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Antiviral Research 76 (2007) 38–47
Antiviral Research
www.elsevier.com/locate/antiviral

A polyphenol rich plant extract, CYSTUS052, exerts anti influenza virus activity in cell culture without toxic side effects or the tendency to induce viral resistance

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Received 20 November 2006; accepted 7 May 2007

Abstract

Infections with influenza A viruses still pose a major threat to humans and several animal species. The occurrence of highly pathogenic avian influenza viruses of the H5N1 subtype capable to infect and kill humans highlights the urgent need for new and efficient countermeasures against this viral disease. Here we demonstrate that a polyphenol rich extract (CYSTUS052) from the Mediterranean plant *Cistus incanus* exerts a potent anti-influenza virus activity in A549 or MDCK cell cultures infected with prototype avian and human influenza strains of different subtypes. CYSTUS052 treatment resulted in a reduction of progeny virus titers of up to two logs. At the effective dose of 50 μg/ml the extract did not exhibit apparent harming effects on cell viability, metabolism or proliferation, which is consistent with the fact that these plant extracts are already used in traditional medicine in southern Europe for centuries without any reported complications. Viruses did not develop resistance to CYSTUS052 when compared to amantadine that resulted in the generation of resistant variants after only a few passages. On a molecular basis the protective effect of CYSTUS052 appears to be mainly due to binding of the polymeric polyphenol components of the extract to the virus surface, thereby inhibiting binding of the hemagglutinin to cellular receptors. Thus, a local application of CYSTUS052 at the viral entry routes may be a promising approach that may help to protect from influenza virus infections.

Keywords: Influenza A virus; CYSTUS052; Polyphenols; Plant extract; Virus entry

1. Introduction

Influenza A viruses are negative strand RNA viruses with a segmented genome that belong to the family of *orthomyxoviridae* (Lamb and Krug, 2001). While the natural reservoir of these viruses is within wild living water fowl, influenza A viruses also infect humans and several animal species (Webster et al., 1992). In fact, influenza A viruses still pose a major burden to human health and cannot be eradicated due to their large natural reservoir. Thus, introduction of avian virus genes into the human population can happen at any time and may give rise to a new pandemic. There is even the likeliness of a direct infection of humans by avian viruses, as evidenced by the emergence of highly pathogenic avian influenza viruses of the H5N1 subtype that were capable to infect and kill humans (Shortridge et al., 2000; Webby and Webster, 2001).

Although vaccination is the best option to protect from influenza virus infections, this approach is difficult with regard to pathogenic avian strains or human reassortants with avian glycoprotein genes. To date only two classes of anti-influenza drugs have been approved; inhibitors of the M2 ion channel such as amantadine and rimantadine or neuraminidase inhibitors such as oseltamivir or zanamivir (De Clercq, 2004). Treatment with amantadine and derivatives rapidly results in the emergence of resistant variants and is not recommended for a general and uncontrolled use (Fleming, 2001; Hayden and Hay, 1992). Among H5N1 isolates from Thailand and Vietnam 95% of the strains have been shown to harbor genetic

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doi:10.1016/j.antiviral.2007.05.002

mutations associated with resistance to the M2 ion channel-blocking adamantine derivatives, amantadine and rimantadine (Cheung et al., 2006). Furthermore influenza B viruses are not sensitive to amantadine derivatives (Pinto and Lamb, 2006). Recent reports have described that resistance development also occurs to neuraminidase inhibitors (Hatakeyama and Kawaoka, 2006; Hurt et al., 2007). According to a recent study, oseltamivir-resistant mutants in children being treated for influenza with oseltamivir appear to arise more frequently than previously reported (Kiso et al., 2004). The authors concluded that children can be a source of viral transmission, even after 5 days of treatment with oseltamivir (Kiso et al., 2004). In addition, there are several reports that suggest that resistance of H5N1 viruses can emerge during the currently recommended regimen of oseltamivir therapy and may be associated with clinical deterioration (de Jong et al., 2005). Thus, it has been stated that the strategy for the treatment of influenza A (H5N1) virus infection should include additional antiviral agents. This highlights the urgent need for new and amply available anti-influenza agents.

Polyphenols are a large family of natural compounds widely distributed in plants and characterized by the presence of more than one phenol group per molecule. Thus, polyphenols constitute one of the most numerous groups of substances with more than 8000 phenolic structures currently known. The structure can vary from simple molecules, such as phenolic acids, to highly polymerized compounds (as reviewed by Harborne, 1980). Natural polyphenols have been attributed to exert an array of biological effects. Plant derived polyphenols have been shown to be strong antioxidants with potential health benefits (Urquiaga and Leighton, 2000). Reports on an antiviral and antibacterial potency of polyphenolic compounds are numerous (reviewed by Cos et al., 2003, 2004; Scalbert, 1991; Taguri et al., 2006). A variety of studies described the antiviral action of natural flavonoids/flavanoids on rhinoviruses and other picornaviruses (reviewed by Conti et al., 1988; Selway, 1986; Vlietinck et al., 1988). This work has also led to the synthesis of derivatives of the natural products and there detailed examination as antiviral drugs (reviewed by Vlietinck and Vanden Berghe, 1991).

While a variety of mechanisms of action of different polyphenolic plant products have been proposed in different studies, it has been suspected quite early on that antiviral activities of many phenolic compounds may be due to direct interactions with the viral particle (reviewed by Haslam, 1996). In an early study by Konishi and Hotta the authors showed that the polyphenolic agent tannic acid inactivates Chikungunya virus (CHIKV), a positively stranded enveloped RNA virus, most likely due to preferential binding to viral proteins on the particle (Konishi and Hotta, 1979). While anti-HIV activity of the biphenolic agent L-chicoric acid was initially thought to be due to integrase inhibition, it was later found that the compound targets the HIV glycoprotein gp120 at the surface of the particle (Pluymers et al., 2000). More recent data show that flavonoids and proanthocyanidins from *Crataegus sinaica* exhibit significant inhibitory activity against herpes simplex virus type I (HSV-1) due to an extracellular mechanism (Shahat et al., 2002).

Finally, it has been demonstrated that the polyphenols (-) epigallocatechin gallate and theaflavin digallate are able to bind hemagglutinin of influenza viruses and agglutinate the particles and therefore reduce their infectivity and binding at the cell surface (Nakayama et al., 1993).

Bioavailability differs greatly from one polyphenol to another (Manach et al., 2004; Urquiaga and Leighton, 2000). The current knowledge of absorption, biodistribution and metabolism of polyphenols is poor, however, in general it can be stated, that some low-molecular-weight polyphenols are absorbed and exhibit bioactive properties, while only few polymeric polyphenols are metabolized (Manach et al., 2004). Thus, it was suggested that polyphenols might exert direct effects when present in high concentrations (Halliwell et al., 2005).

CYSTUS052 is a plant extract that is very rich in highly polymeric polyphenols. Major constituents include flavan-3-ols and proanthocyanidins (Danne et al., 1993; Petereit et al., 1991). The total content of monomeric components is lower than 2% and the polymeric components, especially the proanthocyanidines with a molecular weight range of 500–1200 Da are dominant. The extract is a preparation of a selected variety of the biochemical polymorphic species *Cistus incanus* (Pink Rockrose). The genus *Cistus* consists of a group of about 20 shrub species found in wide areas throughout the whole Mediterranean region up to the Caucasus (Comandini et al., 2006). *Cistus* species are used as an anti-diarrheic, as general remedies in folk medicine for treatment of various skin diseases, and as anti-inflammatory agents (Danne et al., 1993; Petereit et al., 1991).

Given that some purified polyphenolic compounds have been demonstrated to show anti-influenza virus activity (Nakayama et al., 1993; Palamara et al., 2005; Song et al., 2005) and that *Cistus* plant preparations have been attributed to exert bactericidal and fungicidal effects (Bouamama et al., 1999), we were prompted to analyze whether CYSTUS052 may have anti-influenza virus activity in cell culture.

2. Experimental/materials and methods

2.1. CYSTUS052 extract

CYSTUS052 Extract Charge-Number 40121T01B/04 was supplied and originally developed by Dr. Pandalis NatUrprodukte GmbH & Co. KG (8-2004; Glandorf, Germany). The extract is a special preparation from a distinct variety of *Cistus incanus* (*Cistus incanus* PANDALIS). CYSTUS052 extract was defined by the producer to exhibit a polyphenolic content of more than 26% (determined after Singleton et al., 1999; Singleton and Rossi, 1965). The total content of monomeric components (gallic acid, gallocatechin, catechin, epicatechin) is lower than 2%.

CYSTUS052 was solved in sterile PBS (1mg/ml) at 100 °C for 1 h and was employed from 1 to 100 μg/ml and added directly to the medium or virus-stock.

2.2. Viruses, cells and viral infections

Avian influenza virus A/FPV/Bratislava/79 (H7N7) (FPV) has been propagated and passaged in Madin Darby canine kidney (MDCK) cells. Human influenza virus A/Puerto Rico/8/34

(H1N1) (PR8) was grown in 10-days-old embryonated chicken eggs. After incubation at 37 °C for 2 days the allantois fluid was harvested and used for infection as described below (Ehrhardt et al., 2004, 2006; Wurzer et al., 2004).

The human influenza isolate A/Thailand/1(KAN-1)/2004 (H5N1) was provided by P. Puthavathana (Mahidol University, Bangkok, Thailand) (Puthavathana et al., 2005) and has been passaged in MDCK cells. Human Rhinovirus serotype 14 (HRV14) was purchased from ATCC (ATCC #284) and was amplified in HeLa cells. HRV was propagated at 33 °C. For infection cells were washed with PBS, incubated with virus diluted in PBS/BA (PBS containing 0.2% BSA, 1mM $MgCl_2$, 0.9mM $CaCl_2$, 100U/ml penicillin and 0.1mg/ml streptomycin) for 30min at 33 °C or 37 °C at the indicated multiplicities of infection (MOI). The inoculum was aspirated and cells were incubated with MEM, DMEM or HAM's F12 containing 0.2% BSA and antibiotics. In the case of PR8-infection medium was supplemented with 2 μg/ml trypsin. MDCK cells were grown in MEM, human cervical carcinoma cells (HeLa) were grown in DMEM and the human lung epithelial cell line A549 was grown in HAM's F12, respectively. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). For proapoptotic or proinflammatory stimulation of cells staurosporine dissolved in DMSO (2.5 μM, 8 h) (Sigma) or recombinant TNFα (Sigma) (30 ng/ml, 15 min) were directly added into the medium.

2.3. Plaque titrations and resistance assays

Supernatants, collected at the indicated time points, were used to assess the number of infectious particles (plaque titers) in the sample. Briefly, MDCK-cells grown to 90% confluency in 6-well dishes were washed with PBS and infected with serial dilutions of the supernatants in PBS/BA for 30min at 37 °C. The inoculum was aspirated and cells were incubated with 2 ml MEM/BA (medium containing 0.2% BSA and antibiotics) supplemented with 0.6% Agar (Oxoid), 0.3% DEAE-Dextran (Pharmacia Biotech) and 1.5% $NaHCO_3$ at 37 °C, 5% CO_2 for 2–3 days. Virus plaques were visualized by staining with neutral red. Generation of resistant virus variants to antiviral treatment was assessed as recently described (Ludwig et al., 2004). Briefly, MDCK cells were infected with the influenza A virus strain FPV (MOI = 0.01) and were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine (Sigma–Aldrich). At 24 h post infection supernatants were taken and employed for infection in the second round of investigation. After infection cells were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine again. This procedure was repeated six times. Supernatants were assayed for progeny virus yields by standard plaque titrations. Virus yields of mock-treated cells were arbitrarily set as 100%.

2.4. Hemagglutination-inhibition-test

Influenza viruses are characterized by their ability to agglutinate erythrocytes. This hemagglutinating activity can be visualized upon mixing virus dilutions with chicken erythrocytes in microtiter plates. The chicken erythrocytes (Lensing–Baeumer, Ibbenbueren) supplemented with 1.6% sodium citrate (Sigma) in sterile water were separated by centrifugation (800 × *g*, 10 min, room temperature) and washed three times with sterile PBS (Invitrogen). The lowest amount of virus particles able to agglutinate the chicken erythrocytes was determined in a serial virus dilution and used to investigate the inhibitory effect of CYSTUS052 onto the hemagglutinating activity. Untreated erythrocytes precipitate to the bottom of the plate, while upon preincubation with virus the blood cells show an even and diffuse distribution. Briefly, CYSTUS052 was serially diluted as indicated. From virus stocks (H5N1 or H7N7, HA titers: 2^9) 1/128 dilutions were made, and 50 μl/well of this virus dilution was added as indicated. After preincubation of 45min, chicken erythrocytes (1/20 in PBS) were mixed with the solution. In the samples where viruses were preincubated with CYSTUS052, up to a certain dilution the viral particles were no longer capable of agglutinating erythrocytes, indicating an interaction of CYSTUS052 with the viral HA.

2.5. MTT-assay

A549 cells were left untreated or treated with the indicated amounts of CYSTUS052 for the indicated time points. Cell-proliferation/metabolism was measured in the MTT-assay. Briefly, cells were treated with 5 mg/ml thiazolylblue-tetrazoliumbromid/PBS (Sigma/Invitrogen) and incubated for 3 h at 37 °C. Reaction was blocked by DMSO (Sigma) and cells were further incubated for 20 min at 37 °C. The color reaction was measured in an Emax precision microplate reader at 562 nm. The untreated control was arbitrarily set as 100%.

2.6. Western blotting

For Western blotting cells were lysed on ice with Triton lysis buffer (TLB; 20 mM Tris–HCL, pH 7.4; 137 mM NaCl; 10% Glycerol; 1% Triton X-100; 2 mM EDTA; 50 mM sodium glycerophosphate, 20 mM sodium pyrophosphate; 5 $\mu g ml^{-1}$ aprotinin; 5 $\mu g ml^{-1}$ leupeptine; 1 mM sodium vanadate and 5 mM benzamidine) for 30 min. Cell lysates were cleared by centrifugation and directly subjected to SDS-PAGE and subsequent blotting on nitrocellulose membrane. For protein detection PARP-specific mouse antibody (BD Transduction Laboratories) or an IκBα-specific rabbit antibody (Santa Cruz Biotechnologies) were employed and loading controls were performed with pan-ERK2 antiserum (Santa Cruz Biotechnologies). Protein bands were visualized in a standard enhanced chemiluminescence reaction.

2.7. Indirect immunofluorescence microscopy

A549 cells were directly seeded onto 15 mm glass-plates. Twenty-four hours later cells were infected with FPV (MOI = 200) for 1 h or (MOI = 50) for 1.5 h. Cells and/or virus were incubated with CYSTUS052 as indicated. Cells were

washed twice with PBS and then fixed for 30 min with 3.7% paraformaldehyde (in PBS) at room temperature. After washing, cells were permeabilized with acetone, washed with PBS and blocked with 1% bovine albumin or 10% fetal bovine serum in PBS for 20 min at 37 °C. Upon blocking, cells were incubated with a mouse monoclonal antibody against viral NP (Serotec) (1:400) in PBS for 30 min. Following several washing steps, cells were incubated with a nucleoprotein-specific mouse antibody and an Alexa fluor 488 chicken anti mouse IgG (H + L) (1:300) in PBS for 1 h. Finally, cells were washed. Fluorescence was visualized using a Leitz DMRB fluorescence microscope.

2.8. Flow cytometry analysis

For cell viability assays MDCK or A549 cells were grown to near confluency in 6-well or 12-well dishes and either left untreated or incubated with the indicated amounts of CYSTUS052 for the indicated time points. For analysis both adherent and attached cells were collected, washed with PBS and incubated with 250 μl propidium iodide (PI) solution (50 μg/ml PI in PBS) for 10 min. The proportion of dead *versus* viable cells was then analyzed by flow cytometry analysis using a FACScalibur (BD Biosciences). All FACS analyses were repeated twice and revealed essentially similar results.

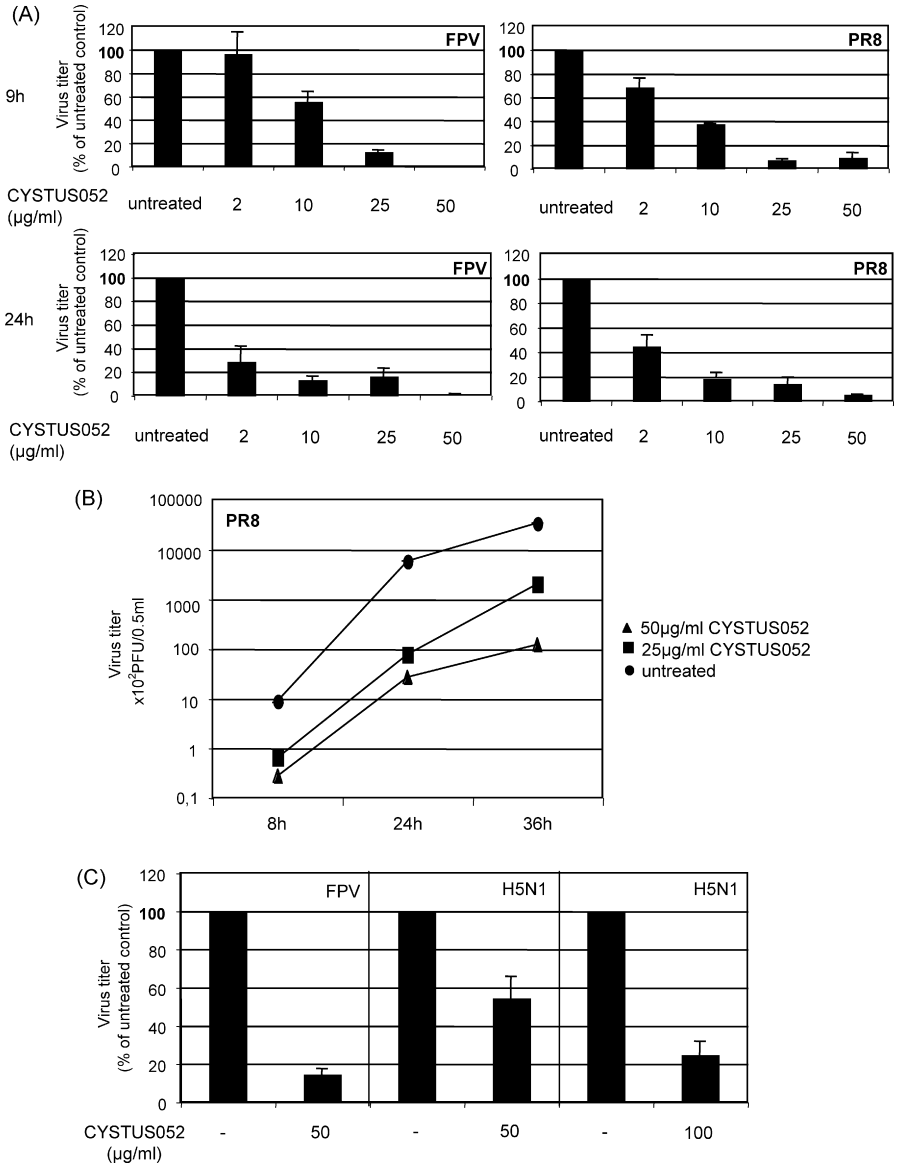

Fig. 1. CYSTUS052 inhibits influenza virus propagation. A549 (A, left panel; B) or MDCK (A, right panel) cells were left untreated or treated with the indicated amounts of CYSTUS052 before and during infection with the influenza A virus strains FPV (MOI = 0.001) (A, left panel) or PR8 (MOI = 0.01) (A, right panel; B) which were either left untreated or pretreated with CYSTUS052 as well. Supernatants were assayed for progeny virus yields 9 and 24 h (A) or 8, 24 and 36 h (B) post infection in standard plaque titrations. Virus titers (plaque forming units/ml) of mock-treated cells in (A) were 1.3×10^2pfu/ml (9 h) or 9.1×10^6pfu/ml (24 h) (FPV, left panel) and 3.2×10^2pfu/ml (9 h) or 1.1×10^3pfu/ml (24 h) (PR8, right panel), respectively, and were arbitrarily set as 100%. In (B) the pfu are given on the axis of the graph. (C) A549 cells were treated with the indicated amounts of CYSTUS052 before and during infection with FPV or the human isolate of the avian H5N1 Asia subtype (A/Thailand/1(KAN-1)/2004 (H5N1)) (MOI = 0.001). The virus was pretreated with CYSTUS052 as well. 20 h post infection supernatants were taken and assayed for progeny virus yields in standard plaque titrations. Virus yields of mock treated cells were arbitrarily set as 100%.

3. Results

3.1. CYSTUS052 treatment results in an efficient and concentration-dependent reduction of progeny virus titers

In a first set of experiments we attempted to assess whether CYSTUS052 may exert an antiviral effect on influenza virus propagation in cultured cells. We used A549 lung epithelial cells (A549) or Madine Darby canine kidney (MDCK) cells, both standard host cell lines for influenza virus propagation. These cells were incubated with the plant extract at various concentrations and subsequently infected with different influenza A virus strains. This included the human prototype isolate A/Puerto-Rico/8/34 (H1N1) (PR8), the highly pathogenic avian influenza virus (HPAIV) A/FPV/Bratislava/79 (H7N7) (FPV) as well as a human isolate of the HPAIV of the H5N1 subtype (A/Thailand/1(KAN-1)/2004 (H5N1)). The concentrations of the various extract dilutions were kept constant throughout the experiment in each sample (Fig. 1A and B) and showed a dose dependent reduction of progeny virus titers of FPV and PR8. Maximum reduction of progeny virus titers in the order of two logs was observed with both viruses in A549 cells using CYSTUS052 at a concentration of 50 μg/ml (Fig. 1A and B). This reduction is not due to a simple delay in virus replication since it can be consistently observed in growth kinetics of PR8 over a 36 h observation period (Fig. 1B). CYSTUS052 was also effective against the HPAIV of the H5N1 subtype; however, compared to FPV, an HPAIV of the H7 subtype, a higher concentration was needed to achieve the same degree of reduction of progeny virus titers (Fig. 1C). These data indicate that CYSTUS052 has broad antiviral properties towards different influenza viruses.

3.2. CYSTUS052 treatment does not affect cell morphology and viability and does not negatively interfere with cellular proliferation and metabolism

A major prerequisite for an antiviral agent is safety. Thus, we tested whether CYSTUS052 in the concentrations used would have any harming effect on the healthy cell. In an initial approach cells treated with CYSTUS052 for different time points (up to 72 h) were examined morphologically (data not shown). No differences in cell shape or cell numbers could be observed compared to control cells. The same cells were stained with propidium iodide (PI) to detect the numbers of dead cells in each sample (Fig. 2A). Again no significant changes in the number of dead cells could be detected over a 72 h observation period. CYSTUS052 did also not negatively interfere with the proliferative and metabolic capacity of cells as determined in MTT assay (data not shown). In this assay the activity of a mitochondrial metabolic enzyme that is only active in proliferating healthy cells is monitored. Consistent with these data, CYSTUS052 did also not induce apoptosis as evidenced by a lack of cleavage of Poly-ADP-ribose-polymerase (PARP), a prominent substrate of apoptotic caspases (Fig. 2B).

While the extract did not show any effects on cell proliferation or cell survival, it may still be that cellular responses may be significantly altered in CYSTUS052 treated cell. Thus, the effect of CYSTUS052 on cellular gene transcription and

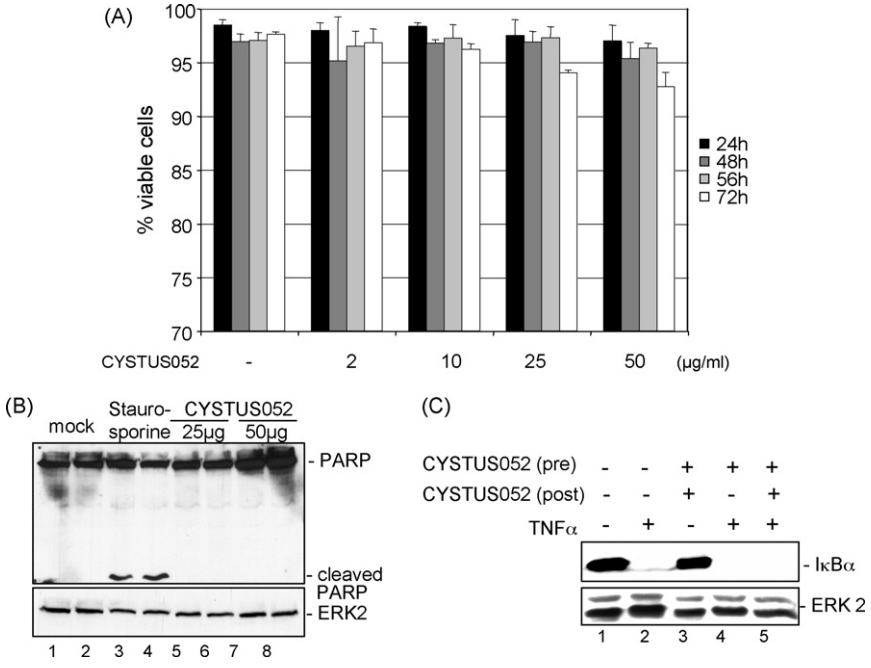

Fig. 2. CYSTUS052 is not toxic or proapoptotic and does not interfere with cellular responses to cytokines. MDCK (A) cells were left untreated or treated with the indicated amounts of CYSTUS052 for the indicated time points and were subjected to propidium iodide (PI, 1 μg/ml) staining and subsequent flow cytometry analysis using a FACScan (BD FACScalibur). (B) A549 cells were left untreated or were treated with 2.5 μM staurosporine for 8 h or 25 μg/ml and 50 μg/ml CYSTUS052 for 48 h, respectively. Upon stimulation cell lysates were prepared and subjected to Western blots, and caspase mediated PARP cleavage was determined as a measure for apoptosis induction. (C) A549 cells were pretreated with CYSTUS052 for 30 min or left untreated. Subsequently cells were stimulated with TNFα for 15 min in the absence or presence of CYSTUS052. Cell lysates were subjected to SDS Page and Western-blot using an anti-IκBα antiserum to detect TNFα-induced degradation of IκBα as a measure for NF-κB activity. Equal protein loads in (B) and (C) were verified in ERK blots.

translation was assessed in a reporter gene assay using cells that were transfected with a vector expressing luciferase under the control of a constitutively active CMV promoter element (data not shown). CYSTUS052 treatment did not alter transcription or translation in this system. Finally, it was analyzed whether CYSTUS052 may alter the response to ligands that bind to cellular receptors. As an example, the cellular response to the cytokine TNFα was analyzed (Fig. 2C). A549 cells were treated with CYSTUS052 or left untreated. Subsequently cells were stimulated with TNFα, a strong activator of the cellular transcription factor NF-κB (Fig. 2C, lanes 2, 4 and 5). Activation of NF-κB was then monitored in Western blots by examination of degradation of IκBα, the inhibitor of NF-κB (Fig. 2C). In these assays IκBα was nearly fully degraded in TNFα-stimulated cells regardless whether the cells were pretreated with CYSTUS052 or not (Fig. 2C, lanes 4 and 5). This is indicative of an unimpaired response to TNFα stimulation and may stand as a general example for cellular ligand/receptor systems. In addition, these assays also demonstrated that CYSTUS052 alone is inert towards activation of NF-κB (Fig. 2C, lane 3) again indicating that the compound does not harm living cells.

3.3. CYSTUS052 affects early viral entry

In a next set of experiments we aimed to identify the step in the virus life cycle that is affected by CYSTUS052. Therefore, supernatants from cells that were treated with CYSTUS052 at different time points pre- and post-infection were analyzed for their content of progeny virus. A titer reduction could only be observed if cells were either pre-incubated with CYSTUS052 or

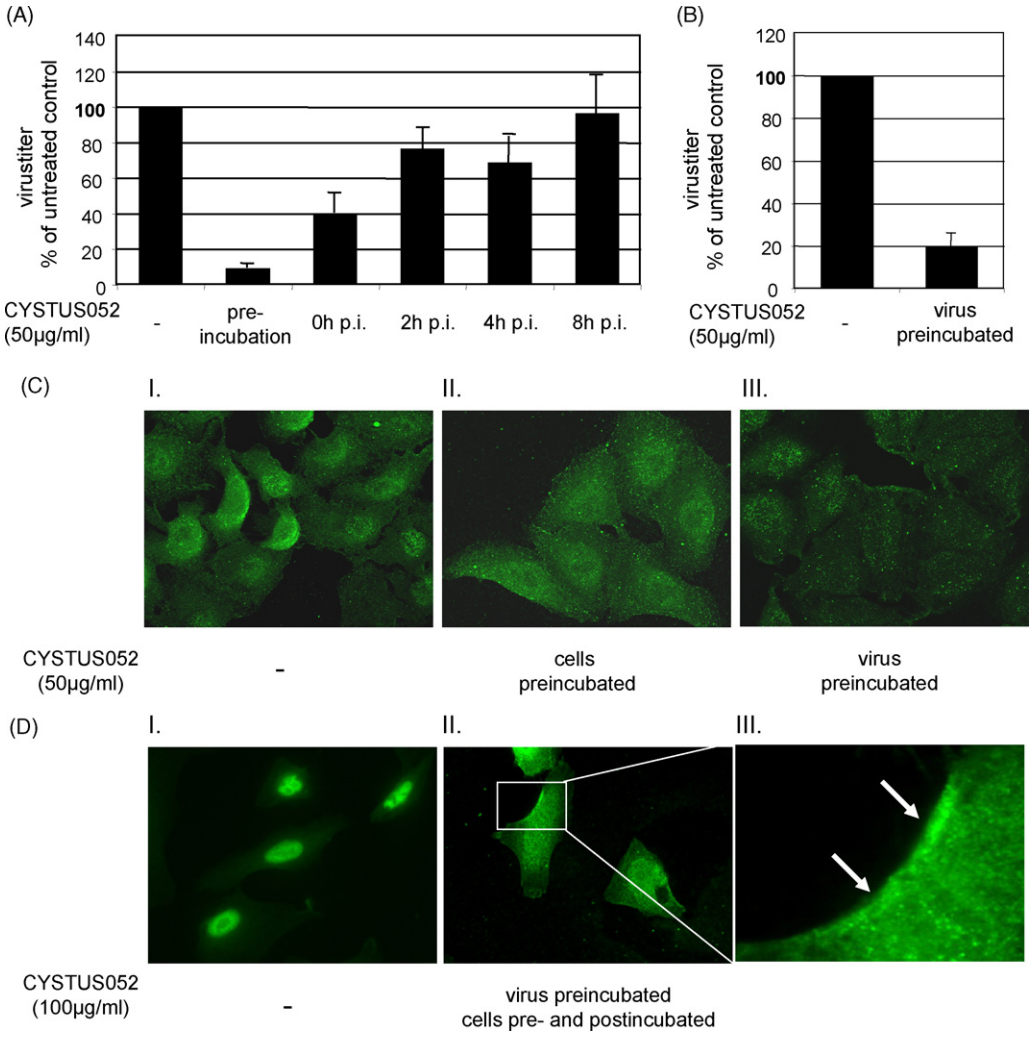

Fig. 3. CYSTUS052 appears to primarily inhibit the viral entry process. (A) MDCK cells were left untreated or treated with 50 μg/ml of CYSTUS052 at the indicated time points during infection with the influenza virus strain FPV (MOI = 0.001). (B) Influenza virus FPV was left untreated or pre-incubated for 2 h. 24 h post infection supernatants were taken and assayed for progeny virus yields in standard plaque titrations. Virus yields of mock-treated cells were arbitrarily set as 100%. (C) A549 cells were left untreated (I.) or were preincubated (II.) with 50 μg/ml CYSTUS052 for 30 min and infected with the influenza virus strain FPV (MOI = 200) for 1 h. In (III.) FPV was pre-incubated with 50 μg/ml CYSTUS052 overnight and subsequently used for infection. (D) A549 cells were left untreated (I.) or treated with CYSTUS052 (100 μg/ml) (II., III.) before and during infection with FPV (MOI = 50). FPV was left untreated or was preincubated (30 min) with CYSTUS052 (100 μg/ml) as well. 1 h (C) or 1.5 h (D) after infection RNP localization was determined by indirect immunofluorescence using a NP-specific mouse monoclonal antibody and an Alexa Fluor 488 chicken anti-mouse IgG (H + L). Picture (III.) represents an enlargement of the inlay of the middle picture. The white arrowhead shows virus particles lining on the cell borders.

if the extract was added simultaneously with infection (Fig. 3A). This argues for a very early step in the virus life cycle that is affected by CYSTUS052. Interestingly, a pronounced titer reduction of progeny virus could also be detected if untreated cells were infected with a virus lot that was preincubated with CYSTUS052. This is a first indication that components of the CYSTUS052 extract may interfere with the virus particle itself to prevent infection. Thus, we analyzed whether CYSTUS052 would affect viral uptake. Cells were infected with a very high multiplicity of infection (MOI) to detect incoming virus according to an established immunofluorescence protocol to study entry of influenza A virus (Ehrhardt et al., 2006; Sieczkarski et al., 2003). Briefly, the ribonucleoprotein (RNP) complexes of virus particles are stained with an antibody against the nucleoprotein, the major constituent of the viral RNPs. In untreated cells newly synthesized NP is already visible 2 h post infection

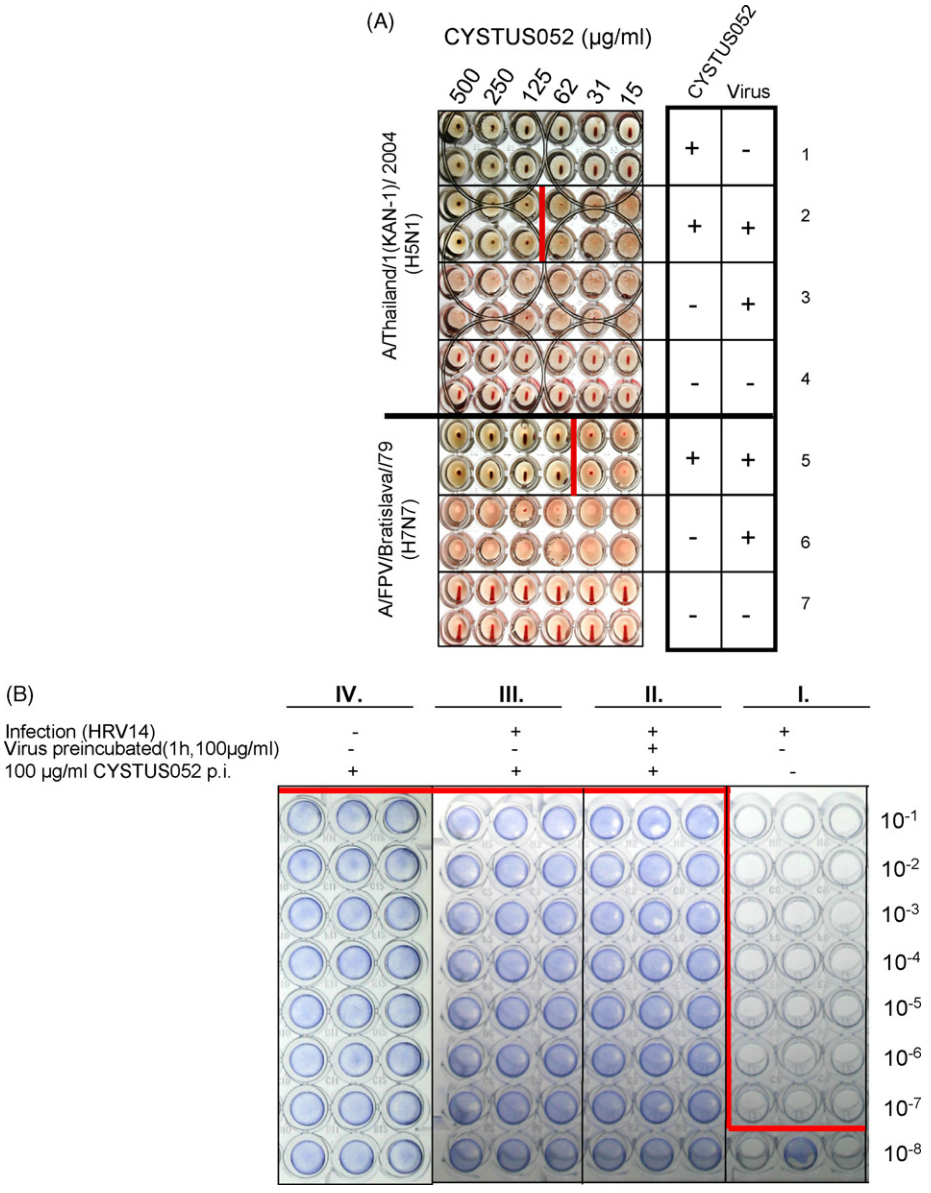

Fig. 4. CYSTUS052 inhibits hemagglutinating activity of influenza virus and blocks propagation of human rhinovirus HRV14 (A) CYSTUS052 was diluted as indicated. Virus stocks (H5N1 or H7N7) (4×10^4 pfu/well) were dissolved in PBS/BA and 50 µl was added per well of a 96-well plate. After preincubation of 45 min chicken erythrocytes (1/20 in PBS) were mixed with the solution. In the samples where viruses were preincubated with CYSTUS052, up to a certain dilution the viral particles are no longer capable of agglutinating erythrocytes, indicating an interaction of CYSTUS052 with the viral HA. (B) 5×10^4 HeLa cells in D-MEM medium were seeded into a 96-well plate to achieve 70% confluency 1 day later. The medium was then removed and replaced by 90 µl of the infection medium (D-MEM, 2% FCS, 10–20 mM $MgCl_2$) per well. The HRV14 virus titer was determined to 10^{-7}/ml CCID (I.). 10 µl of the virus suspension (treated (II.) or not treated (I., III., IV.) with 100 µg/ml CYSTUS052 extract as indicated) was mixed with 90 µl of the infection medium (supplemented (II., III., IV.) or not supplemented (I.) with 100 µg/µl CYSTUS052 extract, as indicated) in the first well (triplicates) and then was serially diluted in 1:10 steps in the subsequent wells. The 96-well plate was then incubated for 5 days at 33 °C in a cell culture incubator. At day 5 the plate was washed twice with PBS before staining cells in each well with 100 µl a crystal-violet staining solution (0.07% in EtOH) for 5 h at room temperature. The plate was subsequently washed several times with water and dried. The blue staining indicates non-lysed cells, while a white stain indicates that cells were destroyed by the lytic virus infection.

in the cell nucleus (Fig. 3C and D, left). If cells were pretreated with CYSTUS052 nuclear NP staining was more diffuse and there was enhanced detection of bright spots, which most likely represent RNPs of viral particles sticking to the cell surface or in intracellular compartments (Fig. 3C, middle). This effect was even more pronounced if the virus lot used for infection was pre-treated with CYSTUS052 (Fig. 3C, right) Here, newly synthesized NP is only poorly visible in the cell nucleus, however, a massive spot-like staining indicates the presence of virus particles that did not effectively enter the cell. For some cells a staining pattern could be observed that is consistent with virus particles lining-up in close proximity to the membrane without the ability to fully enter (Fig. 3D, right, white arrowheads). Taken together, CYSTUS052 treatment of virus particles appears to inhibit virus uptake.

3.4. CYSTUS052 blocks hemagglutinating activity of pre-treated virus particles

The data so far suggest that components of the CYSTUS052 extract directly interferes with the virus particle itself to inhibit infection. To study whether CYSTUS052 would prevent the ability of the virus particle to bind to cell surface receptors, we employed hemagglutination inhibition (HI) assays. Influenza A viruses are able to agglutinate red blood cells (RBC) *via* the viral glycoprotein, the hemagglutinin, that binds to N-acetylneuraminic acid at the cell surface. The RBC get cross-linked by the virus and will form a type of lattice in this case. This results in a diffuse appearance of the RBC in a round-bottom vial (Fig. 4A panel 3 and 6) in contrast to a spot-like appearance of precipitated RBC (or line shaped when plate is tilted) in the absence of any virus (Fig. 4A, panel 1, 4 and 7). CYSTUS052 pretreatment prevented binding of different viruses to RBC in this assay (Fig. 4A, panel 2 and 5) indicating that components of the CYSTUS052 extracts are capable of directly interfering with the viral HA to block binding to cellular receptors. Interestingly, a lower concentration of CYSTUS052 was sufficient to prevent binding of FPV compared to the H5N1 isolate, which is consistent with the previous observation that higher concentrations of CYSTUS052 are required to inhibit propagation of A/Thailand/1(KAN-1)/2004 (H5N1) (Fig. 1C).

3.5. CYSTUS052 inhibits infection of cells with human rhinoviruses

Direct interaction of CYSTUS052 components with proteins at the surface of the virus particle would suggest a rather unspecific physical mode of antiviral action. If this would be the case, CYSTUS052 should not only block infectivity of influenza A viruses but also other viruses by interference with surface proteins. This hypothesis was tested using a different respiratory virus, namely human rhinovirus (HRV14). Since rhinoviruses, in contrast to influenza viruses, are non-enveloped viruses, this experiment further allowed to discriminate whether presence of a glycoprotein containing viral envelope is required for CYSTUS052 action, HRV14 was used in a concentration sufficient for full lysis of infected HeLa cells up to a dilution of 10^{-7}.

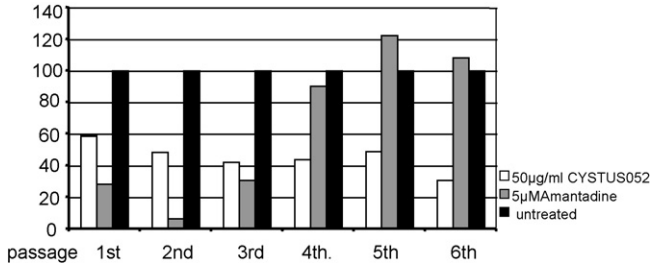

Fig. 5. CYSTUS052 shows no tendency to induce virus drug resistance. MDCK cells were infected with the influenza A virus strain FPV (MOI = 0.01) and were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine. At 24 h post infection supernatants were taken and employed for infection in the second round of investigation. After infection cells were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine again. This procedure was repeated six times. Supernatants were assayed for progeny virus yields in standard plaque titrations. Virus yields of mock-treated cells were arbitrarily set as 100%.

If viruses or cells were preincubated with CYSTUS052, cell lysis that indicates infectivity of HRV14 is completely prevented even at the highest virus concentration used (Fig. 4B). This indicates that CYSTUS052 also efficiently blocks propagation of the non-enveloped HRV14, presumably by direct interaction with its viral protein surface.

3.6. CYSTUS052 does not show any tendency to induce viral resistance

A most crucial issue regarding an anti-influenza virus agent is the emergence of resistant variants to the drug. According to the data presented so far, components of the CYSTUS052 extract appear to act in a rather unspecific physical manner to interfere with the surface of viruses. In this case, the virus should not easily overcome the inhibitory effect by mutations of single amino-acid residues at its surface proteins. This was indeed the case as assessed in an established multi-passaging experiment to detect emergence of resistant viruses in cell culture (Ludwig et al., 2004). As shown in Fig. 5, in this assay virus titers from cells treated with amantadine, a blocker of the viral M2 ion channel that rapidly results in the generation of resistant variants, were up to levels of untreated cells after the fifth passage, indicating that this pool of viruses had become fully resistant to the drug. This was different for infected cells treated with CYSTUS052. While the initial inhibition of virus propagation was less efficient than in amantadine-treated cells, titers did not rise with increasing passage numbers but even appeared to slowly decrease. This indicates that in contrast to amantadine, influenza virus does not easily become resistant to CYSTUS052 treatment.

4. Discussion

Infections with influenza A viruses are still a major health burden and the options for control and treatment of the disease are limited. Here we show that an extract derived from a special variant of the Mediterranean plant *Cistus incanus*, CYSTUS052, exerts a potent antiviral activity in cell culture. On a molecular basis the extract appears to interfere with the virus

surface proteins and inhibits binding of the virus particle to cellular receptors. We cannot completely rule out an additional contribution of binding of CYSTUS052 components directly to the cell surface, e.g. to proteins, oligosaccharides or sialic acids (SA), that are receptors for influenza virus. However, since SA are widely distributed on cellular proteins on the surface, a pronounced binding to these components should harm some of the cellular functions examined (which was not the case) or binding of ligands to the cell surface, as analyzed in Fig. 2C.

CYSTUS052 has a very high polyphenol content. It is well known that polyphenols exhibit protein-binding capacity, suggesting that ingredients of CYSTUS052 may interact with pathogens via such a physical and unspecific interaction. This is not only supported by our own finding that CYSTUS052 also blocks propagation of rhinoviruses but also by data from the literature showing that the extracts from *Cistus* plants exert antibacterial and antifungal activities (Bouamama et al., 1999). The advantage of such an unspecific action may be that resistant variants cannot easily emerge and that the compound may also act against bacterial co-infections that represent a major complication in severe influenza virus infections. An unspecific interaction with the viral HA has been reported for the polyphenolic compound epigallocatechin-gallate (Nakayama et al., 1993). However, polyphenols are a very large group of chemical compounds and to date the active moiety of CYSTUS052 is not known. Nevertheless, our experimental evidence suggests that the high content of polymeric polyphenols in CYSTUS052 is required for its antiviral activity. Extracts of other polyphenol rich plants but with a lesser content of polymeric forms, e.g. from the sage plant did not exert any anti-influenza virus activity (data not shown). In fact CYSTUS052 was chosen as the most potent antiviral acting extract from an array of extracts that not only differed in plant species but also in the biochemical variety of the highly polymorphic genus *Cistus* (data not shown). Another indication of the polymeric nature of the active component comes from first attempts to fractionate the extract. None of five different soluble fractions representing around 60% of the original extract showed an antiviral or hemagglutination-inhibition activity. A final indirect indication that the active components of CYSTUS052 are of higher molecular weight and may not enter the cell comes from experiments shown in Fig. 2. Although polyphenols are known as protein binders and antioxidants, no negative effect on proliferation, metabolism, transcriptional/translational activity or responsiveness of the cell to ligands could be attributed to CYSTUS052, which may be due to the simple reason that the active component is not taken up into the cell. This would of course imply, that CYSTUS052 as an antiviral agent has to be applied locally, e.g. as an aerosol into the lung of the patients. Consistent with that view, experiments by Planz and coworkers (Droebner et al., 2007) have recently demonstrated the potent antiviral activity of CYSTUS052 by this application route in a mouse infection model.

Although the efficacy of CYSTUS052 action in infected individuals has still to be proven, the plant extract may have several advantages with regard to its use as an antiviral agent. First, *Cistus* plant extracts have been used for centuries in traditional medicine without reports of side effects or allergic reactions. Besides a therapeutic application, this would even allow prophylactic use in risk groups. Furthermore, by its rather unspecific and broad action directly on the pathogen, CYSTUS052 may not easily result in the emergence of virus drug resistance and may also be active against opportunistic bacterial infections.

Given that there is an urgent need for new and amply available anti-influenza drugs, an inhalable formulation of the CYSTUS052 appears to be a promising option as a replacement or supplementary strategy to currently available anti-influenza therapeutics.

Acknowledgements

We thank Andrea Stadtbäumer, Carmen Theseling, and Sandra Neumann for excellent technical assistance. This work was supported by grants of the Deutsche Forschungsgemeinschaft (DFG), the DFG Graduate Schools GRK1045 and GRK1409 and the fund "Innovative Medical Research (IMF)" of the University of Muenster Medical School. Furthermore, this work is part of the activities of the EUROFLU Consortium and the VIRGIL European Network of Excellence on Antiviral Drug Resistance supported by grants from the Priority 1 "Life Sciences, Genomics and Biotechnology for Health" program in the 6th Framework Program of the EU.

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