The polyphenol rich plant extract CYSTUS052 is highly effective against H5N1 and pandemic H1N1v influenza A virus

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Introduction

The 2009/2010 H1N1influenza A virus pandemic clearly demonstrates that influenza is still a major risk for the public health. Although the pandemic swine origin influenza A virus (SOIV) caused only mild symptoms, the control of the outbreak still remains difficult. Even as vaccine is available against this virus, the possibility of reassortment between the pandemic and a seasonal or avian A/H5N1 influenza virus strain is indeed a frightening, but a likely event. This reassortant strain might be able to transmit easily between humans causing fatal infections, and the current SOIV vaccine might no longer be sufficient to protect against the reassorted virus. In such a case, we can only rely on effective antiviral drugs. Today, neuraminidase-inhibitors, such as oseltamivir, represent the most common clinically approved medication against influenza A viruses. Unfortunately, the frequency of reports describing the appearance of drug-resistant seasonal H1N1 and also H5N1 influenza A viruses dramatically increased in the recent past.[1–4] Drug resistance to the known antivirals highlights the urgent need for alternative antiviral compounds with novel defense mechanisms. Recently, we have reported that a polyphenol rich plant extract, CYSTUS052, which showed antiviral activity against influenza A viruses in cell culture and in mice.[5,6] Moreover, the antiviral activity of CYSTUS052 against seasonal influenza virus and common colds was also demonstrated in humans.[7] However, the efficiency of CYSTUS052 against SOIV and A/H5N1 isolates was unknown so far. Therefore, we investigated CYSTUS052 effectiveness against the pandemic strain and seven natural influenza A/H5N1 isolates detected in several avian species during 2006/2007 avian influenza outbreak. Additionally, the potency of the most common neuraminidase inhibitor oseltamivir was also investigated against these isolates. Here, we show that CYSTUS052 treatment was effective in *in vitro* studies against SOIV and A/H5N1 influenza virus.

Material and methods

Viruses

Avian H5N1 isolates were originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany. The SOIV A/Hamburg/4/2009 was obtained from the Robert-Koch-Institut, Berlin, Germany. All H5N1 viruses were further propagated in embryonated chicken eggs or MDCK II (H1N1v) cells at the Friedrich-Loeffler-Institut, Tübingen, Germany.

Antiviral compounds

CYSTUS052 extract was supplied and originally developed by Dr. Pandalis NatUrprodukte GmbH & Co. KG (Charge-Nr.:40121T01B/04; Glandorf, Germany). CYSTUS052 granulate was dissolved in sterile PBS (10 mg/ml) at 60°C for 1 hour. Oseltamivir carboxylate was obtained from Toronto Research Chemicals Inc. (TCR, North York, Canada) and dissolved in sterile PBS.

Viral cytopathological effect inhibition screening

For the cytopathological effect (CPE) inhibition screening, in accordance with Sidwell,[8] MDCK II cells were infected with different viruses at MOI of 0·005. Virus-infected cells were then treated with antiviral compounds CYSTUS052 from 0·1 to 1000 μg/ml or oseltamivir from 0·01 nm to 1 mm. After incubation for 48 hours at 37°C and 5% CO_2, cells were fixed, and viable cells were stained with crystal violet. After extraction of crystal violet from viable cells with 100% methanol, the extinction was measured with an ELISA reader.

Infectivity assay

Immediately before infection, MDCK II cells (8×10^4 cells/well) were washed with PBS and subsequently incubated with virus diluted in PBS/BA (0·2% BA) 1 mm $MgCl_2$, 0·9 mm $CaCl_2$, penicillin and streptomycin to a multiplicity of infection (MOI) of 0·001 for 30 minutes at 37°C. CYSTUS052 was added in a concentration of 50 μg/ml directly to the virus-stock and on the cell monolayer simultaneously with the infection. After 30 minutes incubation period, the inoculums were aspirated and cells were incubated with either MEM or MEM containing 1 μm oseltamivir. At indicated time points, supernatants were collected. Infectious particles (plaque titers) in the supernatants were assessed by a plaque assay under Avicel as described previously.[9]

Results

In order to investigate the antiviral potential of CYSTUS052, EC_{50} values based on the inhibition of the CPE on MDCK II cells were determined for CYSTUS052 and in addition for oseltamivir. The EC_{50} values for CYSTUS052 ranged from 1·53 to 18·88 μg/ml. CYSTUS052 demonstrated the highest sensitivity against the SOIV, SN1 and MB1 isolates with EC_{50} values below 5 μg/ml. Compared to these virus strains, CYSTUS052 showed a slightly increased EC_{50} value for GSB1 (18·88 μg/ml). In contrast the EC_{50} values for BB1 and BB2 were notably elevated (65·68 and 76·22 μg/ml). Thus, the weakest antiviral effect of CYSTUS052 was observed against these two isolates.

The EC_{50} values evaluated for oseltamivir ranged from 0·07 to 512·76 μm (Table 1), indicating that BB2 (512·76) and GSB1 (356·92 μm) can be considered resistant against oseltamivir. To confirm these results we investigated the ability of CYSTUS052 to block virus replication as published before.[1] As a control, virus infected cells were treated with oseltamivir as described earlier.[6] In the absence of the drugs all influenza strains showed similar growth properties (Figure 1, black squares).

First progeny viruses were detectable between 8 and 20 hours post infection (Figure 1, black squares). Treatment with CYSTUS052 resulted in reduction of virus titers of all influenza virus strains (Fig. 1A–H, open triangles). Surprisingly, oseltamivir failed to inhibit the replication of two H5N1 influenza virus strains (GSB1 and BB2), supporting the data of EC_{50} values (Figure 1D+H, grey rhombes).

Discussion

We assessed the antiviral activity of CYSTUS052 against the newly emerged SOIV and seven avian H5N1 influenza viruses. CYSTUS052 showed efficient antiviral activity against the pandemic H1N1v strain and was effective to a wide range of H5N1 viruses. Furthermore, CYSTUS052 demonstrated a broader and more efficient antiviral potential than oseltamivir. CYSTUS052 treatment leads to a stronger reduction of progeny virus titers, and more importantly, CYSTUS052 was effective against all tested viruses, while oseltamivir was unresponsive against two of seven A/H5N1 viruses. Even though the pandemic strain in general is still sensitive to oseltamivir treatment, there are increasing numbers of reports of emerging resistant variants. The treatment with CYSTUS052 does not result in the emergence of viral drug resistance since the mode of action is an unspecific physical binding of the virus particle that is also beneficial to reduce opportunistic bacterial

Table 1. In vitro effect of oseltamivir and CYSTUS052 on different influenza A viruses

Isolate	Abbreviation	EC_{50}*	
		CYSTUS052 (μg/ml)	Oseltamivir (μm)
A/Hamburg/4/2009	SOIV	3·58 ± 0·42	$0{\cdot}07 \pm 4{\cdot}14^{-03}$
A/mute swan/Germany/R1349/07	SN1	1·53 ± 0·61	0·49 ± 0·001
A/mallard/Bavaria/1/2006	MB1	2·51 ± 0·82	0·51 ± 0·004
A/common buzzard/Bavaria/11/2006	BB1	65·68 ± 1·59	11·09 ± 0·01
A/common buzzard/Bavaria/2/2006	BB2	76·22 ± 1·62	512·76 ± 10·21
A/great crested grebe/Bavaria/22/2006	CGB1	15·78 ± 2·68	8·05 ± 0·003
A/goldeneye duck/Bavaria/19/2006	GEB1	16·87 ± 2·72	9·5 ± 0·004
A/goosander/Bavaria/20/2006	GSB1	18·88 ± 1·61	356·92 ± 2·59

*The percent of cell viability after treatment with the antiviral compound was calculated after correction for the background values (virus-infected cell control) as follows: Percent inhibition = 100/[(OD 590) cell-control sample: (OD 590) treated sample]. The EC_{50} value (i.e. the concentration of compound required to reduce the viral cytopathological effect on MDCKII cells to 50%) was determined with the GraphPad Prism 5 Software by plotting the percent cell viability as a function of compound concentration.

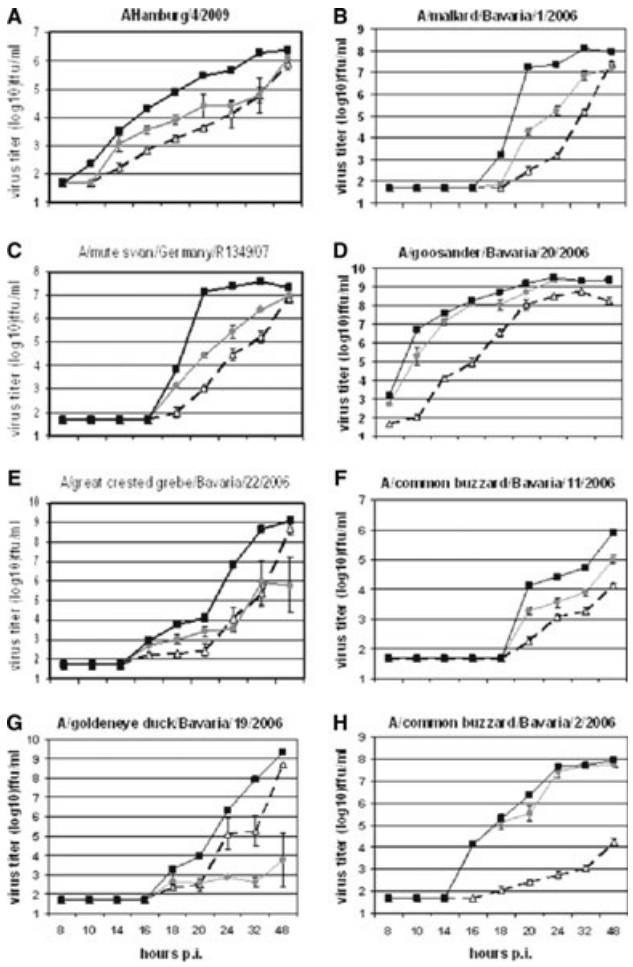

Figure 1. Infection of MDCK II cells with SOIV (A) and various H5N1 isolates (B–H) either untreated (black squares), treated with CYSTUS052 (50 μg/ml) (open triangles) or oseltamivir (1 μm) (grey rhombs). For (A)–(F) and (H) CYSTUS052 treatment of the virus inoculum was performed with 50 μg/ml 30 minutes prior to infection, whereas treatment of the virus inoculum in (G) was performed with the standard protocol, using 100 μg/ml following supplementation of the culture medium with CYSTUS052 (100 μg/ml).

infections.[5,7,10] CYSTUS052 is an extract from a special variety of the plant *Cistus incanus*, and it is very rich in polymeric polyphenols.[11] It is well known that polyphenols exhibit protein-binding capacity.[12] However, CYSTUS052 exhibited no neuraminidase inhibiting activity. Therefore, ingredients of CYSTUS052 may act in a rather unspecific physical manner by interfering with the viral hemagglutinin at the surface of the virus particle as demonstrated before.[5] While this prevents binding of the virion to cellular receptors, it does not block accessibility and action of the viral neuraminidase. Since, infections with influenza A viruses are still a major health burden and the options for control and treatment of the disease are limited, plant extracts such as CYSTUS052 should be considered as a new candidate drug for a save prophylactic and therapeutic use against influenza viruses.

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