Phytomedicine 16 (2009) 1127–1136

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Pomegranate (*Punica granatum*) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir

Mehran Haidari [a,b], Muzammil Ali [a], Samuel Ward Casscells III[a,b], Mohammad Madjid [a,b,*]

[a] *University of Texas Health Science Center at Houston, USA*
[b] *Texas Heart Institute, 6770 Bertner Ave., MC 2-255, Houston, TX 77030, USA*

ARTICLE INFO

Keywords:
Influenza
Pomegranate
Punica granatum
Punicalagin
Anti-influenza
Synergism

ABSTRACT

Influenza epidemics cause numerous deaths and millions of hospitalizations each year. Because of the alarming emergence of resistance to anti-influenza drugs, there is a need to identify new naturally occurring antiviral molecules. We tested the hypothesis that pomegranate polyphenol extract (PPE) has anti-influenza properties. Using real time PCR, plaque assay, and TCID 50% hemagglutination assay, we have shown that PPE suppresses replication of influenza A virus in MDCK cells. PPE inhibits agglutination of chicken red blood cells (cRBC) by influenza virus and is virucidal. The single-cycle growth conditions indicated that independent of the virucidal effect PPE also inhibits viral RNA replication. PPE did not alter virus ribonucleoprotein (RNP) entry into nucleus or translocation of virus RNP from nucleus to cytoplasm in MDCK cells. We evaluated four major Polyphenols in PPE (ellagic acid, caffeic acid, luteolin, and punicalagin) and demonstrated that punicalagin is the effective, anti-influenza component of PPE. Punicalagin blocked replication of the virus RNA, inhibited agglutination of chicken RBC's by the virus and had virucidal effects. Furthermore, the combination of PPE and oseltamivir synergistically increased the anti-influenza effect of oseltamivir. In conclusion, PPE inhibited the replication of human influenza A/Hong Kong (H3N2) in vitro. Pomegranate extracts should be further studied for therapeutic and prophylactic potential especially for influenza epidemics and pandemics.

Introduction

Influenza virus is documented to cause epidemics and pandemics in human population over several centuries. Influenza A virus has several zoonite hosts, therefore cannot be eradicated from human populations. Despite widespread access to vaccines and antiviral therapies, influenza continues to be a major cause of morbidity and mortality. About 31,000 deaths each year in the US are associated with influenza A infections (Thompson et al. 2003).

Frequent antigenic drifts necessitate a change in vaccine composition almost every single year. Vaccines with a good antigenic match have an efficacy of 60% to 80% in healthy adults; however, the rate of protection is lower in certain high risk groups particularly the elderly and the immuno-compromised. Furthermore, vaccines are even less effective when they are not matched with new strains which were not expected at time of vaccine development. Examples are H5N1 strains that caused an outbreak in Hong Kong in 1997 and emerging avian influenza strains causing epidemic in 2006.

Annual vaccination is the mainstay strategy for preventing influenza infections and antiviral drugs offer additional preventive and therapeutic benefits (Hayden 2006a). Currently, there are two classes of anti-influenza agents available for influenza management and are under consideration for stockpiling in the event of an influenza pandemic; one class targets the M2 ion channel (e.g., amantadine and rimantadine) and the other inhibits neuraminidase (e.g., oseltamivir and zanamivir). In the absence of an abundant supply of an effective vaccine, neuraminidase inhibitors will be considered the drug of choice for controlling an emerging influenza pandemic until vaccine supplies become available. Post-Exposure use of oseltamivir has an efficacy of 70% to 90% and can shorten the duration of illness by 1.5 days when used within the first 48 hours (Hayden et al. 1999). Due to continuous genetic variations in influenza virus genome, development of resistance against anti-influenza drugs has been a serious problem. Usage of amantadine and rimantadine, are limited by a lack of inhibitory effect against influenza B viruses, side effects, and a rapid emergence of antiviral resistance (Hayden 2006b).

Emergence of resistance to oseltamivir in human influenza A viruses (Ison et al. 2006) and the H5N1 subtype in Vietnam (de Jong et al. 2005) is a cause for concern. However, resistance has not been reported for the other neuraminidase inhibitor, Zanamivir (Moscona 2005). Nevertheless, expanding the range of

* Corresponding author at: Texas Heart Institute, 6770 Bertner Ave., MC 2-255, Houston, TX 77030, USA. Tel.: +832 355 9330; fax: +832 355 9595.
E-mail address: mmadjid@gmail.com (M. Madjid).

0944-7113/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2009.06.002

antiviral drugs that effectively inhibit replications of the influenza A virus, or potentially act in synergy with neuraminidase inhibitors is a matter of urgency.

Several novel agents that may be effective against influenza virus, specifically the avian flu virus strain, are currently under development. Naturally occurring anti-viral nutrients may be of special interest because they are widely available and may be used as part of the diet to combat diseases, including influenza infection. Plant-derived flavonoids found in fruits, leaves, and vegetables have recently been the focus of many studies because of their beneficial health effects in several disease models (Williamson and Manach 2005). Among fruits, pomegranate (*Punica granatum*, Punicaceae) is a rich source of anthocyanins and other phenolic compounds that have strong antioxidant activity (Gil et al. 2000). Pomegranate, native to Persia, is an edible fruit cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia, and the United States (California). Edible parts of pomegranate fruit (about 80% of total fruit weight) comprise 80% juice and 20% seed. Pomegranate fruit extract is a rich source of 2 types of polyphenolic compounds: anthocyanins (derived from delphinidin, cyanidin and pelargonidin), which give red color to the fruit and juice, and hydrolysable tannins (i.e. punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose), which account for 92% of the antioxidant activity of the whole fruit (Gil et al. 2000).

In an HPLC fingerprint analysis, pomegranate-specific polyphenol classes were identified as punicalin (2%), punicalagins (15.7%), oligomers (77%) and ellagic acid (3%). (Numbers in parenthesis are percent area on HPLC peak area distribution) Punicalagin and punicalin are considered pomegranate-specific and can be used as a standard of pomegranate product authenticity (Martin et al. 2008).

Pomegranate has been widely used for several centuries in traditional medicine for a wide variety of diseases including upper respiratory tract infections and influenza (Nonaka et al. 1990). The potential effect of pomegranate on the replication of the influenza virus has not been systemically studied before and very little data is available in english language literature on this subject. In the present study, we have tested the efficacy of a purified flavonoid-rich extract of pomegranate fruit (e.g. POMx compoud) against influenza A virus.

Material and methods

Reagents

Pomegranate juice, commercially available for human consumption was used in a concentrate form (70° Brix Extract). Pomegranate polyphenol extract (PPE), punicalagin (in powder form), and liquid pomegranate concentrate were provided by POM Wonderful (LLC, Los Angeles, CA). PPE and punicalagin powders were dissolved in DMSO (100 mg/ml stock). MDCK cells were purchased from ATCC (Manassas, VA). Hoechst 33342, ellagic acid, luteolin and caffeic acid were purchased from Sigma Aldrich (St. Louis, MO). Oseltamivir carboxylic acid (active form of oseltamivir) was purchased from Toronto Research chemicals (Toronto, Ontario, Canada). Tyramide signal amplification kits (TSA, Fluorescein) were obtained from PerkinElmer (Waltham, Massachusetts). Anti-influenza monoclonal antibody against nucleoprotein (NP) protein was purchased from Chemicon International Inc (Temecula, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary and anti-FITC–HRP antibodies were purchased from Abcam (Cambridge, MA). Chicken red blood cells (RBCs) were obtained from Lampire Biological Laboratories (Pipersville, PA).

Viral stock and cell culture

Influenza virus A/Hong Kong/2/68; H3N2 [A/HK (H3N2)] was used as the primary influenza virus strain in our experiments. Influenza A/HK (H3N2), A/USSR/90/77 (H1N1) and influenza B/Harbin/07/94 were kindly provided by Dr. Philip Wyde (Baylor College of Medicine, Houston, Texas). MDCK cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Cell viability assay

The effect of PPE and other chemicals on the viability of MDCK were determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide (MTT) assay. The cells were plated in 200 µl of complete culture medium containing serial dilutions of the chemicals in 96-well microtiter plates for 48 h. After incubating the cells for specified times at 37 °C in a humidified incubator, we added (MTT) (5 mg/ml in PBS) to each well and incubated the cells for 2 h. The absorbance was recorded on a microplate reader at the wavelength of 540 nm.

Viral infection

When 90% confluent, MDCK cells were washed twice with phosphate-buffered saline (PBS) to remove residual FBS and infected with virus at an MOI of 10 or 0.05 to allow single-cycle (evaluated 4–12 h after infection) or multicycle (24 h after infection) replication, respectively. Viral stock was used in serum-free DMEM for 60 minutes in 4 °C to inoculate the cells (absorption phase). Cells were then washed with PBS and cultured in DMEM+2% FBS, and TPCK trypsin (4 µg/ml, Worthington Biochemicals, Tryp-MEM) either with or without drug treatment, for 12-48 h (post infection phase).

Extracellular virus yield reduction assay

The extracellular virus yield reduction assay was performed in 24-well plates containing confluent MDCK monolayers. Three experiments were conducted for each assay. Drugs, alone or in combination, were added to cells in 24-well plates, and the plates were incubated for 16 h at 37 °C. The cells were then inoculated with virus first for 1 h at 4 °C, and after washing for 24 h at 37 °C in DMEM+2% FBS, and TPCK trypsin. The medium was removed and centrifuged at 3,200 g for 5 min to remove the floating cells and used for RNA extraction and quantification of virus using RT-PCR technique. The media were also used for tissue culture infectious dose (TCID) 50%, or plaque assay. To determine TCID 50%, we titrated the supernatant by adding serially diluted samples to four wells (each) in 96-well plates of MDCK cells (4 °C, DMEM). The medium was replaced with DMEM+2% FBS, and TPCK trypsin 60 minutes after the virus inoculation to remove residual compound. Virus replication was detected by a hemagglutination assay 72 h after the inoculation and titers were expressed as log10 of the 50% TCID (Reed and Muench 1938). To perform hemagglutination assays Chicken RBC's were added to the medium to make a final dilution of 0.5% for RBCs. After 60 min incubation at room temperature, chicken RBCs in negative wells settled to form red button pellets, whereas positive wells had a diffuse appearance with no sedimentation.

Cell-associated virus yield assay

Cell-associated virus yield was quantified by real time PCR assay. After removing the supernatant, we washed the cells twice