Elucidation of the Inhibitory Mechanisms of *Nipponoparmelia laevior* Lichen Extract against Influenza A (H1N1) Virus through Proteomic Analyses

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**Introduction**

The influenza virus genome is segmented, permitting recombination by reassortment, during which the virus repeatedly switches hosts to infect multiple avian and mammalian species, including humans [1]. Among 4 types (A, B, C, and D) of seasonal influenza viruses, only influenza A virus (IAV) is considered the most common among infectious respiratory diseases, causing epidemics and pandemics globally [1–3]. According to a World Health Organization report, IAV seasonal epidemics result in about 3 to 5 million serious human illnesses, leading to an estimated 290,000 to 650,000 deaths annually worldwide [4]. For the control and treatment of influenza viruses, several antiviral drugs, including amantadine and rimantadine (Matrix-2 protein inhibitors), oseltamivir, zanamivir, peramivir, and laninamivir octanoate (Neuraminidase inhibitors), and favipiravir (T-705, Viral polymerase inhibitor), have been developed and commercialized [5, 6]. However, no current medication can efficiently prevent...
IAV [6]; thus, there has been extensive interest in developing safe, effective and selective antiviral drugs against IAV [5–7].

Lichens are stable composite organisms, formed through the symbiosis of mycobionts (normally a fungus) and photobionts (algae or cyanobacteria), and sometimes, with other microorganisms [8, 9]. Lichens are highly diverse, ranging from 18,500 to 20,000 species, and produce >1,000 unique secondary metabolites [10–12], reported to have a number of biological functions, primarily including anticancer [13, 14], antibacterial [15, 16], antioxidant [17, 18], antiviral [19–22], and anti-inflammatory activities [23, 24]. So far, lichen extracts have been explored for activity against various viruses, such as herpes simplex type 1 virus and polio virus type 1 [19], respiratory syncytial virus [20], and hepatitis C virus [22]. Recently, usnic acid, a novel compound from lichens, showed antiviral activity against influenza viruses in vitro and in vivo [25–27]. Several active molecules present in lichens have been investigated; however, the antiviral mechanisms of lichen secondary metabolites against influenza A virus have not been elucidated. Therefore, we evaluated the antiviral activity of the molecules isolated from different lichens found in South Korea and investigated the underlying mechanisms of the possible antiviral effect of the lichen, *Nipponoparmelia laevis* (LC24), against influenza A (H1N1) virus infection in MDCK cells through proteomic analysis.

Materials and Methods

Extraction and HPLC Analysis of the Lichen Substances

Thirty-two species of natural lichens were collected from different sources in the provinces of South Korea (Table S1). The lichen species were identified by the Korean Lichen Research Institute (Sunchon National University, Korea). All samples were ground to fine powder in a mortar. The dried powder was weighed and extracted using methanol and acetone solvents in a Soxhlet apparatus. The extracts were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extract powders were stored at −20°C until further use. Phytochemical analysis of acetone (AcOH) and methanol (MeOH) lichen extracts was performed using an HPLC–UV (SHIMADZU, LC-20A) system as described previously [14].

Cells and Virus

Madin–Darby canine kidney (MDCK) cells were purchased from the Korean Cell Line Bank (Korea; KCBL150034). The cells were grown using a standard method. Low pathogenic human IAV H1N1 strain A/PR/8/34 was obtained from American Type Culture Collection (ATCC VR-1469, USA) and propagated in MDCK according to a protocol described by Vaidya et al. [28].

Cytotoxicity of Lichen Extracts

The cytotoxicity test was performed in MDCK cells using the cell counting kit–8 assay (CCK–8, Dojindo Molecular Technologies, Japan). The compounds extracted from lichens were freshly dissolved in 10% DMSO (Sigma-Aldrich) at a concentration of 10 mg/ml, and two-fold serial dilutions with Dulbecco’s modified Eagle’s medium (DMEM, Welgene, Korea) to obtain the desired concentrations, ranging from 6.25–200 μg/ml. First, MDCK cells were seeded at a density of 2 × 10^4 cells/well in 96–well plates and incubated for 24 h with the standard method. The MDCK cells were then treated with the given dilutions of lichen extracts and incubated for 48 h. Then, the CCK–8 assay was performed according to the manufacturer’s instructions and cytotoxicity was calculated as the half-maximal cytotoxic concentration (CC50) based on nonlinear concentration-response curves using GraphPad Prism (version 5.01, GraphPad Software Inc., USA).

Antiviral Activity of Lichen Compounds against IAV

To measure the antiviral effect of the lichen extracts against IAV infection, MDCK cells were seeded at a density of 2 × 10^4 cells/well in 96–well plates and cultured using the standard method. After the cell monolayer formation, DMEM containing different lichen extracts, at desired concentrations, was used to treat the MDCK cells for 24 h, after which the influenza A virus (H1N1) was infected (MOI = 0.1). The desired DMSO was used as a mock control, and IAV–infected cells were further incubated for 48 h. By 48 h post infection (hpi), when the virus-induced cytopathic effect was observed, the CCK–8 assay was performed according to the manufacturer’s instructions and antiviral activity was calculated as the half–maximal effective concentration (EC50).

Evaluation of Apoptosis Effect

The apoptosis assay was performed in the Muse Cell Analyzer (Merck Millipore, KGaA, Germany) according to the manufacturer’s protocol. MDCK cells were treated with LC24 extract for 24 h and then infected with IAV for 48 h. The MDCK cells were then collected using trypsin–EDTA and stained with Annexin V and Dead Cell Reagent (7-aminoactinomycin D, 7-AAD, Merck Millipore, Germany) according to the manufacturer’s instructions. The apoptosis assay was performed in the Muse Cell Analyzer (Merck Millipore, KGaA, Germany) according to the manufacturer’s protocol.

Quantitative Real-Time (qRT–) PCR of IAV PA–Gene and Selected Genes

At 48 hpi, the cell culture supernatants were collected, and total MDCK cell RNA was extracted using RNAiso plus reagent (Takara Bio, Japan), according to the manufacturer’s instruction. Then, cDNA was synthesized using 10 μg of aliquoted RNA in a reaction mixture containing 1 μl random primer, 2 μl dNTPs, 0.5 μl recombinant RNase inhibitor (Takara Bio), 1 μl MMLV–RT, 2 μl 5x DTT, and 4 μl 5x MMLV–RT buffer (Beams Biotechnology, Korea) on a Takara PCR Thermal Cycler Dice Real-Time System under the following conditions: 67°C for 10 min, 37°C for 60 min,
and 95°C for 5 min. After cDNA synthesis, the qRT-PCR was conducted in a Thermal Cycler Dice Real-Time System using SYBR Premix Ex Taq Kit (Takara Bio). The IAV H1N1 primer sequences were forward 5'-CGTCCTAAATTCCCTGCTGA-3' and reverse 5'-CATTTGCTCCTCCCCATCCA-3'. For the relative quantification of selected genes, the RNA of MDCK cells was extracted using RNAiso plus reagent (Takara Bio), according to the manufacturer’s instruction. The cDNA was synthesized; then, qRT-PCR was performed using the specific primers listed in Table S2. The 18S rRNA primer sequence used for normalization was forward 5'-TGGCAAGTGGGACTTAATC-3' and reverse 5'-ATCGCTTGCCTTT-3'. The relative mRNA expression was calculated using the comparative 2−ΔΔCt method.

Protein Isolation, Cytosol Protein Extract, and Tube-Gel Digestion

MDCK cells of sample groups (Mock: M; LC24–treated and no IAV: LCM; LC24–treated and IAV–infected: LCV; IAV–infected: V) were rinsed and collected for protein extraction using Pierce RIPA lysis buffer (Thermo Scientific, USA), containing 1.0% of Halt proteases inhibitor cocktail 100X (Thermo Scientific, USA), 1.0% phosphatase inhibitor cocktail (Sigma, USA), and 1.0% EDTA 0.5 M (Thermo Scientific). Next, the isolated proteins were then digested using the protocol called “Tube–Gel digestion” as described previously [29].

Nano UPLC-HDMS² Analysis, Protein Identification and Relative Quantification

The tryptic peptide mixtures were separated using a nano–ACQUITY UPLC equipped with a Synapt G2–Si HDMS² (Waters Corp., USA), which was operated in a data–independent manner, coupled with ion mobility [30]. The detailed protocol has been described previously [28]. Proteins were then identified and quantified using the program Progenesis QI for Proteomics (QIP) version 2.0 (Nonlinear Dynamics, UK) [31]. Lists of proteins were identified using the Canis lupus familiaris UniProt database. The criteria for protein identification were set as follows: ≥ 3 fragments per peptide, ≥ 7 fragments per protein, and ≥ 1 peptide per protein.

Bioinformatics and Network Analyses

The up- and down-regulated proteins in MDCK cells of each sample set were collectively used for biological functions, canonical pathways, and interaction networks analysis. Only proteins containing at least one unique peptide (the peptide which has not been assigned to other proteins) were considered as valid identification. To improve the visualization of quantitation, hierarchical clustering of identified proteins was performed based on the pattern of their expression ratio (log base 2), and a heatmap of significantly identified proteins was generated, based on their expression. K–means clustering was performed using the Multi Experiment Viewer (MeV, v4.9.0) software (http://mev.tm4.org/) [32]. Ingenuity Pathway Analysis (IPA, Qiagen, version 44691306, http://www.ingenuity.com) was used to identify biological functions, the canonical pathways of identified proteins from proteome data related to IAV infection.

Statistical Analysis

All experimental samples were performed in triplicates. The statistical difference between groups was considered to be significant when p < 0.05 after Tukey’s test post hoc analysis and one–way ANOVA using IBM SPSS Statistics software (version 22.0; IBM Corp., USA).

Results and Discussion

Cytotoxicity, Antiviral and Apoptotic Effect of Nipponoparmelia laevior Treatment

After cytotoxicity and antiviral activity screening, the methanolic (MeOH) extract of Nipponoparmelia laevior (LC24), which showed the lowest toxicity and the most potent antiviral activity against influenza A virus (Figs. 1A–1C), was selected. The extract did not show any toxicity at the tested concentration (Fig. 1A); however, several AcOH lichen extracts possessed high toxicity in MDCK cells, such as Cladonia dehiscens (CC50 = 63.3 μg/ml), Myelochroa entotheiochroa (CC50 = 71.6 μg/ml), Ramalina sp. 1 (CC50 = 136.1 μg/ml), Cladonia squamosa (CC50 = 139.9 μg/ml), Physconia hokkaidensis (CC50 = 142.2 μg/ml), and Ramalina litoralis (CC50 = 167 μg/ml) (Table S1).

The predominant metabolite of the LC24 MeOH extract was 2-O-methylrhizonic acid (Fig. S1A). However, the major metabolites of the AcOH extract of this lichen were salazinic acid, atranorin and chloroatranorin (Fig. SIB). Besides, the HPLC results of the AcOH and MeOH extracts of 32 lichen samples revealed that the phytochemicals differ by lichen species and the solvents used (Table S2). Several secondary metabolites, such as usnic, fumarprotocetraric, salazinic, norstictic, evernic, homosekikaic, lecanoric, gyrophoric, 2-O-methylrhizonic acid, and stictic acids, and atranorin, were found in common across species.

However, to the best of our knowledge, there is no study on the antiviral activity of 2-O-methylrhizonic acid as a major substance from Nipponoparmelia laevior against IAV.

In addition, based on the CCK–8 assay results (Fig. 1B), the relative cell viability of IAV–infected cells was obviously reduced to 58% compared to the mock cells. Notably, LC24 treatment significantly increased the relative cell viability of the IAV–infected cells, up to 93.3%, as relative quantification of the mock cells. After 48 h, IAV–infected cells highly induced cytopathic effect (CPE) (Figs. S2A–S2C), but LC24–treated cells greatly reduced CPE in comparison. This result indicates that LC24 extract has a...
strong ability to inhibit IAV replication in MDCK cells with almost no virus–induced CPE observed.

To confirm the inhibitory potential of lichen treatment, we measured the relative expression level of viral mRNA PA-gene in IAV–infected cells treated with LC24 extract (Fig. 1C). Consistent with the CCK–8 assay results observed, the relative mRNA expression of the IAV PA–gene was highest in the IAV–infected group, while in comparison, LC24 highly influenced the expression level of IAV PA–gene at 48 hpi. In fact, the lichen treatment reduced ~ 3.2 log-fold viral genome expression. Notably, the insignificant difference between the viral mRNA expression levels of LC24–treated and IAV–infected cells and the mock sample indicates that LC24 can highly inhibit viral replication. The IAV polymerase acidic protein is a component of the viral RNA polymerase complex (PB1, PB2, and PA) [33], which plays crucial roles in the transcription and replication of influenza A virus [2, 6, 33]. Viral RNA–polymerase is highly conserved among different influenza virus strains, for example, the PA domain is similar among different subtypes of influenza viruses A, B and C [6]. Thus, it is an attractive target for developing new anti-viral drugs [5, 6].

In order to investigate the effect of LC24 treatment on the IAV infection–induced apoptosis in MDCK cell, the total apoptotic cells were quantified. As shown in Fig. 1B and Figs. S2D–S2F, the percentage of live cells in the IAV–infected sample (49.3%) was significantly lower than in the LC24–treated sample (77.9%), but the difference between the LC24–treated sample and the mock sample (72.9%) was not significant. The percentage of early apoptotic and late

**Fig. 1.** Effects of *Nipponoparmelia laevior* (LC24) treatment and its antiviral activity against IAV in MDCK cells. (A) Cytotoxicity of methanolic extract of LC24 in MDCK cells; (B) The antiviral and apoptotic effects of LC24 extract on IAV–infected MDCK cells; (C) Effect of LC24 extract treatment on the relative mRNA expression of IAV–PA gene at 48 hpi. Asterisks (*) represent means that are significantly different (NS, no significant difference, **p < 0.01 ***p < 0.005).
apoptotic/dead cells in the IAV–infected sample (22.6% and 19.7%, respectively) was remarkably higher than in the LC24–treated sample (10.4% and 8.7%, respectively). Additionally, apoptosis in LC24–treated cells was not significantly different from that in the mock sample. This result suggests that LC24 treatment promoted the protective effect because it produced lower apoptosis during IAV infection compared to untreated IAV–infected cells. Our observations are consistent with previous studies, which have demonstrated that several lichen metabolites, such as fumarprotocetraric, usnic, and evernic acids, promoted protective effects, and diminished apoptosis in vitro [34, 35].

Effect of *Nipponoparmelia laevior* Extract on the Relative mRNA Expression

Upon IAV infection, several genes are involved in various stages of viral life cycle such as attachment/entry, endocytosis, import/export, and translation, which are critical requirements to establish infection. In the current study, the selection of genes regulating IAV infection was based on previous studies. Amongst heat shock proteins (HSPA5, HSPA8 and HSP90), annexin 1 (ANXA1) and annexin 2 (ANXA2) were already identified in our previous study [28]. Other genes including AKT serine/threonine kinase 1 (AKT1) and hypoxia-inducible factor-alpha 1 (HIF-1α) were also reported to be associated with IAV infection [36, 37]. In order to verify the inhibitory effect of LC24 against IAV, we further measured the expression of the genes using qRT–PCR. As shown in Fig. 2A, heat shock proteins (HSPA4, HSPA5, and HSPA8) were highly upregulated in response to IAV infection. As expected, the LC24 extract highly inhibited the expression of these proteins and reduced viral replication in MDCK cells. The roles of HSPs, the stress–induced genes [38], in virus replication have been discussed in several studies. For example, Kajihara et al. explained that HSP70 was able to modulate the influenza A virus by increasing viral polymerase activity via interaction with PB2 or PB1 monomers and PB2/PB1 heterodimers [39]. HSPs were also considered biomarkers of influenza virus infection, which contribute to inducing nuclear import, nuclear export, and assembly of IAV proteins during viral replication [28]. In addition, Batra et al. reported that HSP40 was also found to be crucial for IAV viral ribonucleoprotein nuclear translocation [40]. Hence, our results suggest that LC24 treatment highly promoted IAV inhibition by inhibiting HSP expression. This can become a potential drug target for inhibiting viral replication.

On the other hand, based on the results in Fig. 2B, IAV significantly induced the expression of two annexin proteins (ANXA1, ANXA2), which gradually decreased with lichen treatment. Annexins (also called lipocortins) are calcium-regulated phospholipid-binding proteins that play a crucial role in the cell cycle, exocytosis, and apoptosis [41]. Consistent with our findings, previous studies found that two annexin proteins (ANXA1, ANXA2) were differentially expressed following IAV infection. Annexin-A1 (ANXA1) enhanced viral replication through various mechanisms in both early and late stages of infection [42], while Annexin-A2 (ANXA2) affected the late stage as it contributed to the translation of the viral protein [43]. In fact, Arora et al. found that ANXA1 plays multiple roles, such as influencing virus binding, inducing IAV-mediated apoptosis, enhancing endosomal trafficking, and improving nuclear accumulation during IAV infection in vivo and in vitro [42]. LeBouder et al. reported that the cellular protein, ANXA2, could bind plasminogen and supports haemagglutinin (HA) cleavage, which is crucial for IAV replication [44].
Furthermore, the current results show that mRNA expression of AKT1 and HIF-1α greatly increased with IAV infection (Fig. 2B), but were highly suppressed with LC24 extract treatment. This may be considered as a critical point in IAV infection prevention. In agreement with our results, Denisova et al. reported that the Akt inhibitor MK2206 could block the influenza H1N1 virus infection in vitro [36]. Similarly, earlier findings demonstrated a similar mechanism for the compound, Ko-Ken Tang, which was able to inhibit influenza virus replication by down-regulating the Akt phosphorylation [45]. It has also been demonstrated that the HIF-1α pathway is activated by pathogen infections through various mechanisms [46], such as the promotion of the secretion of proinflammatory cytokines to inhibit IAV H1N1 infection [37] and the reprogramming of host cellular glucose metabolism towards enhanced glycolysis to support viral replication [47].

Identification of Antiviral Biomarkers through Proteomics

Proteomic analysis was performed to elucidate the biomarkers involved in the antiviral mechanisms of the LC24 extract against IAV infection in MDCK cells. We identified 287 proteins in MDCK cells from all four samples (Mock: M; IAV–infected: V; Lichen-treated + Mock: LCM; Lichen-treated and IAV–infected: LCV). Then, we classified these identified proteins based on the biological functions affected by LC24 treatment, using IPA. Results showed that the top canonical pathways of identified proteins consist of EIF2 signaling, glucocorticoid receptor signaling, 14-3-3-mediated signaling, and unfolded protein response (Table 1). Previous study has reported that the EIF2 signaling pathway is vital for anti-viral responses via pro-inflammatory cytokine regulation [48]. Importantly, among the identified molecules of canonical pathways, several HSPs (HSPA2, HSPA4, HSPA5, HSPA8 and HSP90B1) were found as the common proteins.

We then attempted to compare the proteins’ log2 expression ratios (V/M and LCV/V), which would allow us to explore the antiviral effects of lichens against influenza via upregulation (log ratio > 0) or downregulation (log ratio < 0) of the identified proteins, 214 of which were mapped into human genes by IPA (Table S3). A total of 49 and 136 proteins were found significant in the V/M and LCV/V groups, respectively (p < 0.05); however, there were only 34 common proteins between V/M and LCV/V groups, respectively (p < 0.05); however, there were only 34 common proteins between V/M and LCV/V (Fig. 3A). These 34 proteins were converted into human genes, and 24 proteins were shown to correspond to the same human gene. The expression pattern heatmaps for these proteins, grouped as V/M and LCV/V are shown in Fig. 3B. As shown, all 24 proteins were either significantly
up- or down-regulated between V/M and LCV/V, deemed to be potential biomarkers for the antiviral effect of lichen against IAV infection. IPA also showed the results in relation to diseases and functions of these 24 proteins (Table S4). As shown, the three major categories most related to viral infection and antiviral activity are listed, including survival, infectious diseases, and immune systems. Remarkably, among these proteins, ANXA1 and MX1 were the major regulating factors that were highly influenced by LC24 treatment, and involved in several biofunctions and diseases, indicating that they may play a crucial role in the antiviral mechanism against IAV.

In order to understand how LC24 treatment supported a reduction in viral replication in MDCK cells, we attempted to identify which proteins played roles in virus replication. Based on the IPA result in Fig. S3A, a total of 65 proteins were strongly associated with RNA viral replication/infection. However, only six proteins (MX1, HNRNPH1, HNRNPDL, ANXA1, PDIA3, and VCP) were found in common between Fig. S3A and Fig. 3B, which were significantly regulated between V/M and LCV/V. Thus, these may be potential targets for IAV inhibition. Based on these results, we then generated an interaction network of proteins involved in viral infections through IPA (Figs. 3C and S3B). A strong interaction network was formed among those genes/proteins, and this might highly contribute to

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**Fig. 3.** Summary of proteomic results. (A) Venn diagram of significant identified proteins, (B) their expression pattern heatmaps, and (C) the interaction network of proteins/genes significantly regulated by IAV infection, generated using IPA. TCR, T cell receptor; HNRNPDL, heterogeneous nuclear ribonucleoprotein D-like; HNRNPH1, heterogeneous nuclear ribonucleoprotein H1; VCP, Valosin containing protein; PDIA3, protein disulfide-isomerase A3. Red and green colors represent up- and down-regulation, respectively, in the heat map.
the response of host MDCK cells following LC24 treatment during IAV infection. Taking all these results together, we propose the possible interaction of the proteins/genes identified (ANXA1, AKT1, VCP, HSPA4, HSPA5, HSPA8, Hsp90, MX1, HIF-1α, PDIA3, HNRNPH1, and HNRNPDL) based on the IPA knowledge database. Importantly, the main interaction networks shown consist of three canonical pathways including HIF-1α signaling (AKT1, P38 MAPK, and HIF-1α), unfolded protein response (VCP, Hsp90, HSPA4, HSPA5, and HSPA8) and interferon signaling (MX1).

Previous studies in vitro and in vivo have described the inhibitory mechanisms based on viral-host interactions. The IAV genome contains viral polymerase subunits (PB1, PB2, and PA), neuraminidase (NA), matrix (M1/M2), non-structural protein (NS1/NS2) and nucleoproteins (NP), that all contribute to the viral import/export, transcription, and replication [1, 2] (Fig. 4). Here, the antiviral activity of lichen extract by modulating the proposed biomarkers could be explained based on their identified roles, associated with various steps in the viral life cycle, and several cellular factors regarding the MDCK host cell–IAV interactions (Fig. 4). Indeed, the antiviral mechanism by which the LC24 extract controls IAV may primarily be by blocking or inhibiting viral polymerase activity via HSP modulation (step 2) [28, 39]. LC24 could also inhibit IAV by decreasing the viral NP, mRNA or viral ribonucleoprotein (vRNP) levels (step 3) via Hsp90, AKT1 and MX1 [28, 36, 49] regulation. We, hereby, also suggest a possible involvement of HNRNPH1 and HNRNPDL proteins at this stage to control IAV. Another supported explanation of the antiviral activity of LC24 treatment against IAV is the reduction of viral genome replication/transcription (step 4) via HIF-1α, AKT1 and MX1 inhibition [36, 47, 49], the release of viral mRNA/vRNP (step 5) by downregulating HSP, ANXA1 and ANXA2 expression, and the interference with the translation of viral mRNA, HA and NA (step 6), mainly by reducing ANXA2 and MX1 [43, 49].

In conclusion, we found in the current study that the lichen, *Nipponoparmelia laevior*, effectively inhibited IAV by modulating a range of key genes and proteins associated with viral import/export, transcription, replication and the cellular defense systems. The results presented here suggested that 2-O-methylrhizonic acid found in *Nipponoparmelia laevior* extract may be a new drug target and warrants further studies towards the development of a potential antiviral agent against IAV infection. Therefore, detailed investigations are required to confirm the antiviral mechanisms of the specific secondary metabolite against IAV, both in vitro and in vivo.
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Conflict of interest

The authors have no financial conflicts of interest to declare.

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