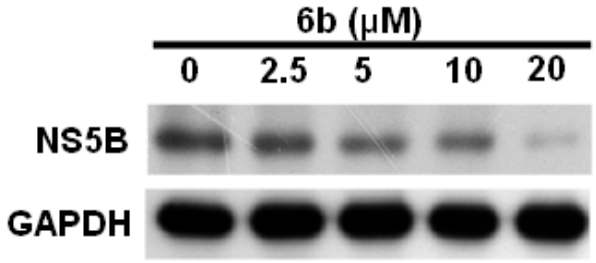


Figure 4. Inhibition of HCV protein synthesis in Ava-5 cells by **6b**. Ava-5 cells were treated with 2.5, 5, 10 and 20 μM of **6b** for 3 days. Total cell lysate was collected for performing western blotting to analyze HCV protein synthesis. Levels of GAPDH were used as equal loading control.

2.2.3. Isoflavones Reduced HCV Replication through Inducing HO-1 Protein Expression

In our previous studies, we found that induction of HO-1 protein level could suppress HCV replication [14,20]. To determine whether compounds **6b**, **6e**, **6h** and **6i** have impact on HO-1 protein expression in Ava-5 cells, we treated these compounds at 10 μM in Ava5 cells. Results indicated that compounds **6b**, **6e**, **6h** and **6i** could induce HO-1 protein level in Ava-5 cells, compared with the DMSO-treated Ava5 cells (Figure 5).

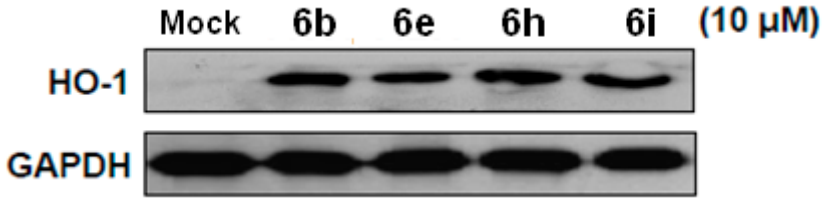


Figure 5. Compounds induced HO-1 protein expression in Ava-5 cells. Compound **6b**, **6e**, **6h** and **6i** induced HO-1 protein expression. Ava-5 cells were treated with compounds at 10 μM for 3 days. The cell lysate was subjected to western blotting with anti-HO-1 and anti-GAPDH antibodies.

2.2.4. Isoflavones Up-Regulates Nrf2 Transactivating HO-1 Expression to Inhibit HCV Replication

Heme oxygenase 1 expression is regulated by the transcription factors Nrf2, Keap1, and Bach1 through the binding of ARE in its promoter region [21–23]; therefore, we determined whether isoflavones-mediated HO-1 induction was dependent on ARE transactivation by treating

p3xARE-Luc-transfected ava-5 cells with increasing concentrations of sulforaphane (SFN) for 3 days. As shown in Figure 6, compounds **6b**, **6e**, **6h** and **6i** increased ARE-mediated luciferase activity. Taken together, these results suggest that the anti-HCV activity of isoflavones were dependent on Nrf2-mediated HO-1 induction.

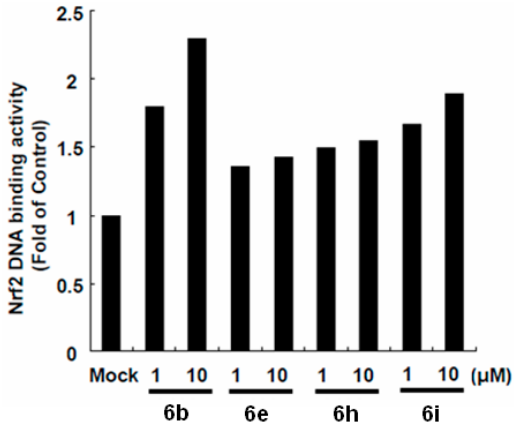


Figure 6. Isoflavones inhibited HCV replication by upregulating Nrf2 expression. Compound **6b**, **6e**, **6h** and **6i** stimulated ARE transactivation in Ava-5 cells. The antioxidant response reporter plasmid, p3xARE-Luc, was transfected into Ava-5 cells and then treated with isoflavones (1 and 10 μM) for 3 days. The relative induction of antioxidant activity was determined by luciferase assay. The activity of untreated Ava-5 cells was considered to be 1.

3. Experimental

3.1. Materials and Methods

3.1.1. Chemical Reactions

General

Melting points were determined on an Electrothermal IA9100 melting point apparatus and are uncorrected. Nuclear magnetic resonance (^{1}H) spectra were recorded on a Varian-Unity-400 spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts were expressed in parts per million (δ) with tetramethylsilane (TMS) as an internal standard. Thin-layer chromatography was performed on silica gel 60 F-254 plates purchased from E. Merck and Co. (Darmstadt, Germany). The elemental analyses were performed in the Instrument Center of Ministry of Science and Technology at National Cheng-Kung University and National Taiwan University using Heraeus CHN-O Rapid EA (Heraeus, Waltham, MA, USA), and all values are within ±0.4% of the theoretical compositions.

General Procedure for the Preparation of 3-Amino-2-Hydroxypropoxyisoflavone Compounds **6a–i**

To a suspension of **5** (1.0 mmol) in ethanol (15 mL) was added substituted amines (3.0 mmol). The reaction mixture was refluxed for 3 h (TLC monitoring). The solvent was removed in vacuo and the residue suspended in H_2O (20 mL). The crude product was purified by flash chromatography on silica gel and recrystallized from MeOH to afford the 3-amino-2-hydroxypropoxyisoflavone products.

3-(3,4-Dimethoxyphenyl)-7-{2-hydroxy-3-[(4-hydroxyphenethyl)amino]propoxy}-4H-chromen-4-one (**6a**): Yield 76%. Mp: 117–118 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.18 (br s, 1H), 8.46 (s, 1H, H-2), 8.04 (d, J = 8.8 Hz, 1H, H-5), 7.21 (d, J = 2.0 Hz, 1H, H-2′), 7.17–7.14 (m, 2H, H-8, H-6′), 7.08 (dd, 1H, J = 8.8, 2.4 Hz, H-6), 7.02 (d, J = 8.4 Hz, 1H, H-5′), 7.01–6.98 (m, 2H), 6.68–6.65 (m, 2H), 5.17 (br s, 1H), 4.15–4.11 (m, 1H), 4.05–4.01 (m, 1H), 3.96–3.92 (m, 1H), 3.79 (s, 6H), 2.75–2.59 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.66 (C-4), 163.16 (C-7), 157.38 (C-8a), 155.48, 153.71 (C-2), 148.66 (C-4′), 148.30 (C-3′), 130.24, 129.46 (2C), 126.98 (C-5), 124.39 (C-1′), 123.48 (C-3), 121.27 (C-6′), 117.58 (C-4a), 115.17 (C-6),

115.06 (2C), 112.74 (C-2′), 111.54 (C-5′), 101.09 (C-8), 71.60, 67.84, 55.54 (3′-OMe-3′, 4′-OMe), 51.99, 51.44, 34.91. Anal. calcd. for $C_{28}H_{29}NO_7 \cdot 1.2H_2O$: C 65.54, H 6.17, N 2.73; found: C 65.50, H 6.06, N 2.59.

7-{3-[(3,4-Dimethoxyphenethyl)amino]-2-hydroxypropoxy}-3-(3,4-dimethoxyphenyl)-4H-chromen-4-one (**6b**): Yield 69%. Mp: 134–135 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (d, 1H, *J* = 8.8 Hz, H-5), 7.94 (s, 1H, H-2), 7.20 (d, 1H, *J* = 2.0 Hz, H-2′), 7.04 (dd, 1H, *J* = 8.4, 2.0 Hz, H-6′), 6.99 (dd, 1H, *J* = 8.8, 2.4 Hz, H-6), 6.92 (d, 1H, *J* = 8.4 Hz, H-5′), 6.86 (d, 1H, *J* = 2.4 Hz, H-8), 6.80 (d, 1H, *J* = 8.0 Hz), 6.77–6.74 (m, 2H), 4.10–4.04 (m, 3H), 3.93 (s, 3H), 3.91 (s, 3H), 3.88 (s, 3H), 3.86 (s, 3H), 2.99–2.74 (m, 6H), 2.23 (br s, 2H, OH and NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.81 (C-4), 162.95 (C-7), 157.73 (C-8a), 152.26 (C-2), 149.10, 148.99 (C-4′), 148.77 (C-3′), 147.57, 132.14, 127.81 (C-5), 124.94 (C-1′), 124.57 (C-3), 121.02 (C-6′), 120.59, 118.62 (C-4a), 114.77 (C-6), 112.48 (C-2′), 111.95, 111.34 (C-5′), 111.16, 100.89 (C-8), 70.98, 67.78, 55.95, 55.93, 55.91 (4′-OMe), 55.86 (3′-OMe), 51.31, 51.02, 35.95. Anal. calcd. for $C_{30}H_{33}NO_8$: C 67.28, H 6.21, N 2.62; found: C 66.91, H 6.31, N 2.57.

7-{3-{[2-(1H-Indol-3-yl)ethyl]amino}-2-hydroxypropoxy}-3-(3,4-dimethoxyphenyl)-4H-chromen-4-one (**6c**): Yield 65%. Mp: 178–179 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.80 (br s, 1H), 8.45 (s, 1H, H-2), 8.04 (d, 1H, *J* = 8.8 Hz, H-5), 7.53–7.51 (m, 1H), 7.34–7.32 (m, 1H), 7.21 (d, 1H, *J* = 2.0 Hz, H-2′), 7.17–7.14 (m, 3H), 7.09–7.04 (m, 2H), 7.01 (d, 1H, *J* = 8.4 Hz, H-5′), 6.98–6.94 (m, 1H), 5.14 (br s, 1H), 4.17–4.13 (m, 1H), 4.06–4.02 (m, 1H), 3.98–3.93 (m, 1H), 3.79 (s, 6H), 2.86 (br s, 4H), 2.77–2.66 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.66 (C-4), 163.19 (C-7), 157.39 (C-8a), 153.70 (C-2), 148.65 (C-4′), 148.30 (C-3′), 136.26, 127.28, 126.97 (C-5), 124.39 (C-1′), 123.47 (C-3), 122.62, 121.27 (C-6′), 120.87, 118.34, 118.16, 117.57 (C-4a), 115.17 (C-6), 112.72 (C-2′), 112.48, 111.54 (C-5′), 111.37, 101.09 (C-8), 71.66, 67.96, 55.56 (4′-OMe), 55.54 (3′-OMe), 52.11, 50.27, 25.51. Anal. calcd. for $C_{30}H_{30}N_2O_6 \cdot 0.2H_2O$: C 69.53, H 5.92, N 5.41; found: C 69.35, H 5.86, N 5.33.

3-{{3-{[3-(3,4-Dimethoxyphenyl)-4-oxo-4H-chromen-7-yl]oxy}-2-hydroxypropyl}amino}-2-(1H-indol-3-yl) propanoic acid (**6d**): Yield 71%. Mp: 221–222 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.91 (br s, 1H), 8.44 (s, 1H, H-2), 8.02 (d, 1H, *J* = 8.8 Hz, H-5), 7.57 (d, 1H, *J* = 7.6 Hz, H-2′), 7.33 (d, 1H, *J* = 7.6 Hz), 7.22–6.94 (m, 8H), 4.07–3.96 (3m, 3H), 3.78 (s, 6H), 3.24–2.74 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.68 (C-4), 172.50, 162.90 (C-7), 157.34 (C-8a), 153.77 (C-2), 148.66 (C-4′), 148.30 (C-3′), 136.21, 127.35 (C-5), 127.00, 124.37 (C-1′), 123.91, 123.50 (C-3), 121.29 (C-6′), 121.00, 118.55, 118.39, 117.69 (C-4a), 115.13 (C-6), 112.69 (C-2′), 111.52 (C-5′), 111.40, 109.69, 101.14 (C-8), 70.96, 66.42, 62.27, 55.57 (4′-OMe), 55.55 (3′-OMe), 49.66, 27.15. Anal. calcd. for $C_{31}H_{30}N_2O_8 \cdot 1.5H_2O$: C 63.57, H 5.69, N 4.78; found: C 63.22, H 5.54, N 4.81.

3-(3,4-Dimethoxyphenyl)-7-(2-hydroxy-3-(tert-pentylamino)propoxy)-4H-chromen-4-one (**6e**): Yield 80%. Mp: 111–112 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (d, 1H, *J* = 9.2 Hz, H-5), 7.95 (s, 1H, H-2), 7.21 (d, 1H, *J* = 2.0 Hz, H-2′), 7.06–7.01 (m, 2H, H-6, H-6′), 6.92 (d, 1H, *J* = 8.4 Hz, H-5′), 6.88 (d, 1H, *J* = 2.4 Hz, H-8), 4.13–4.05 (m, 3H), 3.93 (s, 3H), 3.91 (s, 3H), 2.91 (dd, 1H, *J* = 12.0, 3.2 Hz), 2.70 (dd, 1H, *J* = 12.0, 7.6 Hz), 2.56 (br s, 1H), 1.48 (q, 2H, *J* = 8.0 Hz), 1.11 (s, 6H), 0.90 (t, 3H, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.85 (C-4), 163.04 (C-7), 157.78 (C-8a), 152.27 (C-2), 149.08 (C-4′), 148.75 (C-3′), 127.89 (C-5), 124.92 (C-1′), 124.58 (C-3), 121.01 (C-6′), 118.59 (C-4a), 114.83 (C-6), 112.48 (C-2′), 111.13 (C-5′), 100.87 (C-8), 71.00, 67.98, 55.94 (4′-OMe), 55.92 (3′-OMe), 53.49, 44.05, 33.20, 26.23, 26.18, 8.24. Anal. calcd. for $C_{26}H_{33}NO_6 \cdot 0.5H_2O$: C 68.21, H 7.39, N 3.02; found: C 68.55, H 7.30, N 3.07.

3-(3,4-Dimethoxyphenyl)-7-{2-hydroxy-3-[(2-phenylpropan-2-yl)amino]propoxy}-4H-chromen-4-one (**6f**): Yield 68%. Mp.: 131–132 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (d, 1H, *J* = 8.8 Hz, H-5), 7.94 (s, 1H, H-2), 7.46–7.43 (m, 2H), 7.36–7.32 (m, 2H), 7.25–7.21 (m, 1H), 7.19 (d, 1H, *J* = 2.0 Hz, H-2′), 7.04 (dd, 1H, *J* = 8.0, 2.0 Hz, H-6′), 6.96 (dd, 1H, *J* = 9.2, 2.4 Hz, H-6), 6.92 (d, 1H, *J* = 8.4 Hz, H-5′), 6.83 (d, 1H, *J* = 2.4 Hz, H-8), 4.03–3.96 (m, 3H), 3.92 (s, 3H), 3.91 (s, 3H), 2.65 (dd, 1H, *J* = 12.0, 3.6 Hz), 2.48 (dd, 1H, *J* = 12.0, 7.2 Hz), 2.07 (br s, 1H, NH), 1.51 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.85 (C-4), 162.98 (C-7), 157.73 (C-8a), 152.25 (C-2), 149.01 (C-4′), 148.68 (C-3′), 146.93, 128.30 (2C), 127.72 (C-5), 126.53,

125.73 (2C), 124.88 (C-1′), 124.53 (C-3), 120.97 (C-6′), 118.50 (C-4a), 114.80 (C-6), 112.38 (C-2′), 111.05 (C-5′), 100.76 (C-8), 70.98, 68.73, 55.90 (3′-OMe, 4′-OMe), 55.76, 45.14, 29.68, 29.37. Anal. calcd. for $C_{29}H_{31}NO_6$: C 71.15, H 6.38, N 2.86; found: C 71.01, H 6.34, N 2.57.

3-(3,4-Dimethoxyphenyl)-7-{2-hydroxy-3-[(2-morpholinopropan-2-yl)amino]propoxy}-4H-chromen-4-one (**6g**): Yield 78%. Mp: 108–109 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.19 (d, 1H, *J* = 8.8 Hz, H-5), 7.93 (s, 1H, H-2), 7.19 (d, 1H, *J* = 2.0 Hz, H-2′), 7.04-6.98 (m, 2H, H-6, H-6′), 6.91 (d, 1H, *J* = 8.4 Hz, H-5′), 6.85 (d, 1H, *J* = 2.4 Hz, H-8), 4.32–4.30 (m, 1H), 4.15–4.07 (m, 2H), 3.92 (s, 3H), 3.91 (s, 3H), 3.78–3.72 (m, 4H), 3.15–3.11 (m, 1H), 2.91–2.83 (m, 2H), 2.67–2.52 (m, 5H) 1.17 (s, 3H), 1.10 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.78 (C-4), 162.79 (C-7), 157.72 (C-8a), 152.29 (C-2), 149.07 (C-4′), 148.72 (C-3′), 127.80 (C-5), 124.90 (C-1′), 124.49 (C-3), 121.00 (C-6′), 118.64 (C-4a), 114.72 (C-6), 112.43 (C-2′), 111.10 (C-5′), 100.86 (C-8), 70.56, 67.33 (2C), 66.54, 56.49, 56.14, 55.93 (4′-OMe), 55.90 (3′-OMe), 52.19, 45.82 (2C), 22.37, 20.81. Anal. calcd. for $C_{28}H_{36}N_2O_7 \cdot 0.5H_2O$: C 64.46, H 7.16, N 5.37; found: C 64.26, H 7.18, N 5.20.

3-(3,4-Dimethoxyphenyl)-7-{3-{[1-(4-fluorophenyl)-2-methylpropan-2-yl]amino}-2-hydroxypropoxy}-4H-chromen-4-one (**6h**): Yield 83%. Mp: 151–152 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.46 (s, 1H, H-2), 8.04 (d, 1H, *J* = 8.8 Hz, H-5), 7.23–7.14 (m, 5H), 7.09 (dd, 1H, *J* = 8.8, 2.4 Hz, H-6), 7.07–7.01 (m, 3H), 5.10 (br s, 1H), 4.17 (dd, 1H, *J* = 10.0, 4.0 Hz), 4.06 (dd, 1H, *J* = 10.0, 6.0 Hz), 3.89–3.86 (m, 1H), 3.79 (s, 6H), 2.77–2.65 (m, 2H), 2.62 (s, 2H), 0.96 (s, 3H), 0.95 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.63 (C-4), 163.20 (C-7), 160.77 (*J* = 240.3 Hz), 157.36 (C-8a), 153.68 (C-2), 148.48 (C-4′), 148.31 (C-3′), 134.84 (*J* = 3.1 Hz), 132.06 (2C, *J* = 7.6 Hz), 126.96 (C-5), 124.38 (C-1′), 123.47 (C-3), 121.27 (C-6′), 117.55 (C-4a), 115.15 (C-6), 114.26 (2C, *J* = 20.5 Hz), 112.76 (C-2′), 111.57 (C-5′), 101.08 (C-8), 71.52, 68.87, 55.56 (4′-OMe), 55.55 (3′-OMe), 52.49, 45.37, 44.61, 26.66, 26.58. Anal. calcd. for $C_{30}H_{32}FNO_6 \cdot 0.2H_2O$: C 68.61, H 6.22, N 2.67; found: C 68.49, H 6.21, N 2.26.

3-(3,4-Dimethoxyphenyl)-7-{2-hydroxy-3-{[(1-morpholinocyclohexyl)methyl]amino}propoxy}-4H-chromen-4-one (**6i**): Yield 74%. Mp: 67–68 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.19 (d, 1H, *J* = 8.8 Hz, H-5), 7.93 (s, 1H, H-2), 7.19 (d, 1H, *J* = 2.0 Hz, H-2′), 7.04–6.99 (m, 2H, H-6, H-6′), 6.91 (d, 1H, *J* = 8.0 Hz, H-5′), 6.86 (d, 1H, *J* = 2.4 Hz, H-8), 4.34–4.32 (m, 1H), 4.15–4.07 (m, 2H), 3.92 (s, 3H), 3.91 (s, 3H), 3.76–3.69 (m, 4H), 3.14–3.05 (m, 2H), 2.84 (dd, 1H, *J* = 12.0, 8.8 Hz), 2.69–2.57 (m, 5H), 1.69–1.19 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.78 (C-4), 162.80 (C-7), 157.72 (C-8a), 152.28 (C-2), 149.07 (C-4′), 148.73 (C-3′), 127.81 (C-5), 124.90 (C-1′), 124.49 (C-3), 121.00 (C-6′), 118.64 (C-4a) 114.72 (C-6), 112.44 (C-2′), 111.11 (C-5′), 100.87 (C-8), 70.60, 67.75 (2C), 66.52, 58.22, 55.92 (4′-OMe), 55.90 (3′-OMe), 52.33, 50.01, 45.41 (2C), 29.99, 29.63, 25.77, 22.07, 21.98. Anal. calcd. for $C_{31}H_{40}N_2O_7 \cdot 2.6H_2O$: C 62.10, H 7.60, N 4.67; found: C 61.88, H 7.20, N 4.47.

3.1.2. Cytotoxicity and Antiviral Activity Assays

Compounds

Compounds were dissolved in DMSO at 10 mM and then diluted in culture medium.

Cell

Ava5 cells, an engineered HCV subgenomic replicon cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, 1% antibiotic–antimycotic, and 1% non-essential amino acids. Ava5 cells were maintained in DMEM with 1 mg mL^{-1} G418 to maintain the stable expression of replicon.

Cytotoxicity Assays

For cytotoxicity tests, run in parallel with antiviral assays, plates at an initial density of (5×10^3 cells/well) were treated with or without serial dilutions of test compounds. Cell viability was determined after 72 h at 37 °C in a humidified CO_2 (5%) atmosphere by the (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide) (XTT) method [24].

Transfection and Luciferase Activity Assay

Ava5 cells were transfected with the HO-1 promoter-driven luciferase plasmid, pHO-1-Luc, using the T-pro^{TM} transfection reagent (Ji-Feng Biotechnology Co., Ltd., Taipei, Taiwan) according to the manufacturer's instructions. The transfected cells were treated with compounds at various concentrations for 3 days. Each transfection complex contains 0.1 μg pSEAP, a secreted alkaline phosphatase (SEAP) expression vector, for normalization luciferase activity serving as a transfection control. The luciferase activity assay was performed using the Bright-Glo Luciferase assay system (Promega) (Madison, WI, USA) according to the manufacturer's instructions.

Immunoblot Analysis

Ava5 cells were seeded in 24-well plates at a density of 5×10^4 cells per well overnight and treated with indicated reagent at proper concentrations for 3 days. Cells were washed with cold phosphate buffer saline (PBS) and lysed by radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and stored at −20 °C. The protein concentration was determined by the Bradford method. Ten μg protein were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluorid (PVDF) membrane. The membrane was blocked with 5% non-fat dried milk and incubated with specific antibodies against NS5B (1:5000; Abcam Cambridge, MA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-HO-1 (1:3000, Abcam Cambridge, MA, USA). Antibodies were diluted in 5% milk containing Tris-buffered saline (TBS) and 0.5% Tween. The blotting signal was developed using an ECL detection kit (PerkinElmer, Norwalk, CT, USA) and was counted by the software Quantity One (Bio-Rad, Foster, CA, USA).

4. Conclusions

We have synthesized and evaluated 3-amino-2-hydroxypropoxyisoflavone derivatives for their inhibitory activities of anti-HCV replication. These compounds exhibited better EC_{50} and SI values than ribavirin upon the antiviral experiment. Among them, 7-{3-[(3,4-dimethoxyphenethyl)amino]-2-hydroxypropoxy}-3-(3,4-dimethoxyphenyl)-4*H*-chromen-4-one (**6b**) exhibited the most potent activity against HCV replication. By the determination of antiviral mechanism, the results indicated that compounds **6b**, **6e**, **6h**, and **6i** reduced HCV replication through Nrf2-mediated HO-1 induction. Further studies on the structural optimization are ongoing.

Supplementary Materials: The supplementary materials are available online.

Author Contributions: J.-C.L. participated in the biological activity, the interpretation of the results and in manuscript writing; C.-K.L. and C.-K.T. participated in the biological activity; Y.-L.C. and C.-C.T. participated in synthesis; C.-H.T. participated in synthesis, purification and characterization of the chemical compounds and suggested the research idea, participated in the interpretation of the results and in manuscript writing.

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Sample Availability: Samples of the compounds reported herein are available from the authors.