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Antiviral effects of *Psidium guajava* Linn. (guava) tea on the growth of clinical isolated H1N1 viruses: Its role in viral hemagglutination and neuraminidase inhibition

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ABSTRACT

Rapid evolution of influenza RNA virus has resulted in limitation of vaccine effectiveness, increased emergence of drug-resistant viruses and occurrence of pandemics. A new effective antiviral is therefore needed for control of the highly mutative influenza virus. Teas prepared by the infusion method were tested for their anti-influenza activity against clinical influenza A (H1N1) isolates by a 19-h influenza growth inhibition assay with ST6Gal I-expressing MDCK cells (AX4 cells) using fluorogenic quantification and chromogenic visualization. Guava tea markedly inhibited the growth of A/Narita/1/2009 (amantadine-resistant pandemic 2009 strain) at an IC_{50} of 0.05% and the growth of A/Yamaguchi/20/06 (sensitive strain) and A/Kitakyushu/10/06 (oseltamivir-resistant strain) at similar IC_{50} values ranging from 0.24% to 0.42% in AX4 cells, being 3.4- to 5.4-fold more potent than green tea (IC_{50} values: 0.27% for the 2009 pandemic strain and 0.91% to 1.44% for the seasonal strains). In contrast to both teas, oseltamivir carboxylate (OC) demonstrated high potency against the growth of A/Narita/1/09 (IC_{50} of 3.83 nM) and A/Yamaguchi/20/06 (IC_{50} of 11.57 nM) but not against that of A/Kitakyushu/10/06 bearing a His274-to-Tyr substitution (IC_{50} of 15.97 μM). Immunofluorescence analysis under a confocal microscope indicated that both teas inhibited the most susceptible A/Narita/1/2009 virus at the initial stage of virus infection. This is consistent with results of direct inhibition assays showing that both teas inhibited viral hemagglutination at concentrations comparable to their growth inhibition concentrations but inhibited sialidase activity at about 8-times higher concentrations. Guava tea shows promise to be efficacious for control of epidemic and pandemic influenza viruses including oseltamivir-resistant strains, and its broad target blockage makes it less likely to lead to emergence of viral resistance.

1. Introduction

None of the 17 known hemagglutinin (HA) and 10 known neuraminidase (NA) subtypes of influenza A viruses usually cross the host species barrier, but they are occasionally transmitted to other animal species, resulting in devastating outbreaks in that naïve animal population (Tong et al., 2012; Kuiken et al., 2006; Sriwilaijaroen et al., 2011b). There are currently two circulating H1N1 viruses in humans: one originates from the 1918 Spanish flu widely spread in humans until 1957 and restarted in 1977 and the other is derived from swine flu 2009. After the pandemic period, the viruses still occur at high levels in winter as seasonal flu strains. The repetitive occurrence of influenza continues annually due to antigenic mutations in viral surface proteins HA and NA, resulting in morbidity and mortality globally in humans of all ages (WHO, 2009). The high mutation rate of influenza RNA virus also limits the effects of influenza vaccine. Only two classes of licensed anti-influenza drugs acting at different stages of the influenza life cycle have so far been available for influenza therapy (De Clercq, 2006). The first class targets M2 ion-channel protein, which plays a crucial role in uncoating of the virus, resulting in release of viral content to the host cytoplasm; however, M2 inhibitors including oral amantadine and its derivative rimantadine have encountered widespread resistance. The other class interacts with the active site of influenza NA enzyme rendering progeny virions that are unable

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to escape from infected host cells to new host cells. Although inhaled zanamivir (Relenza), an NA inhibitor, has rarely been reported to cause influenza virus resistance in clinical use (Hurt et al., 2009), its adverse effects on respiratory function of patients with underlying respiratory disease have restricted its use (FDA). Unlike inhaled zanamivir, oral oseltamivir (Tamiflu, Roche) has been widely used worldwide for prophylaxis and treatment of influenza A viruses, and resistance to oseltamivir has a tendency to increase yearly in circulating H1N1 viruses (Lackenby et al., 2011; WHO, 2011).

Plants are natural sources of valuable materials for medicine. Shikimic acid, originally extracted from fruits of Chinese star anise (*Illicium verum* in Illiciaceae (anise) family), is a key starting material for oseltamivir synthesis (Dewick, 2009; Kramer et al., 2003). Tea is a source of medicinal compounds that have not only beneficial physiological effects on human health but also inhibitory effects against a wide range of microorganisms, and it has been considered a healthy drink (Zhen et al., 2002). Black tea, which is popular in most of the Western world, and green tea, which is popular in the Orient, have been reported to act against influenza virus replication (Green, 1949; Imanishi et al., 2002; Kuzuhara et al., 2009; Nakayama et al., 1993; Song et al., 2005). By screening of different kinds of teas as influenza antivirals in cultured cells, we found that guava tea, which is mainly consumed in Oriental countries, has a stronger inhibitory activity than that of green tea against H1N1 clinical isolates, and we elucidated the mechanism of its anti-influenza virus action.

2. Materials and methods

2.1. Compounds and antibodies

Oseltamivir carboxylate ((3R,4R,5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate) was provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Acetylated trypsin, type V-S from bovine pancreas, for influenza growth inhibition assay and ethyl gallate (EGA) used as a standard for tannin content determination were purchased from Sigma Chemical Co. (St. Louis, MO). Two fluorogenic substrates, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNA) for sialidase activity assay and 4-methylumbelliferyl-β-D-galactoside (MU-Gal) for influenza growth inhibition assay, were purchased from Toronto Research Chemicals, Inc. (TRC, Ontario, Canada) and Calbiochem (San Diego, CA), respectively. Mouse IgG-conjugated anti-influenza nucleoprotein (NP) monoclonal (4E6) antibody was obtained using A/Memphis/1/71 (H3N2) as an antigen (Takahashi et al., 2008). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody and Alex 488-labeled goat anti-mouse IgG antibody were purchased from Jackson Immuno Research (West Grove, PA) and Molecular Probes (Eugene, Oregon), respectively. 4′,6-Diamidine-2-phenylindole dihydrochlroride (DAPI) was obtained from Boehringer Mannheim (Mannheim, Germany).

2.2. Cells

Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Tissue Culture Biological, Tulare, CA) and antibiotics (250 U/mL penicillin and 250 μg/mL streptomycin; GIBCO-BRL, Rockville, MD). β-Galactoside α2,6-sialyltransferase I (ST6Gal I)-expressing MDCK cells with the puromycin *N*-acetyltransferase gene (called AX4 cells), which have recently been developed to have a high expression level of sialyl-α2,6-galactose moieties (Hatakeyama et al., 2005), were grown in Dulbecco's modified essential medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated FBS and 7.5 μg/mL puromycin (Nacalai Tesque).

2.3. Viruses

Information on influenza virus isolates used in this study is shown in Fig. 1. The viruses were amplified in MDCK cells, and viral culture supernatants were harvested, concentrated, and stored at −80 °C as described previously (Sriwilaijaroen et al., 2011a).

2.4. Preparation of teas

Dried leaves of green tea (*Camellia sonensis*) and of guava tea (*Psidium guajava* Linn.) were purchased from a local market in Okinawa, Japan. To make tea, 20 g of each ground sample was steeped in 1000 mL of 85 °C water for 8 min, stirred gently for 10 s, filtered through filter paper, and left to cool down on ice. Tea extracts were filtered through a 3-μM polytetrafluoroethylene (PTFE) filter to remove remaining tea leaf particles prior to tannin analysis as described below. Each tea sample was diluted with cold water to obtain the same tannin value of 40 mg/100 mL and adjusted with sodium bicarbonate powder to obtain a final pH of 6.5.

2.5. Determination of tannin content

A colorimetric assay using the ferrous-tartrate method, the Japanese official analytical method for tea tannin, was used for quantitative analysis of tannin in tea samples (Iwasa and Torri, 1962). Each sample or water (background) was mixed with ferrous tartrate solution, containing 0.1% ferrous sulfate and 0.5% sodium potassium tartrate, and 1 M phosphate buffer, pH 7.5, at a 1:1:3 ratio. After incubation for 30 min at room temperature, the purple color that had developed in the mixture was measured at absorbance of 540 nm. The background subtracted absorbance was converted to tannin equivalent by the tannin standard curve using ethyl gallate as a standard. Tannin content was expressed as mg of tannin equivalents per 100 mL of sample.

2.6. Cytotoxicity assay

AX4 cell viability in the presence of various concentrations of an inhibitor was determined by using a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, the cells were incubated with an inhibitor for 24 h at 37 °C. After removal of inhibitor overlays, 10 μl of CCK-8 solution containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium-monosodium salt (WST-8) in 100 μl of medium was added to the cells. Following incubation at 37 °C for 45 min, absorbance of the formazan product directly proportional to the number of live cells was measured at 450 nm by a microplate reader (Bio-Rad Model 680, Hercules, CA).

2.7. Influenza growth inhibition assay

AX4 cells were washed and pretreated with DMEM alone or containing serial diluted inhibitors for 1 h at 37 °C. Influenza virus at a multiplicity of infection (MOI) of 0.03 was preincubated with a medium containing 2 μg/mL acetylated trypsin in the absence or presence of the inhibitor at the same concentrations as those used for pretreating cells for 1 h at 4 °C. Then the medium in the plate was replaced with the inhibitor-virus mixture and incubated for 19–20 h at 37 °C. Virus titer in infected cells was determined by a galactosidase-based fluorescent assay, and viral formation was observed by a peroxidase-based chromogenic assay. The infected cells were fixed and permealized with methanol and were then