

Inhibitory effect of traditional herbal (kampo) medicines on the replication of human parainfluenza virus type 2 *in vitro*

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SUMMARY Thirteen herbal medicines, Kakkonto (TJ-001), Kakkontokasenkyushin'i (TJ-002), Hangekobokuto (TJ-016), Shoseiryuto (TJ-019), Maoto (TJ-027), Bakumondoto (TJ-029), Hochuekkito (TJ-041), Goshakusan (TJ-063), Kososan (TJ-070), Chikujountanto (TJ-091), Gokoto (TJ-095), Saibokuto (TJ-096), and Ryokankyomishingeninto (TJ-119) were tested for human parainfluenza virus type 2 (hPIV-2) replication. Eight (TJ-001, TJ-002, TJ-019, TJ-029, TJ-041, TJ-063, TJ-095 and TJ-119) out of the thirteen medicines had virus growth inhibitory activity. TJ-001 and TJ-002 inhibited virus release, and largely inhibited genome, mRNA and protein syntheses. TJ-019 slightly inhibited virus release, inhibited gene and mRNA syntheses, and largely inhibited protein synthesis. TJ-029 slightly inhibited virus release, largely inhibited protein synthesis, but gene and mRNA syntheses were unaffected. TJ-041 only slightly inhibited virus release, the gene and mRNA syntheses, but largely inhibited protein synthesis. TJ-091 largely inhibited gene, mRNA and protein syntheses. TJ-095 largely inhibited gene synthesis, but NP and HN mRNAs were slightly detected, and protein syntheses were observed. TJ-119 inhibited gene, mRNA and protein syntheses. TJ-001, TJ-002, TJ-091, TJ-095 and TJ-119 inhibited multinucleated giant cell formation derived from cell-to-cell spreading of virus. However, in TJ-019, TJ-029 and TJ-041 treated infected cells, only small sized fused cells with some nuclei were found. TJ-019 and TJ-041 slightly disrupted actin microfilaments, and TJ-001 and TJ-002 destroyed them. TJ-041 slightly disrupted microtubules, and TJ-001 and TJ-002 disrupted them. In general, the medicines effective on common cold and bronchitis inhibited hPIV-2 replication.

Keywords virus replication, recombinant green fluorescence protein-expressing hPIV-2 without matrix protein, negative-strand RNA virus

1. Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possesses a single-stranded, non-segmented, negative stranded RNA genome of 15,654 nucleotides (1). hPIV-2 has seven structural proteins, nucleoprotein (NP), the fifth (V), phospho (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large (L) proteins. The gene

order of hPIV-2 is 3'-(leader)-NP-V/P-M-F-HN-L-(trailer)-5'. All genes of hPIV-2 were sequenced by our group (2-7). Monoclonal antibodies (mAbs) were made, and antigenic diversity of clinical isolates was investigated, by Tsurudome (8). The infectious hPIV-2 from cDNA clone was constructed by Kawano, and it was shown that its growth property was the same as that of control natural hPIV-2 (9).

Traditional herbal medicines have had an important role in Far East countries, especially in China, Korea and Japan for many years. Shoseiryuto inhibited mouse

adapted influenza A/G/(H3N2) or B virus replication in nasal cavity and lung of ~6 month-old BALB/c mice after oral administration from 7 days to 4 days after infection, suggesting that it is useful for influenza virus infection in aged persons and protects against both subtypes A and B (10). Maoto has clinical and virological efficacy for influenza virus by inhibiting uncoating of influenza virus A (11). Hochuekkito also has protective effects against influenza virus, and it stimulates the upper respiratory mucosal immune system (12). However, there are no reports on the effect of traditional herbal medicines on human parainfluenza viruses. We here report the effects of traditional herbal medicines on the replication of hPIV-2.

In the present investigation, thirteen herbal medicines were tested for hPIV-2 growth, and it was found that eight had inhibitory effects on hPIV-2. To investigate the effects of the medicines on viral genome synthesis, virus RNA was prepared and analyzed by PCR and real-time PCR. To elucidate the effects of the eight drugs on mRNA synthesis, cDNA was synthesized using oligo(dT) primer and PCR was carried out. Virus protein expression was observed by indirect immunofluorescence study using mAbs against NP, F and HN proteins of hPIV-2 (8). The inhibitory effects of the drugs on cell-to-cell spreading of hPIV-2 were analyzed using a recombinant green fluorescence protein (GFP)-expressing hPIV-2 without matrix protein (rhPIV-2ΔM-GFP) (9,13,14). The number of viruses released from infected cells was determined. Cytoskeleton was reported to have an important role in paramyxovirus replication. Actin microfilaments are important in the hPIV-3 life cycle, specifically at the level of viral transport and replication (15). Tubulin also acts as a positive transcription factor for *in vitro* RNA synthesis by Sendai virus (16). The effects of the drugs on actin microfilaments and microtubules were analyzed using rhodamine phalloidin and anti-tubulin α mAb, respectively.

2. Materials and Methods

2.1. Herbal medicines

Thirteen herbal medicines were chosen because they are effective on common cold and respiratory tract disease. Kakkonto (TJ-001: mainly effective on common cold, fever, headache), Kakkontokasenkyushin'i (TJ-002: snuffle, nasal inflammation), Hangekobokuto (TJ-016: neurosis), Shoseiryuto (TJ-019: nasal inflammation, common cold), Maoto (TJ-027: common cold, rheumatoid arthritis), Bakumondoto (TJ-029: cough, bronchitis), Hochuekkito (TJ-041: tuberculosis, bad appetite, common cold), Goshakusan (TJ-063: gastroenteritis, headache, common cold), Kososan (TJ-070: an early stage of common cold with poor digestion), Chikujountanto (TJ-091: sleeplessness in the convalescent stage of influenza or cold), Gokoto (TJ-095:

cough, asthma), Saibokuto (TJ-096: asthma, bronchitis), Ryokankyomishingeninto (TJ-119: bronchitis, asthma) were tested for hPIV-2 growth inhibition. The medicines were kind gifts of Tsumura & Co. (Tokyo, Japan) and Akatsuka Pharmacy and Minami Kampo Pharmacy in Tsu City, Mie, Japan.

They were suspended in phosphate-buffered saline (PBS) (100 mg/mL), autoclaved for 15 min, centrifuged at 2,000 rpm for 5 min, and the supernatants were added to the cell culture. The supernatant was stored at -80°C until use.

2.2. Virus and recombinant virus

The virus and the recombinant virus were approved by the relevant biosafety committees of Suzuka University of Medical Science. hPIV-2 (Toshiba strain) was used. rhPIV-2ΔM-GFP was constructed according to the method described previously (9,13,14), and it was shown that it did not produce infectious virus particles without addition of M protein gene *in trans* (data not shown). The virus titer was determined using Vero cells and the titer was about 1×10^5 TCID₅₀/mL.

2.3. Cell line and cultivation of cells

LLCMK₂ cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. Minimum essential medium α (MEM α : FUJIFILM Wako Pure Chemical, Osaka, Japan), supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin (Nacalai Tesque, Kyoto, Japan), was used. The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. After three days, when the cells became confluent (5×10^5 cells), the medium was changed to MEM α with 0.5% FCS and 0.1 mg/mL kanamycin. The medicines were added to the cells, and the cells were infected with hPIV-2 (3×10^2 TCID₅₀).

2.4. Cytopathogenic assay

Cell fusion was observed at four days post infection under a cell culture microscope.

2.5. RNA preparation, cDNA synthesis, real-time PCR and PCR

RNA was extracted from the cells (2×10^6 cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's method. cDNA was synthesized with 1 μ g RNA using Reverse Tra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and NP gene specific primer (nucleotide number 1661-1679: 5'-CAACATTCAATGAATCAGT-3'). Real-time PCR was performed on the ABI PRISM 7700 Sequence Detection System (Thermo Fisher Scientific, Waltham,

MA, USA) using TaqMan Probe (1932-1956: 5'-FAM-AAGCACC GGATTCTAACCCGTC CG-TAMRA-3'), forward primer (1851-1875: 5'-ACACACTCATCCAG ACAAATCAAAC-3'), and reverse primer (1958-1980: 5'-TGTGGAGGTTATCTGATCACGAA-3').

cDNA was synthesized with 1 µg RNA using forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') and superscript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100), F (5,821-5,840) and HN (7,741-7,760), and reverse primers for NP (1,466-1,489: 5'-CCTCC GAGTATCGATTGGATTGAA-3'), F (6,661-6,681: 5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq (Takara BIO, Kusatsu, Shiga, Japan).

2.6. Detection of messenger RNA (mRNA)

cDNA was synthesized with 1 µg RNA using oligo(dT) primer and superscript II reverse transcriptase, and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') genes of hPIV-2, and reverse primers for NP (1,466-1,489: 5'-CC TCCGAGTATCGATTGGATTGAA-3'), F (6,661-6,681): 5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq.

2.7. Immunofluorescence study

To detect virus proteins in the infected cells, the cells were fixed with 3.7% formaldehyde solution in PBS at room temperature for 15 min. The cells were further incubated with 0.05% Tween-20 in PBS at room temperature for 15 min to detect NP protein that exists mainly in the cytoplasm, or 3 min to detect F and HN proteins that are both in the cytoplasm and in the cell membrane, washed with PBS, and incubated with mouse mAbs against NP, F and HN proteins of hPIV-2 at room temperature for 30 min. After washing with PBS, the cells were incubated with Alexa 488 conjugated secondary antibody to mouse IgGs (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 30 min, and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Actin was detected using rhodamine phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) and microtubules were observed using anti-tubulin α mAb against sea urchin tubulin α (clone B-5-1-2, Sigma-Aldrich, St Louis, MO, USA) at four days of cultivation.

The cells were fixed with 3.7% formaldehyde solution in PBS at 37°C for 15 min, washed with PBS, and the cells were further incubated with 0.05% tween20 in PBS at 37°C for 3 min to detect actin and for 15 min to detect microtubules.

2.8. Cell-to-cell spreading of hPIV-2

The drugs were added to the cells, and immediately after the addition, the cells were infected with rhPIV-2ΔM-GFP (1×10^4 TCID₅₀), and cultured for four days. They were then fixed with 1.2% formaldehyde solution in PBS at room temperature for 15 min and observed under a fluorescence microscope.

3. Results

3.1. Inhibitory effects of the herbal medicines

Different doses of the supernatants of the thirteen medicines were added to the cell culture, and the cells were infected with hPIV-2, and the cell fusion was observed and a hemadsorption test was carried out at four days post infection. Eight exhibited dose-dependent inhibitory effects and three caused cell toxicity, while two had no inhibitory effect. Table 1 shows the amount (µL) of supernatants of the herbal medicines that had an inhibitory effect on hPIV-2 replication. TJ-001, TJ-002, TJ-019, TJ-029, TJ-041, TJ-091, TJ-095 and TJ-119 had an inhibitory effect on hPIV-2 induced cell fusion and hemadsorption. TJ-027 and TJ-70 had no inhibitory effect. TJ-016, TJ-063 and TJ-096 showed cell toxicity at low concentration. Mainly the medicines which are effective on common cold and bronchitis inhibited hPIV-2 replication. The eight medicines were used in the following experiments.

3.2. Effect of the eight medicines on the release of hPIV-2

The supernatants of virus-infected cells with the medicines were harvested at four days of virus infection; they were diluted, infected to the cells, and the virus titer was determined by the observation of cell fusion and hemadsorption test at four days of culture. Figure 1 shows the titers (averages ± s.e.m. from five independent experiments) of the medicines. TJ-001, TJ-002 and TJ-119 completely inhibited the release of the virus (below 1/10,000). However, TJ-019, TJ-029, TJ-041, TJ-091 and TJ-095 only partially inhibited the release (1/100 to 1/1,000), though they showed both cell fusion inhibition and hemadsorption inhibition.

3.3. Effect of eight herbal medicines on viral genome RNA and mRNA syntheses

RNA was prepared from the infected cells at four days

Table 1. Inhibitory effect of herbal medicines and the amounts needed to inhibit virus induced cell fusion and hemadsorption

Product number	Herbal medicine	Inhibitory effect	Concentration
TJ-001	Kakkonto	+	140 μ L/mL
TJ-002	Kakkontokasenkyushin'i	+	80 μ L/mL
TJ-016	Hangekobokuto	-	a
TJ-019	Shoseiryuto	+	100 μ L/mL
TJ-027	Maoto	-	b
TJ-029	Bakumondoto	+	160 μ L/mL
TJ-041	Hochuekkito	+	100 μ L/mL
TJ-063	Goshakusan	-	a
TJ-070	Kososan	-	b
TJ-091	Chikujountanto	+	80 μ L/mL
TJ-095	Gokoto	+	80 μ L/mL
TJ-096	Saibokuto	-	a
TJ-119	Ryokankyomishingeninto	+	120 μ L/mL

a: cell toxicity; b: no inhibitory effect.

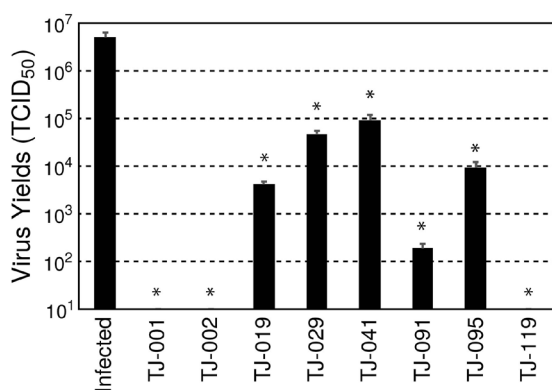


Figure 1. Effect of the medicines on hPIV-2 release from the cells. The supernatants were harvested at four days post infection and the virus titers were determined. Results are means \pm s.e.m. from five independent experiments. Significant differences from the control infected cells are indicated by * ($p < 0.01$). TJ-001, TJ-002 and TJ-119 completely inhibited the release of the virus (below 1/10,000). TJ-019, TJ-029, TJ-041, TJ-091 and TJ-095 only partially inhibited the release (1/100 to 1/1,000).

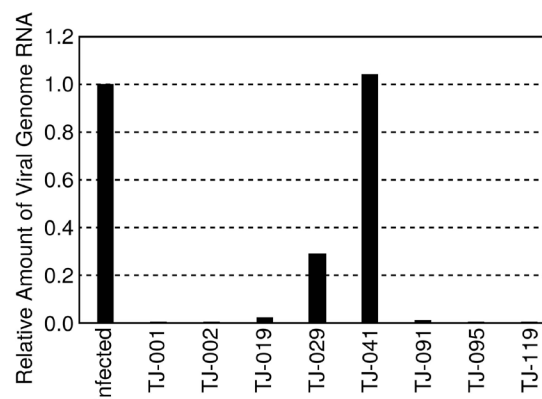


Figure 2. Effect of the medicines on viral genome RNA synthesis analyzed by quantitative real-time PCR. The medicines were added to the cell culture, infected with hPIV-2 and cultured for four days. RNA was extracted and viral genome RNA was analyzed by real-time PCR. The positive control infected cells are shown as 1. TJ-001, TJ-002, TJ-091, TJ-095 and TJ-119 almost completely inhibited viral genome RNA synthesis. TJ-019 and TJ-29 largely inhibited. TJ-041 did not inhibit.

post infection, and viral genome RNA was analyzed by real-time PCR and PCR. Viral mRNA was analyzed by PCR.

Figure 2 shows that TJ-001, TJ-002, TJ-019, TJ-091, TJ-095 and TJ-119 almost completely inhibited viral genome RNA syntheses. TJ-029 reduced the amount of viral genome RNA to about one-third of the control infected cells, but TJ-041 did not inhibit viral genome RNA synthesis.

Similar results were obtained by PCR (Figure 3). In Figure 3, NP, F and HN protein genes were detected in TJ-041 treated infected cells and they were seen as faint bands in TJ-029 treated cells. The other medicines almost completely inhibited NP, F and HN gene syntheses. These results are similar to those of real-time PCR.

Figure 4 shows the seven medicines also inhibited viral mRNA syntheses. NP, HN and F mRNAs were seen in TJ-029 and TJ-041 treated infected cells. In TJ-095 treated cells, NP and HN mRNAs were detected as

faint bands. In TJ-019 and TJ-091 treated cells, only NP mRNA was faintly detected.

These results indicated that six out of eight medicines had inhibitory effects on viral genome and mRNA syntheses.

3.4. Effect on protein syntheses

Indirect immunofluorescence study was carried out using mAbs against NP, HN and F proteins to examine the effects of eight medicines on viral protein syntheses at four days post infection (Figure 5). Negative control non-infected cells had no immunofluorescence (data not shown). In Figure 5, A, B and C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 infected cells, NP, F and HN proteins were observed in almost all the cells: NP protein was observed in many strong fluorescent dots mainly in the cytoplasm, while F and HN proteins were in small dots

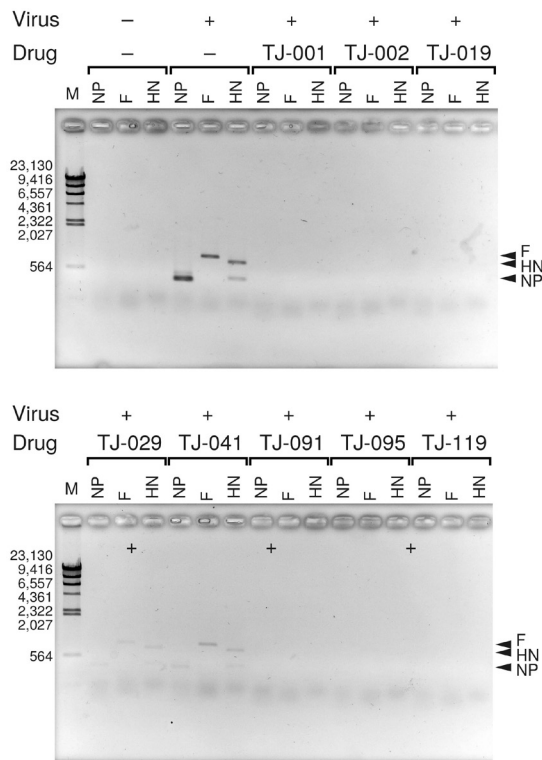


Figure 3. Effect of the medicines on viral genome RNA synthesis analyzed by PCR. NP, F and HN genes were detected using specific primers. TJ-001, TJ-002, TJ019, TJ-091, TJ-095 and TJ-119 inhibited viral genome RNA synthesis. TJ-029 and TJ-041 did not inhibit it. M: size marker.

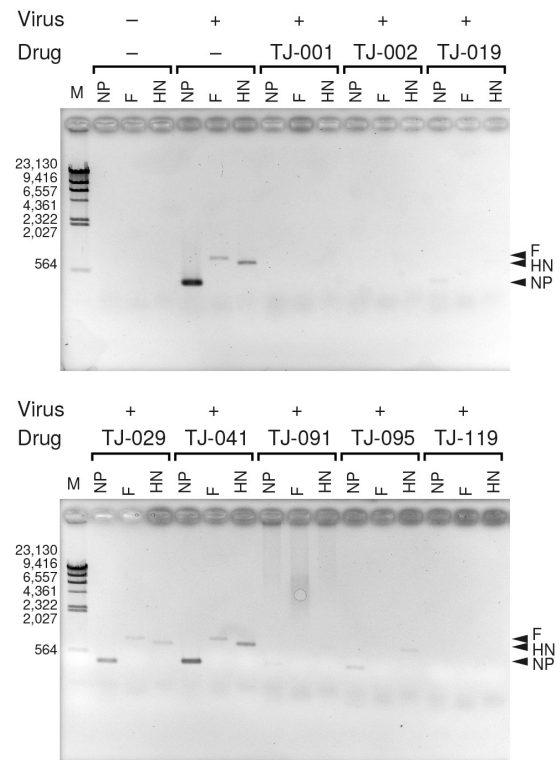


Figure 4. Effect of the medicines on viral mRNA synthesis analyzed by PCR. RNA was extracted and cDNA was synthesized by oligo(dT) primer. NP, F and HN mRNA were detected using specific primers. TJ-029 and TJ-041 did not inhibit mRNA synthesis. In TJ-095 treated infected cells, NP and HN mRNA were slightly observed, and in TJ-019 and TJ-091, only NP mRNA was faintly detected. M: size marker.

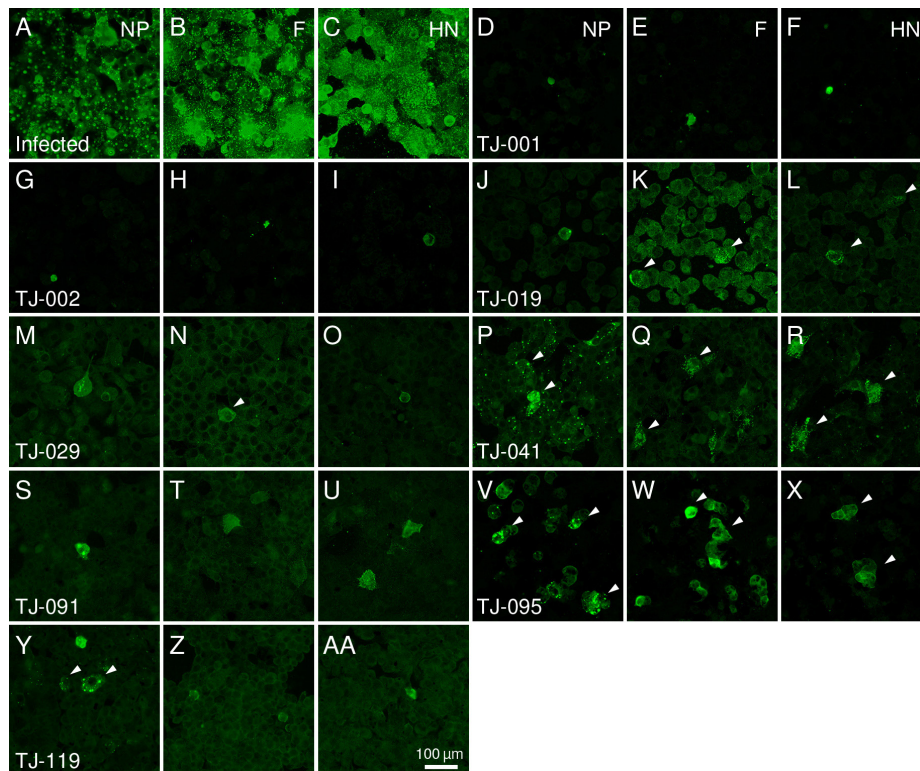


Figure 5. Effect of the medicines on viral protein synthesis. TJ-001 (D-F), TJ-002 (G-I), TJ-029 (M-O) and TJ-091 (S-U) largely inhibited protein synthesis. TJ-119 inhibited F and HN protein synthesis (Z and AA, respectively). In TJ-019 (J-L), TJ-041 (P-R) and TJ-119 (Y) treated infected cells, some positive cells were observed, however, there were no fused giant cells. Arrowheads indicate positive cells. Bar: 100 µm.

in the cytoplasm and on the cell surface. TJ-001 almost completely inhibited the protein syntheses: only a small number of cells were positive in NP, F and HN proteins of infected cells cultured with TJ-001 (Figure 5: D, E and F, respectively). TJ-002 also almost completely inhibited NP, F and HN protein syntheses just the same as TJ-001 treated cells (Figure 5: G, H and I, respectively). TJ-019 also largely inhibited the protein synthesis, but there are some positive cells (arrowheads) especially in F protein (Figure 5: J, K and L: NP, F and HN, respectively). TJ-029 also largely inhibited the protein syntheses (Figure 5: M, N and O: NP, F and HN, respectively). The arrowheads show positive cells. TJ-041 partially inhibited protein syntheses: some positive cells (arrowheads) can be seen (Figure 5: P, Q and R: NP, F, HN, respectively). TJ-091 also largely inhibited protein syntheses (Figure 5: S, T and U: NP, F and HN, respectively). TJ-095 partially inhibited protein syntheses: some positive cells are seen (arrowheads) (Figure 5: V, W and X, respectively). TJ-119 largely inhibited protein syntheses. However, a small number of cells were NP positive (arrowheads in Figure 5Y). Almost no cells were positive on F and HN proteins (Figure 5: Z and AA, F and HN, respectively).

3.5. Effect on the multinucleated giant cell formation

The cells were added with the medicines and immediately after that the cells were infected with rhPIV-2 Δ M-GFP and cultured for four days. The cells were fixed with 1.2% formaldehyde and observed with the fluorescence microscope. Figure 6A is a positive control. There are many multinucleated giant cells with strong fluorescence. TJ-001 (Figure 6B) and TJ-002 (Figure

6C) inhibited the giant cell formation: there was a small number of single cells, but no giant cells. TJ-001 and TJ-002 also inhibited the virus release (Figure 1) and virus genome syntheses (Figure 2). TJ-019 (Figure 6D), TJ-029 (Figure 6E) and TJ-041 (Figure 6F) partially inhibited the giant cell formation: there were small sized fused cells with strong fluorescence. TJ-019, TJ-029 and TJ-041 partially inhibited virus release from the cells (Figure 1). TJ-091 also inhibited the giant cell formation (Figure 6G), but a small number of viruses were released (Figure 1). TJ-095 (Figure 6H) inhibited the giant cell formation, but there were some small fused cells (arrowheads in Figure 6H). TJ-095 only partially inhibited the number of released viruses (Figure 1). TJ-119 (Figure 6I) inhibited the giant cell formation: there are some small sized fused cells with weak fluorescence (arrowhead in Figure 6I). TJ-119 inhibited the number of released viruses (Figure 1). The multinucleated giant cell formation, the number of released viruses from the cells, and genome RNA syntheses were in good accordance.

3.6. Effect on actin microfilaments

The medicines were added to the cell culture without virus infection and cultured for four days. F-actin was stained with rhodamine phalloidin. Figure 7A is the positive control: bundles of actin microfilaments were clearly seen. TJ-001 (Figure 7B), TJ-002 (Figure 7C), TJ-019 (Figure 7D) and TJ-095 (Figure 7H) destroyed actin microfilaments. TJ-041 (Figure 7F) partially destroyed them. TJ-029 (Figure 7E), TJ-091 (Figure 7G) and TJ-119 (Figure 7I) had almost no effect on them. There are some discrepancies among virus titer, genome RNA

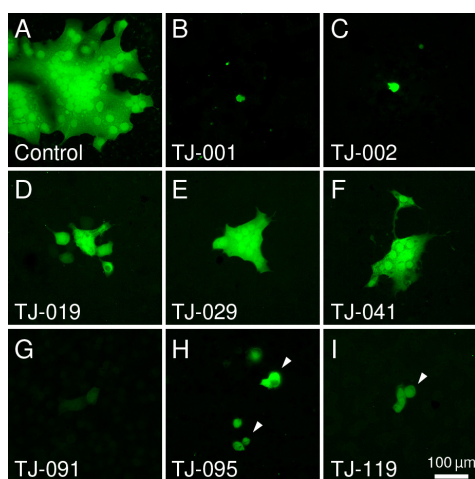


Figure 6. Effect of the medicines on multinucleated giant cell formation. The cells were added with the medicines and infected with rhPIV-2 Δ M-GFP. Immunofluorescence study was carried out at four days post infection. In positive control cells, many multinucleated giant cells with strong fluorescence were observed (A). TJ-001 (B), TJ-002 (C), TJ-091 (G), TJ-095 (H) and TJ-119 (I) largely inhibited giant cell formation. TJ-019 (D), TJ-029 (E), and TJ-041 (F) did not inhibit cell fusion, but the size is small compared with positive control. Bar: 100 μ m.

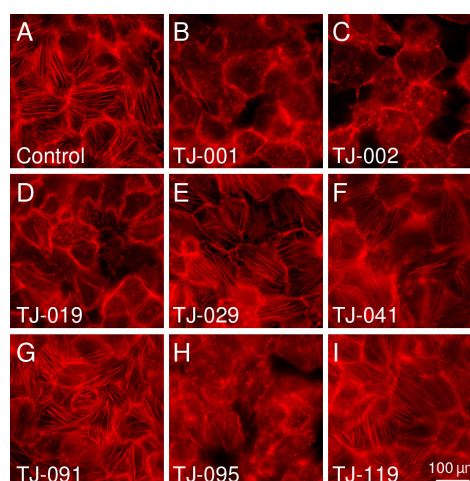


Figure 7. Effect of the medicines on actin microfilaments. The medicines were added to the cell culture without virus infection, and cultured for four days. The cells were stained with rhodamine phalloidin. In non-treated cells (A), actin microfilaments were clearly seen. TJ-001 (B), TJ-002 (C), TJ-019 (D) and TJ-095 (H) almost completely destroyed the filaments. TJ-041 (F) caused partial damage. TJ-029 (E), TJ-091 (G) and TJ-119 (I) showed almost no damage. Bar: 100 μ m.

syntheses, and actin filaments, especially in TJ-041. We cannot explain the discrepancy. It was shown that the damage to actin microfilaments caused some inhibitory effects on the release of virus from the cells to culture medium.

3.7. Effect on microtubules

The cells were added with the medicines without virus infection, and cultured for four days. Microtubules were stained with anti-tubulin mAb. Figure 8A is the positive control: microtubule net was seen in the cytoplasm. TJ-001 (Figure 8B) and TJ-002 (Figure 8C) disrupted microtubules. TJ-019 (Figure 8D), TJ-041 (Figure 8F), TJ-095 (Figure 8H), and TJ-119 (Figure 8I) caused a little damage in microtubules. TJ-029 (Figure 8E) and TJ-091 (Figure 8G) had almost no effect on microtubules. Microtubules are also important for virus replication, so one of the causes of virus replication inhibition had some relation with the disruption of microtubules.

4. Discussion

Thirteen herbal medicines, which are effective on common cold and respiratory diseases, were tested against hPIV-2 replication *in vitro*. Eight out of thirteen medicines had inhibitory effects on hPIV-2 replication. The effects on genome RNA synthesis, viral mRNA synthesis, protein expression, multi-nucleated giant cell formation and cytoskeleton (actin microfilaments and microtubules) were analyzed.

Kakkonto (TJ-001), Kakkontokasenkyushin'i (TJ-

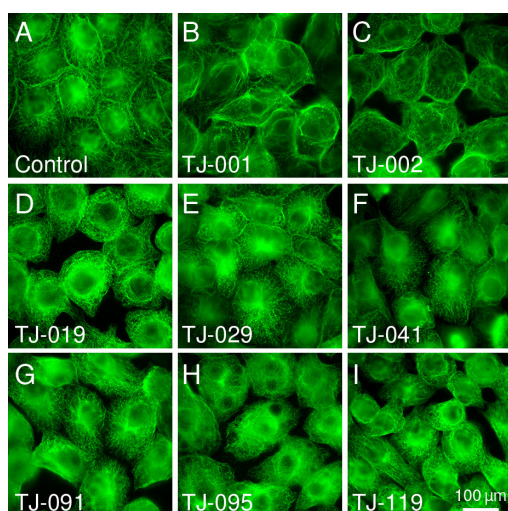


Figure 8. Effect of the medicines on microtubules. The cells were added to the cell culture without virus infection, and cultured for four days. The cells were stained with anti-tubulin α mAb against sea urchin tubulin α . In non-treated cells (A), microtubules were clearly seen in the cytoplasm. TJ-001 (B) and TJ-002 (C) destroyed microtubules. TJ-041 (F) partially destroyed microtubules. TJ-019 (D), TJ-029 (E), TJ-091 (G), TJ-095 (H) and TJ-119 (I) had almost no effect on microtubules. Bar: 100 μ m.

002) and Ryokankyomishingeninto (TJ-119) had strong inhibitory effects on hPIV-2 replication. They inhibited virus genome RNA synthesis, virus mRNA synthesis, virus-induced multinucleated giant cell formation and virus protein synthesis, though in TJ-119 treated infected cells, a small number of cells were NP positive. TJ-001, TJ-002 almost completely inhibited virus induced multinucleated giant cell formation. In Shoseiryuto (TJ-019), Bakumondoto (TJ-029) and Hochuekkito (TJ-041) treated infected cells, small sized giant cells were observed, indicating that these three medicines could not fully inhibit the transmission of virus to the nascent cells. Chikujountanto (TJ-091), Gokoto (TJ-095) and TJ-119 inhibited giant cell formation, but some non-fused fluorescent cells were observed. In TJ-019, TJ-029 and TJ-041 treated infected cells, F protein expression was not fully inhibited. This may be one of the reasons for the appearance of small sized fused cells. In TJ-095 added infected cells, though the expression of NP, F and HN proteins were observed, there were almost no fused cells. In TJ-019, TJ-029 and TJ-041 treated infected cells, F protein was expressed as small dots on the cell surface. However, in TJ-095 treated infected cells, F protein was expressed mainly in the cytoplasm, not in the cell membrane, resulting in the inhibition of cell fusion. TJ-001 and TJ-002 caused damage to actin microfilaments and microtubules, but TJ-119 did not cause damage in actin microfilaments or microtubules. One of the reasons for inhibition by TJ-001 and TJ-002 was damage to actin microfilaments and microtubules, suggesting that the inhibitory mechanisms by TJ-119 may be different from those of TJ-001 and TJ-002.

Many investigations show that the medicines tested in this study inhibit various virus replications by different mechanisms. For instance, Kakkonto inhibits human respiratory syncytial virus infection by preventing viral attachment and internalization to the host cells (17). Kakkonto also possesses inhibitory activity against influenza viral polymerase acidic protein endonuclease (18). Hochuekkito binds to influenza virus particles and forms complexes and can obstruct the entry of the virus into cells (12). Hochuekkito also inhibits rhinovirus infection *in vitro*, partly by inhibiting the expression of its receptor, ICAM-1 (19). Shoseiryuto inhibits human cytomegalovirus replication *in vitro* by inhibiting viral DNA replication (20). Shoseiryuto is also effective against human respiratory syncytial virus infection on airway epithelia by preventing viral attachment and internalization to the host cells and by stimulating interferon secretion (21). Maoto has clinical efficacy for seasonal influenza, and it has equivalent clinical and virological efficacy to oseltamivir and zanamivir (22). It inhibits uncoating of influenza virus by inhibiting acidification and blocks influenza virus entry to cytoplasm (11).

One of the questions is which components of the medicines are effective on virus replication.

Table 2. Crude drugs contained in herbal medicines

TJ-001 Kakkonto	TJ-002 Kakkontokasenkyushin'i	TJ-027 Maoto	TJ-119 Ryokankyomishingeninto
<i>Puerariae radix</i>	<i>Puerariae radix</i>	<i>Ephedrae herba</i>	<i>Poria sclerotium</i>
<i>Ziziphi fructus</i>	<i>Ziziphi fructus</i>	<i>Cinnamomi cortex</i>	<i>Glycyrrhizae radix</i>
<i>Ephedrae herba</i>	<i>Ephedrae herba</i>	<i>Armeniacaee semen</i>	<i>Pinelliae tuber</i>
<i>Glycyrrhizae radix</i>	<i>Glycyrrhizae radix</i>	<i>Glycyrrhizae radix</i>	<i>Zingiberis rhizoma processum</i>
<i>Cinnamomi cortex</i>	<i>Cinnamomi cortex</i>		<i>Armeniacaee semen</i>
<i>Paeoniae radix</i>	<i>Paeoniae radix</i>		<i>Schisandrae fructus</i>
<i>Zingiberis rhizoma</i>	<i>Magnoliae flos</i>		<i>Asiasari radix</i>
	<i>Ligustici chuanxiong rhizoma</i>		
	<i>Zingiberis rhizoma</i>		

Glycyrrhizin is the effective component of *Glycyrrhizae radix*, and has an inhibitory efficacy for various virus replications *in vitro*, such as H5N1 influenza A virus (23), cytomegalovirus (20), rhinovirus (19) and human respiratory syncytial virus (24). Most herbal medicines which are effective on common cold and respiratory diseases contain extract of *Glycyrrhizae radix*. The medicines tested in this study contain *Glycyrrhizae radix* as an ingredient, except for Hangekobokuto (TJ-016). Glycyrrhizin also inhibits hPIV-2 replication *in vitro* (25), but effective concentration is much higher than that in these medicines and there is a significant difference in inhibitory efficacy between the medicines (Table 1). Therefore, glycyrrhizin may have only a small contribution to inhibit hPIV-2 replication in the medicines.

Both Kakkonto (18) and Maoto (11) have inhibitory effects on influenza virus replication. Maoto has inhibitory efficacy in influenza virus replication and its efficacy is partly derived from *Glycyrrhizae radix* (26). Kakkonto (TJ-001) and Kakkontokasenkyushin'i (TJ-002) showed strong inhibitory effects on hPIV-2 replication (Figure 1), although Maoto (TJ-027) did not (Table 1). Three crude drugs, *Ephedrae herba*, *Cinnamomi cortex* and *Glycyrrhizae radix*, are common ingredients to these three medicines (Table 2). Therefore, the inhibitory mechanism for hPIV-2 replication by herbal medicines may be different from that for influenza virus. Four crude drugs, *Puerariae radix*, *Ziziphi Fructus*, *Paeoniae radix* and *Zingiberis rhizoma*, are contained in Kakkonto and Kakkontokasenkyushin'i, but not in Maoto (Table 2). The results in this study suggest that one of the four crude drugs, or combination of two, three or all of them, is effective to inhibit hPIV-2 replication.

Ryokankyomishingeninto (TJ-119) also showed a strong inhibitory effect on hPIV-2 replication (Figure 1). It contains *Glycyrrhizae radix* as an ingredient, but not *Puerariae radix*, *Ziziphi fructus*, *Paeoniae radix* or *Zingiberis rhizoma* (Table 2), which are contained in Kakkonto and Kakkontokasenkyushin'i. Therefore, the inhibitory mechanism on hPIV-2 replication by Ryokankyomishingeninto may be different from that of Kakkonto and Kakkontokasenkyushin'i.

The inhibitory mechanism for hPIV-2 replication by

herbal medicines may be multiple. The medicines have many components, and some can inhibit virus genome synthesis, mRNA synthesis and/or protein synthesis, and at the same time, destroy actin microfilaments and/or microtubules. In the present study, the medicines were suspended in PBS, autoclaved, and the supernatant was added to the cell culture, so some insoluble materials and heat unstable substances that may have inhibitory effects might be missing.

The next aim is to analyze and to extract the compounds that can inhibit not only parainfluenza virus but also respiratory syncytial virus, measles virus, mumps virus and metapneumovirus.

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