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Bioactivity-guided screening identifies pheophytin a as a potent anti-hepatitis C virus compound from *Lonicera hypoglauca* Miq.

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ABSTRACT

Chronic hepatitis C virus (HCV) infection is a worldwide public issue. In this study, we performed bioactivity-guided screening of the *Lonicera hypoglauca* Miq. crude extracts to find for naturally chemical entities with anti-HCV activity. Pheophytin a was identified from the ethanol-soluble fraction of *L. hypoglauca* that elicited dose-dependent inhibition of HCV viral proteins and RNA expression in both replicon cells and cell culture infectious system. Computational modeling revealed that pheophytin a can bind to the active site of HCV-NS3, suggesting that NS3 is a potent molecular target of pheophytin a. Biochemical analysis further revealed that pheophytin a inhibited NS3 serine protease activity with $IC_{50} = 0.89\ \mu M$. Notably, pheophytin a and IFNα-2a elicited synergistic anti-HCV activity in replicon cells with no significant cytotoxicity. This study thereby demonstrates for the first time that pheophytin a is a potent HCV-NS3 protease inhibitor and offers insight for development of novel anti-HCV regimens.

Hepatitis C virus (HCV) belongs to a member of *Flaviviridae* and is a worldwide infectious pathogen causing chronic hepatitis that can progress further to hepatocellular carcinoma [1]. The current therapeutic protocol for HCV infection consists mainly of interferon (IFN) in combination with ribavirin that usually accompanies with strong side effects and moderate successful rate [2,3]. Hence, there is an urgent need to find new regimens to increase the efficacy of anti-HCV therapy.

Natural products metabolized from plants represent desirable sources for novel therapeutic compounds. Both *Lonicera hypoglauca* Miq. and *Lonicera japonica* Thunb are widely used as Jinyinhua in traditional Chinese medicine. Although they have similar geographic distribution, obviously various characteristics are observed [4]. Studies of the phytochemistry and bioactivity of Jinyinhua have mostly focused on *L. japonica* (Japanese honeysuckle) that has been reported to possess properties of anti-inflammation, anti-angiogenic, and anti-nociceptive activities [5,6]. However, the cognate *L. hypoglauca* has barely been studied.

Recently, the subgenomic HCV replicon cells have been developed for mechanistic study of HCV replication [7,8]. In the present study, HCV replicon cells were used to explore whether the extracts from *L. hypoglauca* elicit any anti-HCV activity. We found that *L. hypoglauca* contains an active phytocompound pheophytin a that exhibits strong anti-HCV activity. The inhibition of NS3 protease activity accounts mainly for the anti-HCV activity of pheophytin a. Furthermore, the combination of pheophytin a with INFα-2a elicits synergistic anti-HCV activity with no considerable cytotoxicity. The significance of these findings is discussed.

Materials and methods

Cell culture and viability assay. The subgenomic HCV replicon cells (a kind gift from Professor J.-H. James Ou, University of Southern California), the Huh7/Rep-Feo subgenomic replicon cells containing a luciferase reporter gene, and the Huh7.5 cells (a kind gift from Professor Charles Rice, The Rockefeller University, NY) were cultured as described [9–11]. Cell viability was determined

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by trypan blue exclusion and MTS assay as described by the manufacturer (Promega).

Replicon cell-based assay (Western blot, luciferase, and RT-PCR assay). For HCV replicon cell-based bioactivity-guided screening, the extracts or compounds isolated from *L. hypoglauca* were ectopically applied to the replicon cells for 48 h. The expression of HCV viral proteins was determined by Western blot analysis as described previously [12]. On the other hand, the Huh7/Rep-Feo cells (2×10^5 cells) were seeded in a 6-well plate. At 8 h after seeding, the tested compound was added and incubated for a total of 120 h. The cells were then subjected to luciferase activity assay using the Bright-Glo luciferase assay system (Promega). The IC_{50} was defined as the concentration of compound at which the luciferase activity in the replicon cells was reduced by 50%.

For real-time RT-PCR analysis, total RNA was amplified using the forward primer HCV-F (5′-TGCGGAACCGGTGAGTACA-3′) and the reverse primer HCV-R (5′-CTTAAGGTTTAGGATTCGTGCTCAT-3′) in the presence of SYBR Green I Master Mix (Applied Biosystems). For internal control, RT-PCR was performed using the forward primer β-actin-F (5′-TCACCCACACTGTGCCCATCTACG-3′) and the reverse primer β-actin-R (5′-CAGCGGAACCGCTCATTGCCAATG-3′). The reaction condition was 1 cycle of 48 °C for 30 min., 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min.

Infectious HCV particles production and infection inhibition assay. The production of infectious HCV particles (HCVcc) was performed using the plasmid PFL-J6/JFH (a kind gift from Professor Charles Rice, The Rockefeller University) as described [13]. For infection inhibition assay, 100 μl of HCVcc-containing supernatant (5×10^3 foci forming units) was added to Huh7.5 cells and incubated for 4 h. The virus-containing supernatant was then removed and fresh medium with or without the tested compound was added and incubated for a total of 72 h. The cells were fixed and stained by anti-Core antibody (Affinity BioReagents) and the infectious foci were counted by the fluorescence microscopy.

Extraction. Leaves and stems of *L. hypoglauca* were collected from the Da-kang area of Taichung County in central Taiwan. The species were identified and voucher specimens (*YHT001* (TCF)) were deposited at the Herbarium of the Department of Forestry, National Chung Hsing University, Taiwan. The preparation and purification of crude extracts were performed as mentioned (Supplemental methods).

Molecular modeling of the pheophytin a-HCV NS3/4A complexes. The model of pheophytin a in complex with the HCV-NS3/4A protease was constructed by docking pheophytin a to the crystallographic structure of 1b strain of the HCV-NS3/4A protease (PDB code 1DY8) [14]. Molecular modeling was performed as mentioned (Supplemental methods).

NS3 serine protease activity assay. The NS3 serine protease activity assay was conducted by the FRET-based, SensoLyte™ HCV protease assay kit (AnaSpec). Briefly, HCV-NS3/4A protease was mixed with the tested compound in the assay buffer. After 15 min incubation at room temperature, 50 μl of FRET peptide substrate solution was added and mixed well. For kinetic reading, the fluorescence intensity was measured immediately and continuously at Ex/Em = 490 nm/520 nm. The data was recorded every 5 min for a total of 120 min.

Synergy analysis. To determine whether the effect for the combination of pheophytin a with INFα-2a (Roche) was synergistic, additive or antagonistic, the luciferase-based HCV replicon assay was performed and analyzed according to the classical isobologram analysis [15].

Statistical analysis. The Student's *t* test was used to evaluate the difference between the test sample and solvent control. $p < 0.05$ was considered statistically significant.

Results

Lonicera hypoglauca exhibits potent anti-viral activity in HCV replicon cells

In this study, HCV replicon cells were used to perform bioactivity-guided screening to explore whether the extracts from *L. hypoglauca* elicit any anti-HCV activity. The replicon cells were treated with various concentrations of EtOH-soluble extract (LH-crude) from *L. hypoglauca*. LH-crude caused a dose-dependent inhibition of NS5A expression, a long half-life HCV protein, without affecting cell viability and cell growth (Fig. 1A). Subsequent tracking of LH-crude revealed that the ethyl acetate-soluble fraction (LH-EA) but not the H_2O-soluble fraction (LH-water) was most active (Fig. 1B).

To identify the active component in LH-EA with anti-HCV activity, LH-EA was separated into 20 fractions (LH-EA-1 to -20) by

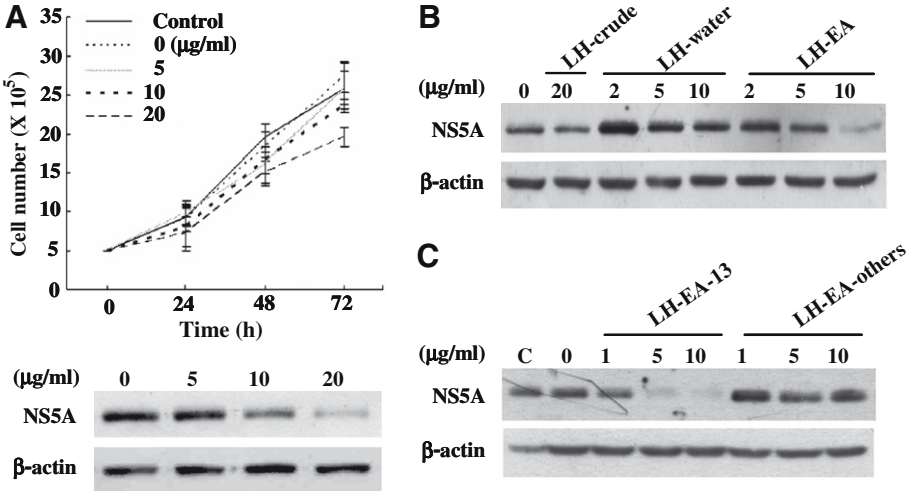

Fig. 1. *Lonicera hypoglauca* exhibits potent anti-viral activity in HCV replicon cells. (A) HCV replicon cells were treated with the indicated concentrations of EtOH extract (LH-crude) from *L. hypoglauca* and viable cells were determined by the trypan blue exclusion analysis. The data represented the means ± SD. ($n = 3$, upper panel). The cell lysates (48 h post-treatment) were subjected to Western blot analysis using the anti-NS5A antibody (lower panel). (B, C) HCV replicon cells were treated with the indicated concentrations of LH-crude, EtOAc-soluble fraction (LH-EA), H_2O-soluble fraction (LH-water), the fraction 13 of LH-EA (LH-EA-13), and the pool of other LH-EA fractions (LH-EA-others), respectively. The cell lysates (48 h post-treatment) were subjected to Western blot analysis using the anti-NS5A antibody. The expression of β-actin was used for the control of equal protein loading. C:No-treated, control replicon cells.

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chromatography. Each fraction was further evaluated for their anti-HCV activity using the same replicon cell-based assay. As shown in Fig. 1C, the fraction of LH-EA-13 exhibited the strongest anti-HCV activity.

Pheophytin a reduces HCV protein expression in replicon cells and the infectivity of HCVcc

To understand which compound exhibits anti-HCV activity, the LH-EA-13 fraction was purified by HPLC to obtain compound **1**. The compound **1** molecular formula was determined to be $C_{55}H_{74}N_4O_6$ (MW = 887.23) by fast atom bombardment mass spectrometry. The ^{1}H NMR spectrum of compound **1** presented 1.68 (t, J = 8 Hz, 3H), 1.79 (d, J = 7.2 Hz, 3H), 2.16 (m, 1H), 2.31 (m, 1H), 2.45 (m, 1H), 2.59 (m, 1H), 3.23 (s, 3H), 3.39 (s, 3H), 3.67 (s, 3H), 3.68 (q, J = 8 Hz, 2H), 3.85 (s, 3H), 4.19 (d, J = 9.2 Hz, 1H), 4.50 (dq, J = 7.2, 5.2 Hz, 1H), 6.17 (dd, J = 11.6, 1.6 Hz, 2H), 6.24 (s, 1H), 6.28 (dd, J = 18.0, 1.6 Hz, 2H), 7.99 (dd, J = 18.0, 11.6 Hz, 1H), 8.54 (s, 1H), 9.38 (s, 1H), and 9.51 (s, 1H). The ^{1}H NMR spectrum of compound **1** was identical to the spectrum of pheophytin a which has been identified by Ina and his coworker [16]. The structure of pheophytin a was shown in Fig. 2A. The purity of pheophytin a was estimated to be greater than 99.5% from the ^{1}H NMR spectrum and HPLC analysis.

We then used compound **1** (pheophytin a) to confirm its anti-HCV activity using HCV replicon cells and Huh7/Rep-Feo cells. Pheophytin a did not affect the cell viability of these cells (Fig. 2B left panel, Fig. 2C upper panel). However, it was more potent than the crude extracts in inhibiting NS5A expression in replicon cells (Fig. 2B right panel) and luciferase expression in Huh7/Rep-Feo cells (Fig. 2C lower panel). Pheophytin a also significantly inhibited replicon cells HCV RNA accumulation (Fig. 2D) and the infectivity of HCVcc (Fig. 2E, $p < 0.05$). The calculated IC_{50} was 4.97 μM. These data thereby unveil pheophytin a as the active component of *L. hypoglauca* with anti-HCV activity.

Computational molecular modeling reveals the interaction of pheophytin a with the active site of HCV-NS3 protease

NS3 protease is an attractive target for development of antiviral agent. To gain more chemical insight for the molecular mechanism involved in the anti-HCV activity of pheophytin a, computational molecular modeling was performed by docking pheophytin a onto the active site of HCV NS3/4A. The best predicted binding mode was illustrated in Fig. 3A and B. The amino acids HIS57, LYS136, SER139, and ALA155 were involved in the formation of four hydrogen bonding with pheophytin a. In addition, the amino acids SER42, GLY137, LYS136, VAL132, SER133, CYS159, PHE154,

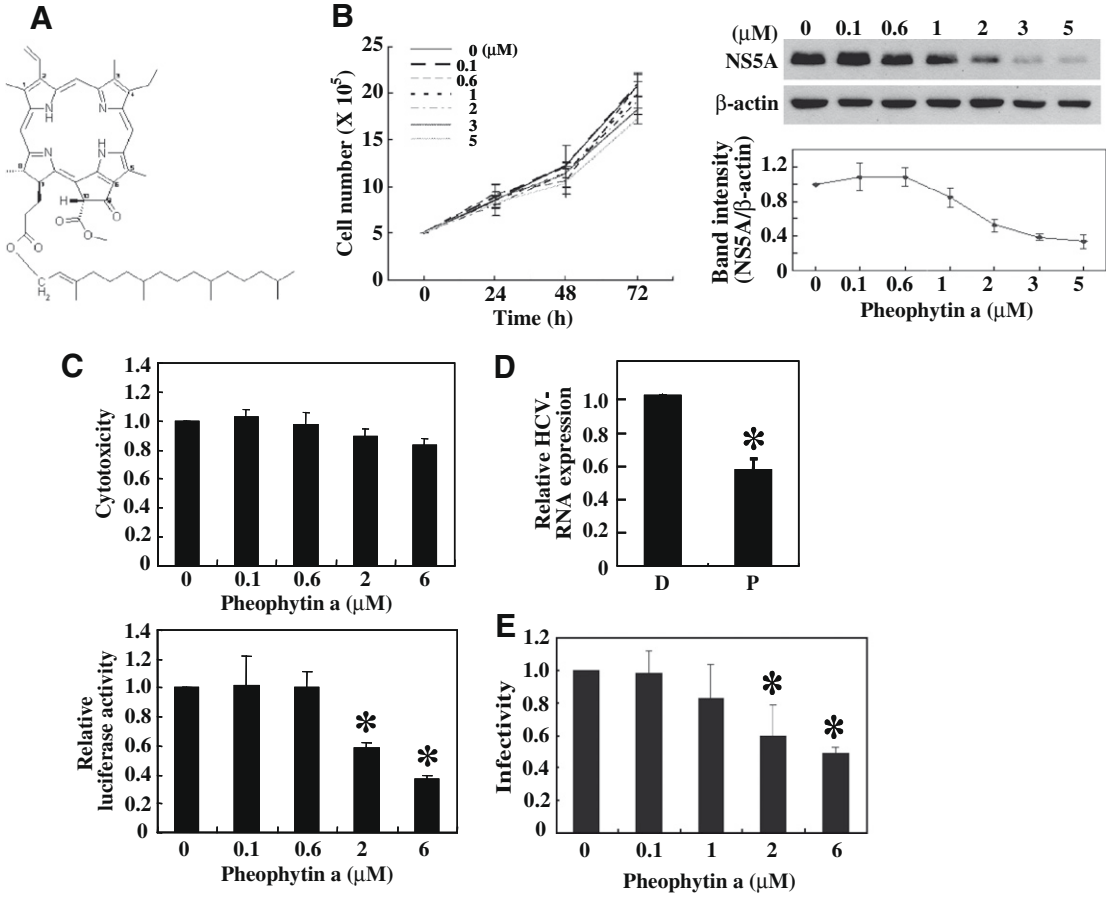

Fig. 2. Pheophytin a inhibits HCV expression in the subgenomic HCV replicon cells and HCVcc. (A) Chemical structure of pheophytin a. (B) HCV replicon cells were treated with the indicated concentrations of pheophytin a and the viable cells were determined by the trypan blue exclusion analysis (left panel). The cell lysates (48 h post-treatment) were subjected to Western blot analysis using the anti-NS5A antibody and the expression of β-actin was used for the control of equal protein loading (right panel). The band intensity of NS5A versus β-actin was determined and the ratios were plotted against the concentration of pheophytin a. (C) Huh7/Rep-Feo cells were treated with the indicated concentrations of pheophytin a. The cell toxicity was determined by MTS assay (upper panel) and the HCV gene expression was determined by luciferase activity assay (lower panel). (D) HCV replicon cells were treated with 6 μM of pheophytin a for 96 h and the total RNA was subjected to real-time RT-PCR assay. The relative HCV-RNA level for pheophytin a- (P) and DMSO-treated (D) control cells was shown. (E) The HCVcc-infected Huh7.5 cells were treated with various concentration of pheophytin a. The cells were stained by the anti-Core antibody and the relative infectivity for the indicated treatment compared to the control treatment cells was shown. * $p < 0.05$ when compared to the control replicon cells. The data represented the mean ± SD (n = 3).

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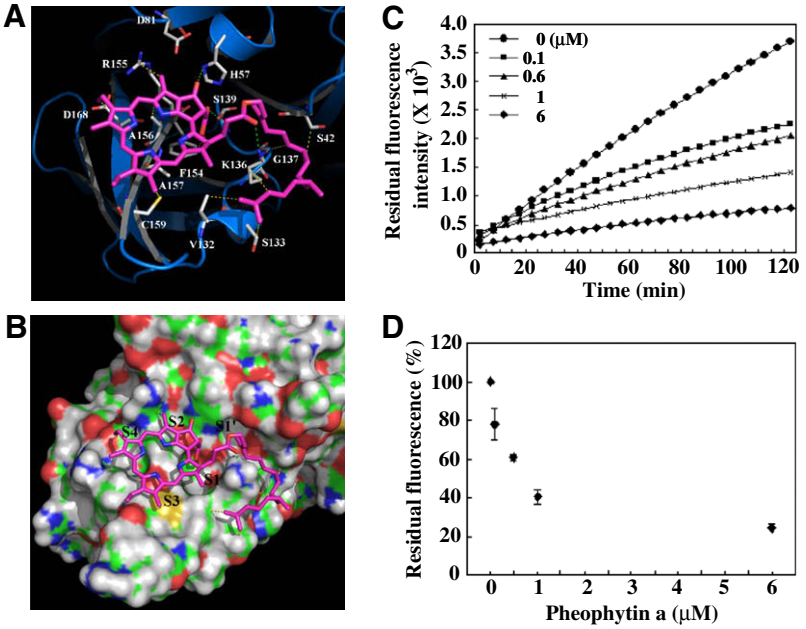

Fig. 3. Computational molecular models and molecular analysis for the interaction between pheophytin a and HCV NS3/4A. (A, B) The computational molecular models of pheophytin a in the active site of the HCV NS3/4A were shown as cartoon and surface in (A) and (B), respectively. The carbon atoms in pheophytin a were colored in magenta. The side-chains of the NS3 amino acid residues within 5 Å radius centered on pheophytin a were shown explicitly. The carbon atoms in NS3 were shown in gray. Nitrogen and oxygen were colored in blue and red, respectively. Trace of the NS3 backbone structure was shown as a blue tube. The secondary structure elements were shown as a ribbon drawing and the important amino acid residues involved in pheophytin a binding were labeled. The green and yellow dotted lines represented tentative donor–acceptor pairs of the hydrogen bonds and the hydrophobic interactions between pheophytin a and NS3, respectively. (C, D) HCV-NS3/4A protease (5 ng/well) was mixed with the indicated concentrations of pheophytin a and the *in vitro* protease activity was determined as described in "Materials and methods". The fluorescence signal was recorded every 5 min (C). The percentage of residual activity at 60 min after initiation of reaction was calculated and was plotted against the concentration of pheophytin a (D). The data represented the means ± SD ($n = 3$).

ALA156, ASP168, and ARG155 also contributed to the hydrophobic interactions and binding with pheophytin a. An important noncovalent hydrogen bond interaction was formed between the hydroxyl group of SER139 of the NS3 catalytic triad and the acyl oxygen in the ester group of pheophytin a. Furthermore, the S1, S2, S3, and S4 sites of NS3 were probably occupied by the porphin group of pheophytin a, and the S′ sites other than S1′ were possibly occupied by a phytol group of pheothytin a. The particular hydrophobic interaction was formed between a phytol group of pheophytin a and a long nonpolar chain of four carbon atoms of LYS136. These data suggest that pheophytin a may have a functional interplay with HCV-NS3.

Pheophytin a elicits its anti-HCV effect by inhibition of NS3 serine protease activity

To further delineate the effect of pheophytin a on HCV-NS3, the NS3 serine protease activity assay was performed in the presence of pheophytin a. The amount of fluorescence signal was decreased with increasing concentration of pheophytin a in a dose-dependent manner (IC_{50} = 0.89 μM), indicating that pheophytin a exhibits the anti-NS3 serine protease activity (Fig. 3C). Plotting the residual protease activity of reactions containing various concentrations of pheophytin a as percentage of activity in the absence of the compound (expressed as 100%) resulted in a titration curve typical of a binding inhibitor (Fig. 3D).

Combination of pheophytin a with INFα-2a significantly enhances anti-HCV activity without an increase in cytotoxicity

To determine whether pheophytin a and INFα-2a have synergistic inhibitory effect on HCV gene expression, the classic isobologram analysis was performed. A typical isobologram used to measure the drug–drug interaction was shown in Fig. 4A with synergy, additivity, and antagonism presented as concave, linear and convex isoeffective curves, respectively [17]. Our results demonstrated that the curve was below the line representing additive effect, indicating the synergy of the two drugs against the replicon cells (Fig. 4B). In addition, MTS assays did not show any difference in cell survival with the drug concentrations used in this isobologram analysis (data not shown), suggesting that the synergistic effect of pheophytin a and INFα-2a on HCV gene expression is not due to cytotoxicity.

Discussion

The global prevalence of HCV infection averages 3% according to the estimation made by the World Health Organization. Through bioactivity-guided screening, structure–activity relationship, and biochemical analysis, we report herein that HCV-NS3 is a potent molecular target of *L. hypoglauca*-derived pheophytin a. As a result, the viral proteins and RNA expression and the HCV infectivity are diminished. Notably, concomitant treatment of HCV replicon cells with pheophytin a and INFα-2a elicits synergistic effect and enhances anti-HCV activity without compensation of cell survival. This study thereby offers insight to the molecular basis for the anti-HCV activity of *L. hypoglauca* and indicates pheophytin a as a potent adjuvant regimen for INFα-2a therapy in the clinical setting.

Among the HCV nonstructural proteins, NS3-mediated processing of the protein junctions is essential for viral replication and therefore provides an attractive target for development of antiviral agents [18]. In addition to pheophytin a, several studies also discovered natural products with anti-NS3 protease activity. These include nature compounds from Galla Chinese and *Rhodiola kirilowii*

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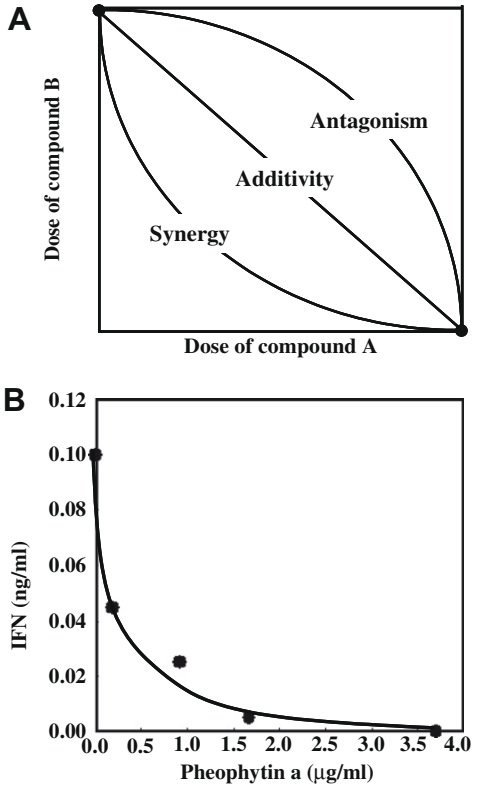

Fig. 4. Pheophytin a and IFNα-2a elicit synergistic inhibitory effect on HCV gene expression. (A) Typical isobologram for analyzing the interaction between two drugs. (B) The isobologram for the combined uses of pheophytin a and IFNα-2a. Luciferase-based replicon cell assays were performed under the indicated treatments. The fixed ratios adjusted by the IC_{50} (FICs) at 50% inhibition were calculated and the FICs for pheophytin a and IFNα-2a were plotted on the *X*-axis and *Y*-axis, respectively.

[19,20]. The potency of pheophytin a appears to be comparable with these compounds and thus pheophytin a is added to a list of natural compounds with potent anti-HCV activity. Nevertheless, pheophytin a represents the only natural compound with the anti-HCV activity directly evaluated by the subgenomic replicon (genotype 1b) and the newly developed HCVcc system.

The mechanism underlying the anti-HCV activity of pheophytin a is also addressed in this study. Laboratory evidence suggests that inhibition of NS3 protease activity accounts mainly for the action of pheophytin a. At first, computational prediction modeling indicates pheophytin a can interact with the active site of NS3 protease. The *in vitro* protease activity assay further demonstrates that pheophytin a is a NS3 protease inhibitor. Notably, using HCV replicon cells for selection of pheophytin a-resistant strains revealed various amino acid substitutions in the NS3 protein coding region (unpublished data). One of the resistant strains carries an NS3 V36A substitution. Similar V36 variant has been found in the telaprevir-treated patient and was thought to loosen binding to Phe^{43} which is part of the S′ substrate-binding pocket of the HCV protease [21]. Hence, inhibition of NS3 activity is pivotal for pheophytin a to elicit its anti-HCV activity.

Recently, natural prevalence of HCV variants with decreased sensitivity to the current use of NS3 protease inhibitors have been found in treatment-naive subjects [22]. For instances, the R155K mutant shows reduced susceptibility to the three protease inhibitors, BILN-2061, ITMN-191 and VX-950 [23]. This raises concern over the potential emergence of these variants as the multidrug-resistant, highly fit mutants in HCV patients treated with protease inhibitors. In contrast to the pure protease inhibitors, pheophytin a not only inhibits NS3 protease activity but also elicits various biological and cellular effects such as the anti-inflammatory activity and the activation of mitogen-activated protein kinase signaling [16,24]. The difference modes of action for pheophytin a may thereby offer advantages in overcoming the drug-resistant variants that is worthy to further investigation.

In conclusion, we demonstrate herein that pheophytin a derived from the extracts of *L. hypoglauca* represents a novel natural compound with strong anti-HCV-NS3 protease activity and little cytotoxicity. This study thereby contributes to our understanding for the anti-HCV activity of pheophytin a and offers new insight for development of novel therapeutic agents.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.043.

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