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CYSTUS052, a polyphenol-rich plant extract, exerts anti-influenza virus activity in mice

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Abstract

Influenza, a respiratory disease caused by influenza viruses, is still a worldwide threat with a high potential to cause a pandemic. Beside vaccination, only two classes of drugs are available for antiviral treatment against the pathogen. Here we show that CYSTUS052, a plant extract from a special variety of *Cistus incanus* that is rich in polymeric polyphenols, exhibits antiviral activity against a highly pathogenic avian influenza A virus (H7N7) in cell culture and in a mouse infection model. *In vitro* and *in vivo* treatment was performed with an aerosol formulation, because the bioavailability of high molecular weight polyphenols is poor. In MDCK cells, a 90% reduction of plaque numbers on cells pre-incubated with the plant extract was achieved. For *in vivo* experiments we used a novel monitoring system for influenza A virus-infected mice that allows measurement of body temperature and gross motor-activity of the animals. Mice treated with CYSTUS052 did not develop disease, showed neither differences in their body temperature nor differences in their gross motor-activity and exhibited no histological alterations of the bronchiolus epithelial cells.

Keywords: Influenza A virus; *Cystus incanus*; Polyphenols

1. Introduction

Influenza is still one of the major plagues worldwide. This respiratory disease is caused by influenza A viruses that are highly contagious pathogens for humans and several animal species. Beside annual epidemics, these pathogens are also capable to cause pandemic outbreaks. Three of these pandemics occurred in the last century, in 1918, 1957, and 1968 (Wright and Webster, 2001; Palese, 2004). The most severe pandemic outbreak known as the "Spanish flu" occurred in 1918 and caused at least 20–40 million deaths worldwide (Reid et al., 2001; Taubenberger et al., 2001). The statistical likeliness of a new pandemic outbreak and the emergence of highly pathogenic avian influenza viruses (HPAIV) of the H5 and H7 subtype that infected and killed humans highlight the urgent need for new and amply available antiviral drugs. At present, even though H5N1 influenza A viruses occasionally infect humans, they are not freely transmissible between humans. Nevertheless, due to mutations and/or reassortment of genes with circulating human influenza A viruses H5 and/or H7 strains might acquire such transmissibility between humans.

Vaccination is the first option for controlling influenza and reducing the impact of pandemics. Changes of the viral proteins require annual adaptation of the influenza vaccine formulation. In addition, antiviral drugs provide a fundamental complementary line of defence, particularly for fast spreading pandemic influenza A virus strains where vaccines might be not available in time (Ferguson et al., 2005; Longini et al., 2005). Only two classes of anti-influenza virus drugs are currently accessible. These are viral neuraminidase inhibitors (oselatamivir and zanamivir) and viral M2 ion channel protein inhibitors (amantadine, rimantadine) (Wright and Webster, 2001; De Clercq, 2004). The emergence of influenza A viruses resistant to the M2 inhibitors occurs at high frequency in treated patients (Cox and Subbarao, 1999; Suzuki et al., 2003). Many of the human isolates of H5N1 viruses are already resistant to these inhibitors (Puthavathana et al., 2005). In addition, a recent study has shown that influenza A viruses resistant to the neuraminidase inhibitor oseltamivir occurred in 20% of the children treated with this

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drug (Kiso et al., 2004). In fact, H5N1 viruses that are partially resistant to oseltamivir have recently been reported (Le et al., 2005). The emergence of influenza A viruses resistant to these two classes of antiviral drugs highlights the need for additional antiviral drugs against these pathogens. Even though several antiviral compounds have been developed against influenza virus, their long-term efficacy is often limited due to toxicity or the emergence of drug-resistant virus mutants (Hayden, 2006).

Phenolic compounds, or polyphenols, constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently known. Polyphenols are products of the secondary metabolism of plants. The structure of natural polyphenols varies from simple molecules, such as phenolic acids, to highly polymerized compounds (Harborne, 1980). Polyphenols exhibit a wide range of biological effects. Bioavailability differs greatly from one polyphenol to another (Manach et al., 2005). The knowledge of absorption, bio-distribution and metabolism of polyphenols is partial and incomplete. Some polyphenols like epigallocatechin (EGC) are bioactive compounds that are absorbed from the gut, while most polymeric polyphenols like flavan-3-ols and proanthocyanidins are not absorbed (Urquiaga and Leighton, 2000; Manach et al., 2004). Due to the overall poor absorption and low plasma concentrations of polymeric polyphenols, it was suggested that phenols might exert direct effects (Halliwell et al., 2005). Plant-derived polyphenols have been shown to be strong antioxidants with potential health benefits as they are able to protect the heart, support the biological activity of vitamin C, protect from premature skin ageing, and bind viruses and bacteria (Urquiaga and Leighton, 2000; Arts and Hollman, 2005; Halliwell et al., 2005). Various reports exist on antiviral and antibacterial potential and the mode of action of polyphenols (reviewed in Cos et al., 2004), including several reports describing the antiviral activity of polyphenols against influenza virus. Epigallocatechin gallate (EGCG) and theaflavin digallate, two polyphenols present in tea are able to bind the hemagglutinin of influenza virus and thus block its infectivity (Nakayama et al., 1993). In another investigation it was shown that the antiviral effect of epigallocatechin gallate, epicatechin gallate (ECG) and epigallocatechin on influenza virus is mediated not only by specific interaction with the hemagglutinin, but also by altering the physical properties of viral membrane (Song et al., 2005). Other reports demonstrate an antiviral activity of a polyphenol-rich extract from the medicinal plant *Geranium sanguineum* L. against influenza virus *in vitro* and *in vivo* (Ivanova et al., 2005; Sokmen et al., 2005). Resveratrol, another polyphenol found in grapes strongly inhibited the replication of influenza virus in cell culture by blockade of the nuclear-cytoplasmic translocation of the viral ribonucleoprotein complex, by reducing expression of late viral proteins and by inhibition of protein kinase C (PKC) activity and PKC-dependent pathways (Palamara et al., 2005).

CYSTUS052 is a plant extract that is very rich in highly polymeric polyphenols. The extract is a preparation of a selected variety of the biochemical polymorphic species *Cistus incanus*. *Cistus incanus* (Pink Rockrose) is one species of the genus *Cistus* L. The genus *Cistus* comprises a group of about 20 shrub species found in wide areas throughout the whole Mediterranean region to the Caucasus. *Cistus* species are used as 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). Being one of the main constituents of the Mediterranean-type maquis, this plant genus is peculiar in that it has developed a range of specific adaptations to resist summer drought and frequent disturbance events, such as fire and grazing (Comandini et al., 2006). CYSTUS052 extract was defined by the producer as having a content of polymeric polyphenols in particular flavan-3-ols and proanthocyanidins of around 26% (determined after Singleton and Rossi, 1965; Singleton et al., 1999). The total content of monomeric components like gallic acid, gallocatechin, catechin, epicatechin was lower than 2% (Danne et al., 1993; Petereit et al., 1991).

In the present communication we raised the question if the polyphenol-rich plant extract CYSTUS052 exert antiviral activity against influenza virus. The bioavailability of flavan-3-ols and proanthocyanidins are poor. Therefore, with regard to an antiviral action against influenza virus a local administration in an aerosol formulation would be more favorable. Here, we demonstrate that CYSTUS052 inhibits influenza A virus replication *in vitro*. Furthermore, CYSTUS052 aerosol treatment of mice infected with a mouse-adapted highly pathogenic avian influenza virus (FPV, H7N7) protected the animals against clinical disease symptoms. From our data we propose a mechanism, where polyphenolic ingredients of CYSTUS052 extract block virus infection by a direct (physical) interaction with the virus particles.

2. Experimental/materials and methods

2.1. Mice

Inbred female Balb/c and C57Bl/6 mice at the age of 6–8 weeks were obtained from the animal breeding facilities at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tübingen, Germany and were used throughout all the experiments.

2.2. Virus and infection

Mouse-adapted avian influenza A/FPV/Bratislava/79 (H7N7) (FPV) virus grown on Madin-Darby canine kidney (MDCK) cells was used throughout this study. The LD_{50} of the virus stock used in the present study was 1×10^2 pfu (plaque forming units). The Bratislava strain of the H7N7 avian influenza A virus (FPV) was originally obtained from the Institute of Virology, Justus-Liebig University, Giessen, Germany and further propagated at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tübingen, Germany.

The *in vitro* infections were performed in an aerosol-chamber (5.0×10^{-2} m^3); 46 cm × 33 cm × 33 cm l/[w h]). Two PARI® aerosol nebulizers (PARI, Starnberg, Germany, Art. No. 73-1963) were connected to either the left or right side of the chamber (Fig. 1A). Ten 3.5 cm Petri dishes with MDCK cells

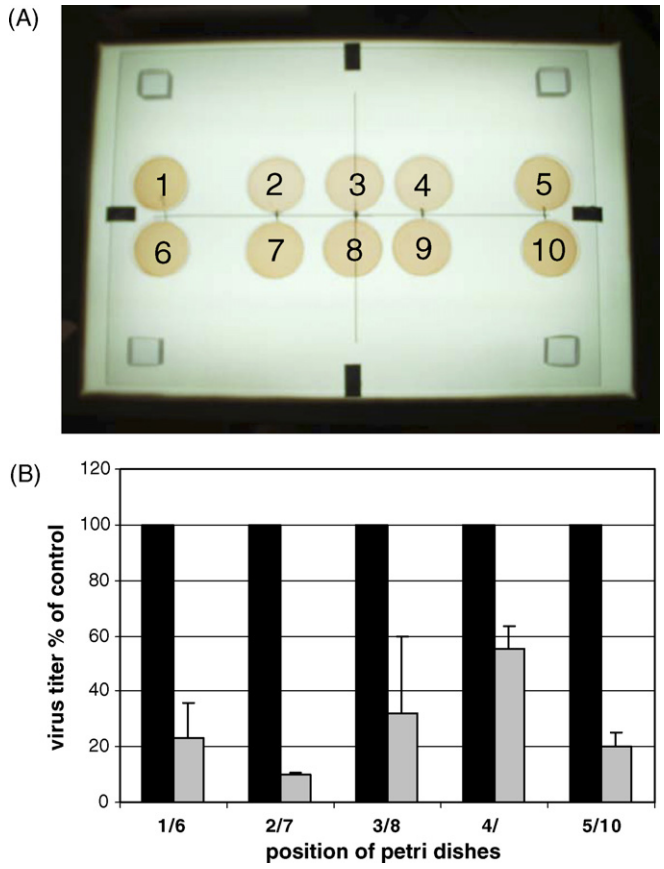

Fig. 1. Reduction of influenza A virus plaque formation after aerosol CYSTUS052 extract treatment. (A) Ten 3.5-cm Petri dishes with a monolayer of MDCK cells were placed at different positions in the aerosol chamber (for detail, see Section 2). (B) Virus plaques on MDCK monolayers after treatment with nebulized PBS were calculated as 100% (black bars). Plaque reduction after CYSTUS052 extract treatment compared to control was given (grey bars). A mean value for positions 1 and 6, 2 and 7, 3 and 8, 4 and 9, 5 and 10 was calculated for each experiment. The data represents the mean value of five independent experiments.

were placed without lid into the chamber as demonstrated in Fig. 1A. Nebulized virus preparation was introduced to the chamber from the left side with 1.5 bar for 10 min (total volume of 2 ml), while either CYSTUS052 extract (1 mg/ml; Charge-Number: 40121T01B/04) or in a different experiment buffer solution with a pressure of 1.5 bar was given for 20 min (roughly 4 ml) from the right side to the chamber starting 10 min prior to virus infection. After infection Petri dishes were placed in a 37 °C incubator with 5% CO_2 for 48 h. Thereafter, the cells were stained for plaque formation as described previously (Ölschläger et al., 2004).

For infection of mice, the animals were anaesthetized by intraperitoneal injection of 150 μl of a ketamine (Sanofi, Germany) rompun (Bayer-Leverkusen, Germany)-solution (equal amounts of a 2%-rompun solution and a 10%-ketamin solution were mixed at the rate of 1:10 with PBS) and infected intranasally (i.n.) with 1×10^2 pfu/50 μl of FPV (H7N7). For infection of mice with FPV pre-incubated with CYSTUS052, either 10^2/25 μl, 10^3/25 or 10^4/25 μl FPV was incubated either with 25 μl CYSTUS052 (1 mg/ml) or with 25 μl PBS for 30 min at room temperature. Thereafter, mice were infected i.n. with 50 μl of FPV/CYSTUS052 or FPV/PBS.

2.3. CYSTUS052 extract

CYSTUS052 extract was supplied and originally developed by Dr. Pandalis NatUrprodukte GmbH & Co. KG (Charge-Number: 40121T01B/04, 8-2004; Glandorf). The extract is a special preparation from a distinct variety of *Cistus incanus* (*Cistus incanus* PANDALIS). CYSTUS052 extract was defined by the producer as having a polyphenolic content of more than 26% (determined after Singleton and Rossi, 1965; Singleton et al., 1999). The total content of monomeric components (gallic acid, gallocatechin, catechin, epicatechin) was lower than 2%. CYSTUS052 granulate was solved either in sterile PBS (for *in vitro* experiments) or in sterile H_2O (for *in vivo* experiments) for 10 min at 60 °C or up to 1 h at 100 °C until the granulate was completely solved. The extract was stored at RT for 1 week.

2.4. Treatment of Balb/c mice with CYSTUS052

Treatment of mice was performed in inhalation chambers. Either five mice were treated at the same time in an inhalation chamber (2.1×10^{-2} m^3) or single mice were treated in an inhalation tube. Five tubes were connected to a central cylinder with an overall volume of 8.1×10^{-4} m^3. A PARI® nebulizer (Aerosol Nebulizer; Art. No. 73-1963) was connected to either the inhalation chamber or the central tube cylinder. CYSTUS052 extract (10 mg/ml; Charge-Number: 40121T01B/04) or buffer solution with a pressure of 1.5 bar was given for 10 min (roughly 2 ml) to the chambers. The CYSTUS052 extract was dissolved in sterile ddH_2O (stock solution: 10 mg Cystus/ml distillate water) and incubated for 1 h at 100 °C in a water bath. Balb/c mice were placed in the tube-cylinder and exposed to 2 ml of aerosolized CYSTUS052 extract for 10 min three times a day at 9:00 a.m., 12:00 a.m. and 3:00 p.m. The treatment was performed for 5 days. Ten minutes after the first treatment mice were infected with FPV. Balb/c controls were treated with the same amount of sterile ddH_2O. After FPV infection, the general health status of the animals was controlled twice a day. Furthermore, the animals were weighted every day. According to the German animal-protection law, the mice were sacrificed after they had lost 25% of their initial weight. All animals were monitored for 15 days after infection.

2.5. Mouse monitoring

The monitoring of body temperature and gross motor activity of the animals was performed with the Vital View® software and hardware system (Mini Mitter U.S.A.). This system allows data acquisition of physiological parameters. The hardware includes a transmitter (E-Mitter)/receiver system. The E-Mitter collects data on temperature and gross motor activity for the lifetime of the animal. The temperature sensitive devices alter their pulse rate in response to temperature changes. The Vital View system records the average rate and converts this to temperature using temperature calibration values specific to each unit. The

vitality or gross motor activity measurement provides a basic index of the movement of mice with implanted E-Mitter. As the mouse moves, movement of the implanted E-Mitter results in subtle changes in the transmitted signal. These small changes are detected by a receiver *via* telemetry and registered by a connected computer as activity counts. In our experiments the Vital View software recorded an index of movement every 5 min to produce a longitudinal record of the activity.

For implantation of the E-Mitters the mice were anesthetized with intraperitoneal injection of 150 μl ketamine/rompun. The ventral surface of the abdomen was shaved and a midline abdominal skin incision was made 0.5–1 cm below the diaphragm with no more then 2 cm in length. The abdomen was opened with a 2 cm incision along the *linea alba* and the E-Mitter was positioned in the abdominal cavity. After the incision was closed with two to three wound clips (autoclip 9 mm; Becton & Dickinson, Germany). The animals were placed into the cage and successful implantation of the E-Mitter was controlled by Vital View software. Health status was controlled for 7 days before infection.

2.6. LCMV infection and footpad swelling

Lymphocytic choriomeningitis virus strain WE was propagated in L-cells. Infection was performed with 10^4 ffu (focus forming unit) in 50 μl buffer solution into the left hind footpad. Delayed-type hypersensitivity reaction (DTH) was determined as local swelling after subcutaneous inoculation of virus into the left hind footpad of C57Bl/6 mice (Moskophidis and Lehmann-Grube, 1989). At daily intervals, the dorsoventral thicknesses of both hind feet were measured with a dial calliper (Oditest; Kroplin, Germany), and swelling was expressed as percent of the factor with which the thickness of the inoculated foot exceeded the thickness of the contra lateral (right) un-inoculated control foot.

2.7. Histology and immunohistology

Mice were treated with nebulized CYSTUS052 (10 mg/ml) three times at 9:00 a.m., 12:00 a.m. and 3:00 p.m for 10 min or H_2O (as control). Immediately after the treatment the mice were killed and lungs were obtained and fixed in buffered 4% paraformaldehyde. The lungs were stained with hematoxylin and eosin as described before (Ölschläger et al., 2004). Lectin staining of lung sections was performed with *Sambucus nigra* agglutinin (SNA; Vector Laboratories) for sialic acid α-2,6 linked to galactose and *Maackia amurensis* agglutinin (MAL II; Vector Laboratories) for sialic acid α-2,3 linked to galactose. Secondary staining was performed with an ABC-kit (Vector) for 30 min at RT. Substrate reaction was performed with DAB kit (Vector Laboratories).

2.8. Hemagglutination assay

Hemagglutination assays were carried out in V-bottomed microtiter plates using 50 μl of 2.5% suspensions of chicken red blood cells in PBS. Fresh chicken blood was supplemented with 1.6% sodium-citrate in sterile H_2O and was centrifuged at 800 × *g* for 10 min at room temperature to separate red blood cells. Thereafter the cells were washed for three times with PBS. Fifty microliters of a 10% lung homogenate was added and serially diluted in PBS. Assays were read following a 1 h-incubation on ice. Untreated red blood cells would precipitate to the bottom of the plate, while red blood cells incubated with influenza virus showed a diffuse distribution on the microtiter plates.

3. Results

3.1. Antiviral effect of CYSTUS052 extract in vitro

To investigate whether the CYSTUS052 extract exerts an antiviral action after being delivered as an aerosol, we performed infectivity studies in aerosol chambers. Both, the virus and the CYSTUS052 extract were applied to MDCK cells as an aerosol as described in detail in Section 2. Cells were cultured in Petri dishes and were placed at different positions in the chamber (Fig. 1A). As a control, MDCK cells were treated with nebulized PBS in a second set of experiment at the same way. When CYSTUS052 extract was nebulized in parallel or after virus infection, no differences regarding plaque formation on MDCK monolayers compared to control cells were detected (data not shown). In contrast, when MDCK cells were treated with CYSTUS052 extract 10 min prior to influenza virus infection with 10^5 pfu/ml, a reduction of plaque numbers on the MDCK cells was found. Depending on the position of the plate in the aerosol chamber the plaque reduction was up to 90% in MDCK monolayers that were pre-treated with CYSTUS052 extract (Fig. 1B). The absolute number of plaques in control cell cultures was $2–7 \times 10^2$ pfu. When an infectious dose of 10^6 pfu/ml was used for aerosol infection, again a reduction of plaque numbers between 70 and 90% was observed in cells that were treated with CYSTUS052 extract starting prior to infection (data not shown).

3.2. Evaluation of toxic effects in Balb/c mice and interference with the immune response after aerosol treatment with CYSTUS052 extract

In a first set of experiments the effects of CYSTUS052 extract on the health and immune status of uninfected mice was determined. Five female Balb/c mice were treated in an aerosol chamber, three times a day for five consecutive days with 2 ml CYSTUS052 extract (10 mg/ml, 1.5 bar) to investigate whether CYSTUS052 extract treatment induces any apparent side effects in the animals. In addition, five female Balb/c mice (littermates) were kept untreated and served as controls. Since mice treated with CYSTUS052 extract exhibited no differences in their body weight during the treatment (Fig. 2A) and showed no differences in the overall health status compared to untreated control animals, it can be concluded that the treatment with CYSTUS052 extract (10 mg/ml aerosol) is non-toxic for the animals. Furthermore, the results also show that the treatment procedure itself (three times a day for five consecutive days in an aerosol chamber) was not harmful to the animals. This indicates that the treatment-protocol is well suitable for experiments to investigate the influence of CYSTUS052 extract in mice after influenza A virus infection.

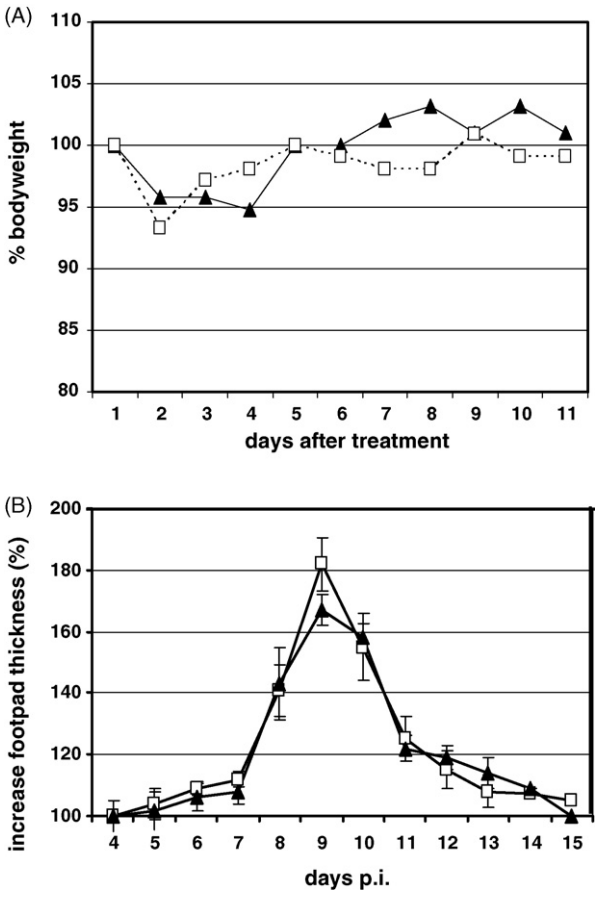

Fig. 2. No differences in the general health status of mice after treatment with CYSTUS052 extract. (A) Five female Balb/c mice were treated with CYSTUS052 extract (open squares) three times a day for five consecutive days or kept untreated (black triangles). For detail, see Section 2. The general health status of the mice was monitored until day 11 after the start of the treatment by measuring the weight of the animals. The graph represents the mean value of five mice (S.E.M. $\leq 5\%$). (B) No alteration of LCMV-induced footpad swelling after CYSTUS052 treatment. Five female C57Bl/6 mice were treated with H_2O (black triangles) and five littermates were treated with CYSTUS052 extract (open squares) three times a day for five consecutive days. The mice were infected into the left footpad with 10^4 ffu LCMW-WE 5 days prior treatment. The graph represents the mean value of five mice (S.E.M. $\leq 5\%$). The experiment was performed twice with similar results.

It is of common knowledge that certain polyphenols including some polyphenols found in CYSTUS052 extract have an anti-inflammatory function. To elucidate if local treatment of CYSTUS052 extract to the lungs would interfere with the systemic cellular immune response, we performed a classical and well-established footpad-swelling assay after lymphocytic choriomeningitis virus (LCMV) infection (Moskophidis and Lehmann-Grube, 1989). LCMV infection into the footpad induces a local delayed-type hypersensitivity (DTH) reaction that consists of phases that are sequentially mediated by class I-restricted cytotoxic $CD8^+$ T cells and class II-restricted $CD4^+$ T cells, cytokines/lymphokines and inflammatory cells. The infiltration of these immune cells and the immune mediators into the footpad results in a footpad-swelling that can be measured with a dial calliper. Interference with the immune system or immune suppression leads to reduced footpad swelling.

LCMV was injected into the left hind foot of C57Bl/6 mice, while the right foot served as a control. Aerosol-treatment with CYSTUS052 extract or H_2O was identically performed as described for the experiments above (see also Section 2). The treatment started 5 days after infection and 1 day prior to the first sign of footpad swelling that was first detectable at day 6 after LCMV infection. Until day 10 p.i., there was a strong increase in footpad swelling in mice that were either treated with CYSTUS052 extract or with H_2O. Thereafter, the swelling declined in both groups and after day 16 p.i. swelling was no longer detectable (Fig. 2B). In comparison of CYSTUS052 extract-treated mice with control animals, there was no difference in footpad-swelling. This experiment clearly demonstrates that CYSTUS052 extract aerosol treatment had neither a positive nor a negative effect on the systemic immune response against a viral pathogen.

3.3. Aerosol-treatment of influenza A virus-infected Balb/c mice with CYSTUS052 extract

To investigate whether CYSTUS052 extract has an antiviral effect *in vivo*, Balb/c mice were treated with CYSTUS052 extract as described above (Fig. 2A; open squares). To allow monitoring of body temperature and the activity of the mice during infection, transponders were implanted into the mice 7 days prior to infection as described in Section 2. To assure that all conditions including physiological stress were equal between the control group and mice that received CYSTUS052 extract, control mice were treated with H_2O, the solvent of CYSTUS052 extract. Treatment and infection were described in detail in Section 2. As expected 1 day prior to infection there was no difference between the two groups of animals regarding body temperature (Fig. 3A left panel) and activity status (Fig. 3B left panel). In general, Balb/c mice showed a circadian rhythm with respect to these two parameters. During daytime a decrease of body temperature and activity was observed. In the evening body temperature and activity increased again. Eight days after infection all control animals showed clinical symptoms, whereas none of the CYSTUS052 extract-treated animals developed disease. While CYSTUS052 extract-treated mice still showed a circadian rhythm of their body temperature (Fig. 3A right panel, black squares) and activity status (Fig. 3B right panel, black squares), a strong decrease of body temperature (Fig. 3A right panel, red squares) and vitality (Fig. 3B right panel, red squares) was found in control animals without any signs of a circadian rhythm. These symptoms correlated with the bodyweight of the animals. During the first 5 days after infection a slight weight loss was found in both groups that was similar to the loss of weight found in the experiment without infection. After 6 days post-infection the H_2O-treated mice developed first signs of clinical symptoms, like ruffled fur, reduced activity and disturbances of the circadian changes in body temperature. Furthermore, the animals of the control group started to loose weight (Fig. 3C). Weight loss increased until day 10 p.i. Thereafter the animals of the control group regained weight. In the described experiment 60% ($n = 3$) of the control mice died. In contrast, CYSTUS052 extract-treated mice did not develop clinical disease symptoms,

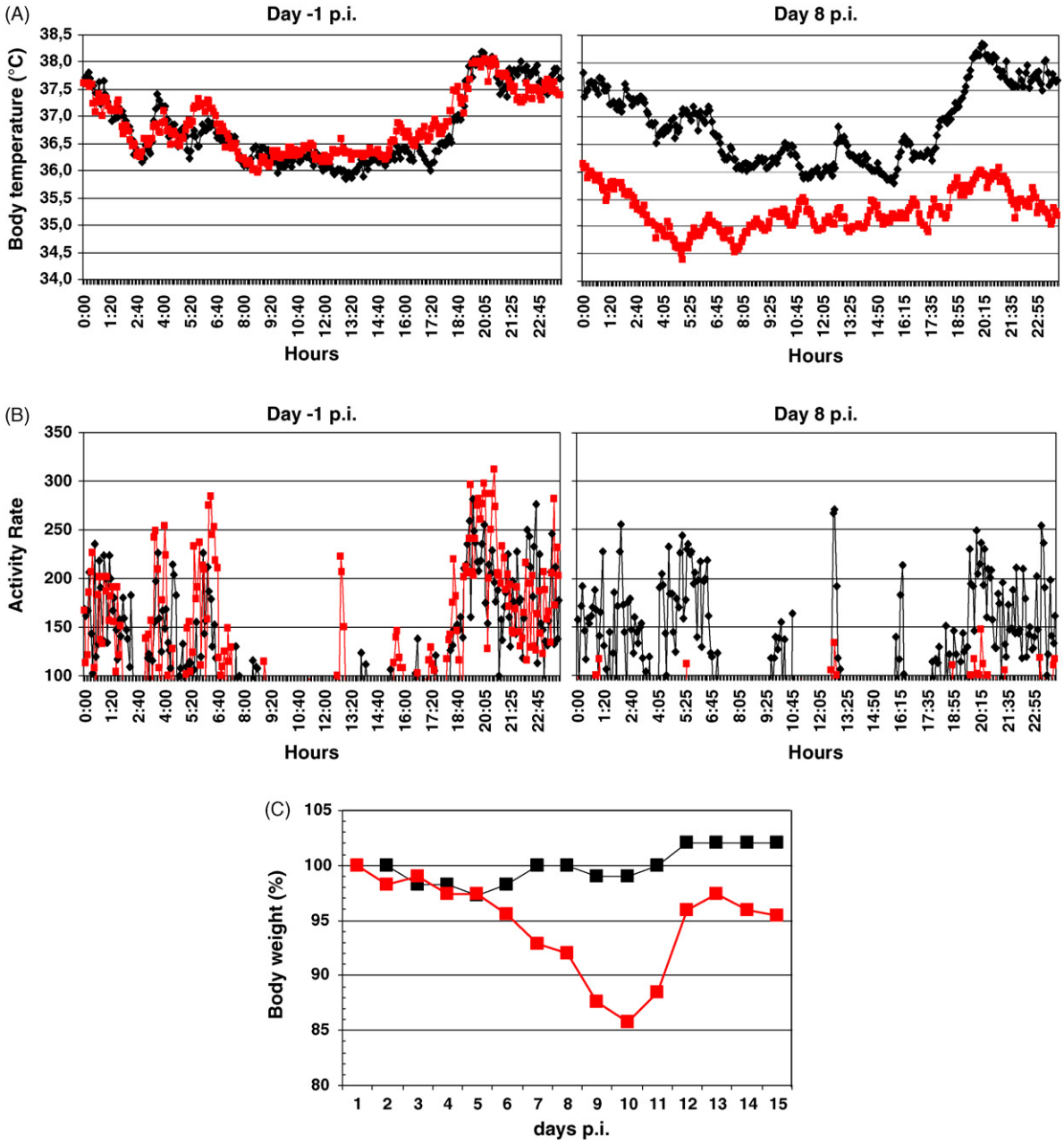

Fig. 3. Antiviral effect of CYSTUS052 treatment in FPV-infected mice. Five female Balb/c mice were treated with H_2O (red squares) and five littermates were treated with CYSTUS052 extract (black squares) three times a day for five consecutive days. Directly after the first treatment the animals were infected with 10^2 pfu/50 μl FPV. (A, left panel) No difference in the body temperature was found 1 day prior to infection. (A, right panel) Loss of circadian rhythm in control mice 8 days p.i. (B, left panel) No difference in the gross body-activity was found 1 day prior to infection. (B, right panel) Absence of gross body-activity in control mice 8 days p.i. (C) Body weight reduction in control mice 7–11 days p.i. All CYSTUS052 extract-treated animals showed no disease symptoms, while H_2O-treated mice developed disease starting at day 7 post-infection and three H_2O-treated mouse died. The H_2O-treated mice recovered between days 10 and 11 post-infection. The graph represents the mean value of five mice (S.E.M. $\leq 5\%$). Three independent experiments were performed with similar results, except one experiment, where two CYSTUS052 extract-treated mice developed disease and died (see also Table 1).

showed no weight reduction and no differences in the body temperature or activity status (Table 1). From this data one might speculate that CYSTUS052-treated mice were completely protected from the infection. Therefore, we looked for the presence of FPV 6 days after infection in the lung of CYSTUS052-treated and control mice. While virus was present in the lung of control mice (240 ± 60 HA units/ml), the amount of virus found in the plant extract-treated mice was drastically reduced (40 HA units/ml), indicating that CYSTUS052-treated mice were partially protected against the infection (Fig. 4A). Taken together, CYSTUS052 extract given three times daily for 5 days *via* an aerosol route resulted in effective reduction of influenza A virus infectivity in mice.

After successful treatment of FPV-infected mice (10^2 pfu/50 μl i.n.) with CYSTUS052 extract as an aerosol, we now raised the question whether oral application of

Table 1
Summary of the mouse-experiments with CYSTUS052 treatment

Treatment	Route	Number of animals	Disease symptoms	Onset of disease (day p.i.)	Survival
CYSTUS052	Aerosol	5	0/5	–	5/5
CYSTUS052	Aerosol	15	2/15[a]	8	13/15[a]
H_2O	Aerosol	15	13/15	7	7/15
CYSTUS052	Oral	10	10/10	7	2/10[*]
H_2O	Oral	10	10/10	7	2/10[*]

[a] Two mice from a single experiment developed disease and died.
[*] In one experiment 5/5 mice died in both groups.

CYSTUS052 extract would also suppress disease in influenza A virus-infected mice. Therefore, mice were treated with CYSTUS052 extract *via* the oral route three times a day for 5 days. Control mice were treated with H_20. In one experiment, already at day 6 p.i., both control mice and CYSTUS052-treated animals developed first signs of disease symptoms and lost weight. The severity of disease symptoms increased until day 8 p.i. By day 10 p.i. mice from both groups showed reduced disease symptoms and gained weight (Fig. 4B). In a second experiment, control mice and CYSTUS052-treated animals developed first signs of disease symptoms by day 6 p.i. and all mice died between days 8 and 10 after infection (Table 1). Taken together, CYSTUS052 extract given three times daily for five days *via* an aerosol route resulted in effective reduction of influenza A virus infectivity in mice, while no antiviral effect of CYSTUS052 extract was found after oral treatment of infected mice.

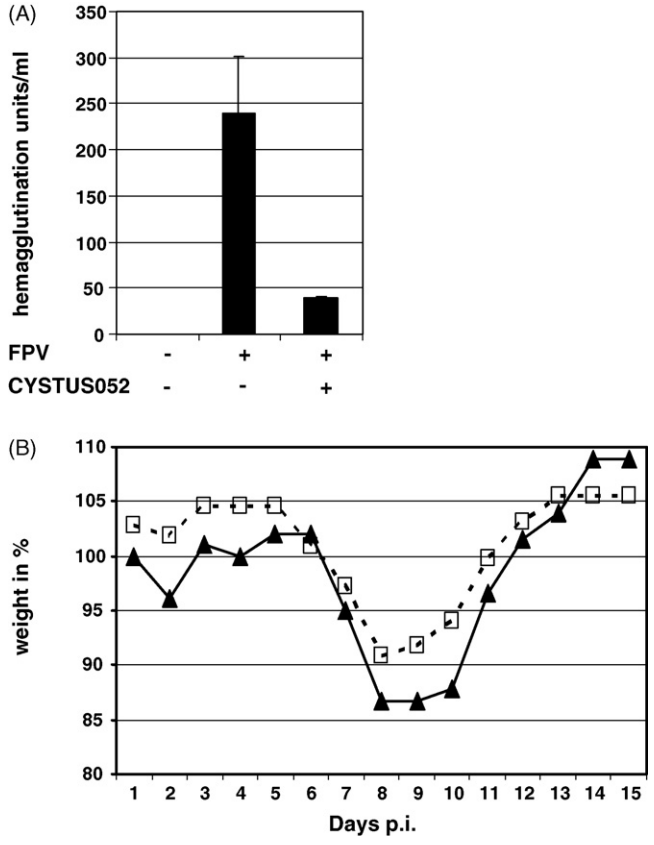

Fig. 4. Hemagglutination units and weight curves. (A) Balb/c mice were infected with 10^2 pfu/50 μl FPV and were treated as three times a day for 5 days either with CYSTUS052 (10 mg/ml) or with H_2O (control) in an aerosol formulation. Six days after infection the animals were killed, lungs were taken and a 10% homogenate of the lungs was produced. Fifty microliters of the lung-homogenate was added to a hemagglutinin assay. Lung-homogenate of uninfected mice showed no reaction with chicken red blood cells. The graph represents the mean value of three mice. (B) No antiviral effect against influenza virus infection after oral treatment of mice with CYSTUS052 extract. Five female Balb/c mice were treated with CYSTUS052 extract (open squares) three times a day for five consecutive days or kept untreated (black triangles). For detail, see Section 2. The health status of the mice was monitored until day 15 after the start of the treatment by measuring the weight of the animals. The graph represents the mean value of five mice (S.E.M. $\leq$ 5%).

We raised the question whether pre-incubation of the virus with the compound would result in an antiviral effect. Therefore, 10^2 pfu/25 μl was either incubated with 25 μl CYSTUS052 (1 mg/ml) or with 25 μl PBS for 30 min at room temperature. Thereafter mice were infected i.n. with 50 μl of either FPV/CYSTUS052 or FPV/PBS. Mice that were infected with FPV pre-treated with PBS developed disease and two out of five mice died. In contrast, none of the mice that were infected with FPV pre-treated with the plant extract developed disease or died. Since a 90% reduction of infectivity was found *in vitro* after pre-incubation of virus with CYSTUS052, we then used 10^3 pfu/25 μl for pre-incubation. All mice that were infected with FPV pre-treated with PBS died. Mice that were infected with FPV pre-treated with CYSTUS052 developed disease or died similarly to mice infected with the LD_{50} of 10^2 pfu (Table 2).

3.4. Histology and immunohistology of lungs sections from CYSTUS052-treated mice

To answer the question if aerosol treatment of CYSTUS052 would lead to alterations on epithelial bronchiolus cells we performed hematoxylin and eosin staining of mice that were either treated with an aerosol formulation of the plant extract (+CYSTUS052) or with H_2O (−CYSTUS052), the dissolvent of the extract as described above. Immediately after three treatments the mice were killed and lungs were fixed in 4% paraformaldehyde. No alterations of epithelial cells were found after CYSTUS052 treatment (Fig. 5; H & E). Moreover we also investigated the expression of sialic acid α-2,6 and sialic acid α-2,3 on epithelial bronchiolus cells after CYSTUS052 staining. Again no alterations of the expression of these two viral receptors were found in CYSTUS052-treated mice (Fig. 5; 2,6 SA, 2,3 SA).

Table 2
Disease in mice after pre-incubation of virus with CYSTUS052

Infectious dose (pfu)	CYSTUS052 pre-incubation	Onset of disease[a] (day p.i.)	Death[a] (day p.i.)	Survival
10^3	–	4	9	0/5
10^3	+	6	11	3/5
10^2	–	7	10	3/5
10^2	+	–	–	5/5

[a] Mean value of five Balb/c mice (S.E.M. ≤ 16%).

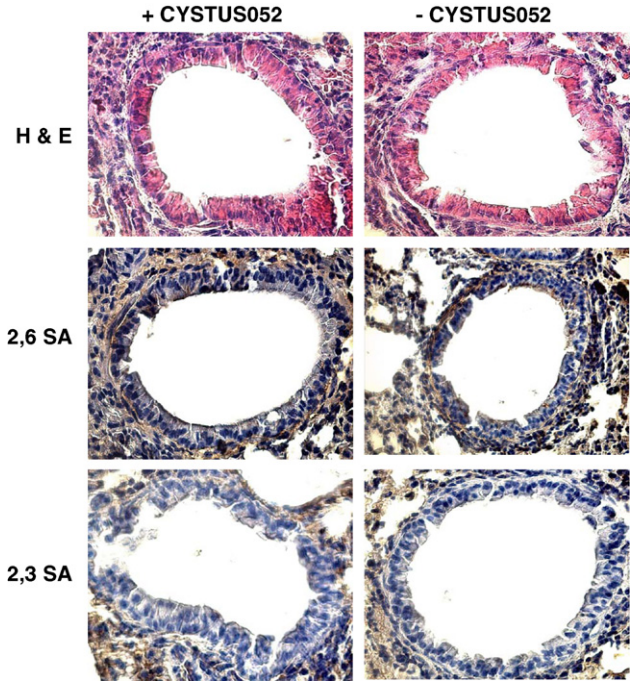

Fig. 5. No alterations of epithelial bronchiolus cells after treatment of mice with CYSTUS052 extract. Female Balb/c mice were treated with CYSTUS052 extract (+CYSTUS052) as an aerosol formulation (10 mg/ml) or were treated with H_2O (−CYSTUS052). No damage of epithelial bronchiolus cells was found in the plant extract-treated mice after H & E staining. Lectin staining of lung sections were performed to detect sialic acid α-2,6 (2,6 SA) and sialic acid α-2,3 (2,3 SA) receptors. Also no different receptor expression was found in CYSTUS052-treated mice (80× magnification).

4. Discussion

The goal of this study was to determine whether the polyphenol-rich plant extract from a distinct variety of *Cistus incanus* would function as an antiviral agent against influenza A virus in mice. Aerosol treatment with CYSTUS052 extract of mice that were infected with a mouse-adapted fowl plague virus (FPV), a highly pathogenic avian influenza virus of the H7 subtype, protected these animals from disease and death. The CYSTUS052 extract-treated mice neither showed changes in their body temperature nor changes in their gross motoric activity, while drastic differences in these parameters were detectable in mice from the control groups.

Our experimental setup to apply CYSTUS052 extract as an aerosol was based on the fact that the bioavailability of high molecular weight polyphenols is very poor. Furthermore, *in vitro* studies demonstrated that CYSTUS052 extract given as an aerosol results in reduced plaque formation after FPV infection of MDCK cells. Interestingly, this antiviral activity of CYSTUS052 extract was only detectable when CYSTUS052 extract was nebulized prior to virus infection. In contrast, when CYSTUS052 extract was nebulized in parallel to or after virus infection, no antiviral effect was detected (data not shown).

One might now speculate about the mechanism that is responsible for the antiviral properties of CYSTUS052 extract. From our findings and from studies of the active compounds in *Cistus incanus* (Petereit et al., 1991), we would like to hypothesize a rather physical mode of action where the content of polymeric polyphenols in the CYSTUS052 extract that by far exceeds other well-known sources of polyphenols, bind to the virus prior to infection and will prevent adsorption of the virus to cells. This hypothesis is based on the fact that in our *in vitro* studies we were able to demonstrate that CYSTUS052 extract showed no antiviral properties, when given after or during the infection (Ehrhardt et al., 2007). Only few polymorphic polyphenols are absorbed and metabolized (Urquiaga and Leighton, 2000; Manach et al., 2004; Halliwell et al., 2005). The active compounds of *Cistus incanus* are most probably flavan-3-ols and proanthocyanidins. This group of polyphenols comprise monomers but also dimers and polymers (Petereit et al., 1991). Proanthocyanidins are active compounds against viral and bacterial pathogens (Cos et al., 2004). It is known that proanthocyanidins downregulate the expression of CCR2b, CCR3 and CCR5 on peripheral blood mononuclear cells. CCR2b, CCR3 and CCR5 function as coreceptors for HIV (Nair et al., 2002). This prompted us to investigate if CYSTUS052 would have an influence on the expression of sialic acid influenza virus receptors. The immunohistochemistry experiments revealed no alterations in the expression of the viral sialic acid receptors on bronchiole epithelial cells after CYSTUS052 treatment.

Some polyphenols of low molecular weight are bioactive compounds that are absorbed from the gut in their native or modified form and show antiviral activity against influenza A virus. One of this bioreactive compounds is Resveratrol, a polyphenol that is synthesized by various plant species including grapes (Fremont, 2000). Resveratrol appears to interfere with several intracellular signalling pathways and showed an antiviral activity against influenza A virus *in vitro* and *in vivo* (Palamara et al., 2005). The fact that oral treatment of CYSTUS052 extract was not effective against influenza A virus infection in mice (Fig. 4, Table 1) argues against a mode of action of CYSTUS052 extract similar to Resveratrol and further supports the mechanism of a direct physically function. The plant *Cistus incanus* is very rich in polymeric polyphenols. This allowed us to perform the

experiments with an extract of the plant without purification of the active component. The great advantage of using the crude plant extract is the lower risk of toxic side effects. Indeed, aerosol treatment of CYSTUS052 extract in a concentration of 10 mg/ml did not lead to toxic effects in mice, when given three times a day for five consecutive days. Furthermore, it is well known that some polyphenols have an anti-inflammatory function. In experiments that examined LCMV-induced footpad-swelling aerosol treatment of CYSTUS052 extract did not result in alterations of the systemic immune reaction against the viral pathogen. These findings are in line with the fact that *Cistus incanus* plant extracts are used in traditional medicine in southern Europe for centuries without complications.

Our studies with the polyphenol-rich extract from the plant *Cistus incanus* demonstrated a pronounced antiviral effect against influenza A virus *in vitro* and *in vivo*. It is commonly accepted that in addition to a direct effect influenza virus alters the lungs through epithelial damage in a way that facilitates superinfections by pneumococci (Mc Cullers, 2006). Since polyphenols are also functional against bacteria (Urquiaga and Leighton, 2000), CYSTUS052 extract may have a significant potential against influenza, where bacterial co-infections contribute to the severity of the disease.

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