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

Sheng-Yang Wang a,1, Ching-Ping Tseng b,1, Keng-Chang Tsai c,2, Chia-Fan Lin d,2, Ching-Ya Wen d, Hsin-Sheng Tsay e, Naoya Sakamoto f, Chin-Hsiao Tseng g, h, Ju-Chien Cheng d, i

a Department of Forestry, National Chung Hsing University, Taichung 402, Taiwan, ROC  
b Graduate Institute of Medical Biotechnology, Chung Gang University, Taoyuan 333, Taiwan, ROC  
c The Genomics Research Center, Academia Sinica, Taipei 115, Taiwan, ROC  
d Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung 404, Taiwan, ROC  
e Graduate Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Taichung 413, Taiwan, ROC  
f Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan  
g Department of Internal Medicine, National Taiwan University Hospital and College of Medicine, Taipei 100, Taiwan, ROC  
h Graduated Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Taichung 413, Taiwan, ROC  
i Department of Medical Research and Development, National Taiwan University Hospital Yun-Lin Branch, Yun-Lin, Taiwan, ROC

**A B S T R A C T**

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 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 IC50 = 0.89 μM. Notably, pheophytin a and IFN-α-2a elicited synergistic anti-HCV activity in replicon cells with no significant cytoxicity. 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 INF-α-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

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.05.043

Please cite this article in press as: S.-Y. Wang et al., Bioactivity-guided screening identifies pheophytin a as a potent anti-hepatitis C virus compound from *Lonicera hypoglauca* Miq., Biochem. Biophys. Res. Commun. (2009), doi:10.1016/j.bbrc.2009.05.043
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 etopically 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 IC50 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′-TGCGAAACCGGTAGTACA-3′) and the reverse primer HCV-R (5′-CTAAGGTTAGCTGCTCAT-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′-TCACCAACTGTGCCCATCTAG-3′) and the reverse primer β-actin-R (5′-CAGCCGAACCGCTCATTGCCAATG-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 × 10^5 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 α-HCV NS3/4A complexes. The model of pheophytin α in complex with the HCV-NS3/4A protease was constructed by docking pheophytin α 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).

NS5 serine protease activity assay. The NS5 serine protease activity assay was conducted by the FRET-based, Sensolyte® HCV protease assay kit (AnaSpec). Briefly, HCV-NS5/4A protease was mixed with the tested compound in the assay buffer. After 15 min incubation at room temperature, 50 μl of FRET peptidic 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 α with INF-α-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 H2O-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
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.

**Phytochytin 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_{4}O_{6} (MW = 887.23) by fast atom bombardment mass spectrometry. The 1H 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 1H NMR spectrum of compound 1 was identical to the spectrum of phytochytin a which has been identified by Ina and his coworker [16]. The structure of phytochytin a was shown in Fig. 2A. The purity of phytochytin a was estimated to be greater than 99.5% from the 1H NMR spectrum and HPLC analysis.

We then used compound 1 (phytochytin a) to confirm its anti-HCV activity using HCV replicon cells and Huh7/Rep-Feo cells. Phytochytin 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). Phytochytin a also significantly inhibited replicon cells HCV RNA accumulation (Fig. 2D) and the infectivity of HCVcc (Fig. 2E, p < 0.05). The calculated IC50 was 4.97 μM. These data thereby unveil phytochytin a as the active component of Lonicera hypoglauca with anti-HCV activity.

**Computational molecular modeling reveals the interaction of phytochytin 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 phytochytin a, computational molecular modeling was performed by docking phytochytin 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 SER155 were involved in the formation of four hydrogen bonding with phytochytin a. In addition, the amino acids SER42, GLY137, lys136, VAL132, SER133, CYS159, PHE154, SER133, and ALA155 were involved in the active site of HCV NS3 protease.

![Fig. 2. Phytochytin a inhibits HCV expression in the subgenomic HCV replicon cells and HCVcc.](image-url)

Please cite this article in press as: S.-Y. Wang et al., Bioactivity-guided screening identifies phytochytin a as a potent anti-hepatitis C virus compound from Lonicera hypoglauca Miq., Biochem. Biophys. Res. Commun. (2009), doi:10.1016/j.bbrc.2009.05.043
Combination of pheophytin a with INF-2a significantly enhances anti-HCV activity without an increase in cytotoxicity.

To determine whether pheophytin a and INF-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 INF-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 INF-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 INF-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...
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 phloretin a may thereby offer advantages in overcoming the drug-resistant variants that is worthy to further investigation.

In conclusion, we demonstrate herein that phloretin 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 phloretin a and offers new insight for development of novel therapeutic agents.

Acknowledgments

This work was supported by Grants 95-AS-6.21-ST-a1(21) and CMU95-335 to J.C.C., NSC97-2317-B-005-007 to S.Y.W., and EMRPD180171 to C.P.T. The SYBLYL computation was conducted at the National Center for High Performance Computing, Taiwan. The GOLD 3.2 computation was conducted at the Genomics Research Center of Academia Sinica.

Appendix A. Supplementary data

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

References


Please cite this article in press as: S.-Y. Wang et al., Bioactivity-guided screening identifies phloretin a as a potent anti-hepatitis C virus compound from Lonicera hypoglauca Miq., Biochem. Biophys. Res. Commun. (2009), doi:10.1016/j.bbrc.2009.05.043.


