NATURAL PIPERINE AS A NEW ALTERNATIVE AGAINST INFLUENZA VIRUSES

ABSTRACT

Influenza viruses have been responsible for highly contagious acute respiratory illnesses, afflicting humans since ancient times and causing high mortality in the elderly. The pandemic of 1918 to 1919 was extremely severe, killing approximately 40 million people worldwide, most probably by a H_{1}N_{1} variant strain. H_{2}N_{1} and H_{3}N_{2} samples were responsible for pandemics occurring in 1957 and 1968 respectively, while H_{5}N_{1}, H_{6}N_{1}, and H_{7}N_{2} have been recently isolated from infected humans in Hong Kong. The evidence of amantadine-resistant, rimantadine-resistant and zanamivir-resistant mutants explains the importance of the continuing search for new antiviral drugs for the treatment of influenza infections. In order to introduce a new option for the treatment of these virus infections, piperine, a natural alkaloid, has been tested. Piperine, purified from the dry fruit of *Piper nigrum*, when used at 0.78 μg/mL concentration, produced an inhibition percentage of 99.8%, showing a virucidal effect on the A/Victoria/3/75 (H_{3}N_{2}) sample of influenza A virus used as a model. This effect is due to interference with the attachment process between virus and host cell membrane.

INTRODUCTION

The family Orthomyxoviridae includes influenza A and B viruses, whose cores are composed of single-stranded negative-sense RNA genomes. Their segmented RNA is associated with a matrix protein (M1) and a nucleocapsid protein (NP), which make up the viral nucleocapsid. Finally, the virus particles are enfolded by a lipoproteic envelope, where a transmembrane protein (M2 or NB) and two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are inserted. Hemagglutinins are antigenically classified from H_{1} to H_{19}, while neuraminidases are classified from N_{1} to N_{14}. Influenza A virus samples classified as H_{1}N_{1}, H_{2}N_{1}, H_{3}N_{2}, H_{5}N_{2} have commonly been recognized in human infections (Lamb & Krug 2001).

These viruses have been responsible for highly contagious acute respiratory human diseases, afflicting humans since ancient times. An epidemic was
recognized by Hippocrates in 412 B.C., while many episodes were observed in the Middle Ages. Episodes from 1500 until nineteenth century were responsible for high mortality among the elderly. The pandemic of 1918-19 was extremely severe, killing approximately 40 million people worldwide, most probably due to a \( H_1N_1 \) variant strain (Potter 1998, Cox & Subbarao 2000). \( H_2N_2 \) and \( H_3N_2 \) samples were responsible for pandemics occurring in 1957 and 1968 respectively, while \( H_1N_1, H_2N_2, \) and \( H_3N_2 \) have been recently isolated from infected humans in Hong Kong (Nicholson et al. 2003). Outbreaks and epidemics, all of them caused by minor mutations in the RNA sequences, have been continuously observed throughout the world (Cox & Subbarao 2000).

The influenza virus replication cycle can be divided into the steps of virus attachment, entry, uncoating, transcription, translation, assembly and release. The virus attachment is cell receptor dependent, where HA uses cell sialated structures as targets. The 120 nm-diameter virus particles are internalized by endocytosis. The particles are then uncoated by a pH-dependent fusion process which in turn is dependent on a HA fusion peptide and M2 protein. In this process, M2 functions as a proton pump, acidifying the endosome environment that contains the endocytosed virus particles, activating the HA fusion peptide and finally liberating the virus particles into the cytoplasm by virus envelope-endosome membrane fusion. RNA transcription occurs in the cell nucleus, where virus polymerases (PB1, PB2 endonuclease and PA) are responsible for the synthesis of virus messenger-RNA and virus-RNA. After the translation process, virus particles are assembled in the cell nucleus and virus particles are liberated by budding. In the budding mechanism, the NA activity permits an efficient virus spreading by cleavage of sialic acid residues on the cytoplasm membrane (Lamb & Krug 2001).

**Antivirals for influenza viruses**

Amantadine and rimantadine are currently licensed for prophylactic and therapeutic uses by the United States Food and Drug Administration (FDA) to treat influenza A virus infections. These compounds block the acid-activated ion channel formed by the M2 protein (Stiver 2003). The related symmetric tricyclic amines were the first drugs to be introduced in order to inhibit fusion pH-dependent processes during infection by influenza viruses. More recently, zanamivir and oseltamivir were licensed to treat influenza virus infections acting as analogues of sialated substrate structures, which bind to the NA active site, blocking enzyme activity responsible for virus spreading (Crumpacker 2001, Colman 2002).

The evidence for amantadine-resistant, rimantadine-resistant and zanamivir-resistant mutants explains the importance of the continuing search for new antiviral drugs to treat influenza virus infections. In order to introduce a new option for the treatment of influenza infections, piperine, a natural amide alkaloid, has been tested. Piperine (Figure 1) is the main secondary metabolite found in *Piper nigrum*, and occurs mainly in the fruits (Semler & Gross, 1988). *Piper nigrum* (popularly known as black pepper) is widely used in folk medicine in India, from where it originated. In other countries it is used mainly as seasoning.

![Figure 1: Structure of piperine, the major chemical component isolated from *Piper nigrum*.](image)

**MATERIAL AND METHODS**

**Compound preparation.**
Piperine was extracted from 50g of black pepper with 500mL of 95% ethanol in a Soxhlet extractor for 2h, as reported previously by Ikan (1991). The solution obtained was concentrated in vacuum. Alcoholic potassium hydroxide was added to the filtrate residue and decanted from the insoluble residue. The alcoholic solution was left overnight, originating 1.5g of piperine as yellow needles which melt at 125-126°C after recrystallization from methanol, exhibiting approximately 98% final purity. The physical and spectroscopic data obtained from an analytical sample are identical to those previously reported (Ikan 1991).

This purified product was diluted in dimethyl sulfoxide (DMSO) to obtained the stock solution of 1000μg/mL (wt/vol). This preparation was diluted in Eagle’s minimum essential medium (MEM-Eagle) to the final concentration of 200μg/mL (DMSO final concentration of 3.12μg/mL), which was adjusted to neutral pH (7.2). The mixture was sterilized by filtration using Millipore membrane (0.22μm) and frozen at -20°C until use.
Cell and virus.

MDCK (Madin-Darby canine kidney) cells were grown in 96-well microtiter plates in MEM-Eagle, supplemented with 10% fetal calf serum (FCS), 50μg/mL gentamicin and 2.5μg/mL fungizone. The Victoria/3/75 (H3N2) strain of influenza A virus was initially obtained in 9-day old embryonated chicken eggs; triplicates of allantoic fluids were evaluated by the hemagglutination reaction. The allantoic fluid presenting a significant hemagglutination titer [≥ 128 hemagglutination units (UHA)/25μL] was finally submitted to cytopathic endpoint assay, where the TCID50 (50% tissue culture infective dose) was calculated by the Reed & Muench (1938) method. The cellular fluids (triplicates) were evaluated for their hemagglutination titer as described in Couceiro et al. (1994).

Cytotoxicity.

This test was performed in duplicate by incubating the MDCK cell monolayers for five days at 37°C, after overlaying with two-fold serial dilutions of piperine (200μg/mL - 0.195μg/mL) in MEM-Eagle. In order to determine the maximum non-toxic concentration (MNTC) that was used in the antiviral assay, the piperine cytotoxicity was evaluated by analysis of cellular morphological alterations (De Clercq et al. 1980) using an inverted optical microscope (American Optical Corporation), by comparison between treated and control cultures.

Cytotoxicity was also evaluated by using XTT (2,3 bis [2-methoxy-4-nitro-5-sulfophenyl]-2-tetrazo- lium-5-carboxinilide), a tetrazolium salt. The XTT assay was carried out by a method described by Roehm et al. (1991). Briefly, 20μl solution of XTT (Sigma, 1mg/mL) in phosphate-buffered saline was added to each well of the microtiter 96-well plate containing MDCK cell monolayers, and then incubated for 4 h at 37°C in a CO2 incubator. After incubation, absorbance was measured at a wavelength of 450 nm in a computer-controlled microplate reader (Model 3550, Bio-Rad Laboratories), in order to determine the percentage of viable cells.

Antiviral assay.

The antiviral activity was evaluated by the reduction of the virus titer using TCID50 determination. A half plate (48 wells) of MDCK cell monolayers cultivated in 96-well microtiter plates was treated with two-fold serial dilutions of the compound starting at the maximum non-toxic concentration (MNTC) and the other half (48 wells) was treated with MEM-Eagle. DMSO controls were prepared using MDCK cell monolayers only treated with DMSO at two-fold serial dilutions (1.56, 3.12 and 6.25μg/mL concentrations) in MEM-Eagle, DMSO at 6.25μg/mL exhibiting cytotoxicity. Immediately after, ten-fold serial dilutions (from 10-1 to 10-7 logarithm virus infectious doses) of the influenza A/Victoria/3/75 virus sample were prepared, added to piperine treated, DMSO treated and untreated cell cultures and then incubated at 37°C in a humidified CO2 atmosphere at 5%. After five days of incubation, the virus titers in the control untreated cells and purified piperine-treated cells were determined. The antiviral activity was expressed as the percentage of inhibition (PI) (Nishimura et al. 1977), using antilogarithm values of TCID50 as follows: PI = 1 - (antilogarithm test value/ antilogarithm control value) x 100 and viral inhibition index (VI) calculated by the following formula: VI = B - A, where B is the virus titer in virus infected control (no compound) and A virus titer in the test sample (Santos et al. 1999). The dose response curve was also built, using methodology already described for antiviral activity. All the assays were developed in duplicate.

Determination of virucidal effect.

The virucidal effect of the extract was determined by addition of 900μL of the purified piperine at the MNTC to 100μL of virus preparation (105.5 TCID50) or MEM-Eagle, incubating the mixture for 1 h at 37°C (Chen et al 1988). After this time, 10-fold dilutions of each mixture were added to MDCK cell monolayers. To show that the virucidal activity of the extract was due to extracellular inactivation of the virus, the virucidal index (VI) was determined on the fifth day by cytopathic endpoint assay (Reed & Muench 1938) using the following formula: VI = virus control titer - virus test titer.

RESULTS

In order to analyse the antiviral activity of the purified product, the maximum non-toxic concentration of piperine alkaloid on MDCK cells was initially evaluated, allowing observation of its MNTC at 3.12μg/mL.

The XTT method was used for complementary determination of the piperine maximum non-toxic concentration on MDCK cells, where the amount of formazan produced is proportional to the number of viable cells. The percentage of viable cells was equal to 97% at 3.12 μg/mL as can be seen in Figure 2.
Figure 2: Cytotoxicity percentage of different concentrations of piperine (µg/mL) on MDCK cells, determining its maximum non-toxic concentration.

Piperine exhibited significant antiviral activity, showing 99.8% inhibition (VII = 2.8) at a concentration of 0.78µg/mL in MDCK cells. Figure 3 shows the dose response curve of the piperine against the influenza A/Victoria/3/75 virus. DMSO controls did not reveal antiviral activity at concentrations used to evaluate the purified piperine for its antiviral activity in cell culture (3.12µg/mL), showing cytotoxicity when used at 6.25µg/mL.

Figure 3: Piperine antiviral activity on the Victoria/3/75 strain of influenza A virus, showing 99.8% inhibition by the maximum non-toxic concentration of 0.78 µg/mL. The virus titer was expressed in log TCID$_{50}$/mL (50% tissue culture infective dose) as calculated by the Reed & Muench (1938) method.

The mechanism of action of piperine was evaluated after direct contact between the virus suspension and the compound at MNTC. The piperine virucidal effect was observed in the MDCK cell line, which exhibits a virucidal index equal to 3.7 on influenza virus particles.

DISCUSSION

In previous studies, flavonoids and esters of phenolic acids present in propolis of different geographical origins (Kujumgiev et al. 1999) and flavonone glycosides from *Citrus junos* (Kim et al. 2001) have shown activity against influenza viruses. The interest in the search for terrestrial plants that present inhibitory properties against viruses is due to their low cytotoxicity and their abundance in nature.

Our target, piperine, is abundant in *Piper nigrum*, being easily extracted from the dry fruits with a yield of 3-7% (Ikan 1991). Various relevant biological activities are attributed to piperine such as insecticidal (DePaula et al. 2000), nematicidal (Kiuchi et al. 1988), and inhibition of liver metabolism (Koul et al. 2000). Kapil and Raay described the results obtained in the investigation of piperine activity against *Leishmania donovani* (Kapil 1993, Raay et al. 1999). More recently, results were described in the evaluation of trypanocidal effects of piperine and another twelve synthetic derivatives against epimastigotes and amastigotes of the protozoan parasite *Trypanosoma cruzi*, the causative agent of the incurable human Chagas' disease (Ribeiro et al. 2004).

The purpose of this paper was to study the in vitro antiviral activity of piperine, which is the major alkaloid isolated from the dry fruit of *Piper nigrum*, using influenza virus as a model for the first time.

In our initial analysis, purified piperine at a concentration of 3.12µg/mL (MNTC) did not induce microscopically detectable alterations in MDCK cell monolayers (Figure 2). The MNTC of the extract was also determined by XTT colorimetric assay, which is currently used as an indirect measure of cell viability, to confirm the analysis based on morphological changes (Borenfreund & Shopsis 1985, Smee et al. 2002).

In order to evaluate the antiviral activity of the purified piperine on influenza viruses (influenza A/Victoria/3/75 virus strain), the dose-response curve was built. Piperine showed an excellent percentage of inhibition equal to 99.8% at 0.78µg/mL (Figure 3). The inhibition of influenza virus samples by alkaloids had already been observed, when Serkedjivea & Velcheva (2003a, 2003b) and Lohézic-Le Dévéhat (2002) analysed extracts from *Thalictrum* species and *Geranium sanguineum* L. respectively.

In a previous work, *Piper aduncum* was only tested against poliovirus (non enveloped RNA virus),
its extract from flowers, which was constituted by contain alkaloids, flavonoids, steroids and tanins, exhibiting more significant activity and selectivity indexes than the extract from leaves (Lohézic-Le Dévéhat et al. 2002).

Natural compounds have exhibited a wide variety of inhibition mechanisms on the influenza virus replication cycle, 5,7,4-trihydroxy-8-methoxyflavone extracted *Scutellaria baicalensis* (Nagai et al. 1995) induced inhibition of the fusion process, while polyphenolic preparations of *Geranium sanguineum* L. inhibited both fusion and budding (Seredjieva & Hay 1998). Natural flutamide and fully substituted analogous pyrazine-2,6-diones exhibit inhibitory activity on virus endonuclease (PB2), which cleaves cellular RNA originating primers for virus messenger-RNA synthesis (Singh & Tomassini 2001).

In our study, analysis on the action mechanism of piperine was developed when the virus suspension was placed in direct contact with the purified compound at MNTC, IV = 3.7. The procedure revealed virus inactivation before attachment to the host cell, a virucidal effect preventing virus infection, which had already been observed using the polyphenolic complex from *Geranium sanguineum* L. (Seredjieva & Hay 1998). However, further investigations are necessary to evaluate possible additional mechanisms of action.

The abundance of the natural product, its structural versatility as an amide that permits its use as a prototype to obtain other molecules, as well as its ease of extraction make piperine a suitable template for the development of new drugs with antiviral activity.

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