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EPs® 7630 (Umckaloabo®), an extract from *Pelargonium sidoides* roots, exerts anti-influenza virus activity *in vitro* and *in vivo*

Linda L. Theisen, Claude P. Muller *

Institute of Immunology, Centre de Recherche Public de la Santé/Laboratoire National de Santé, 20A rue Auguste Lumière, L-1950 Luxembourg, Luxembourg

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ABSTRACT

A prodelphinidin-rich extract from *Pelargonium sidoides* DC, EPs® 7630 (Umckaloabo®), which is licensed to treat respiratory tract infections such as acute bronchitis, was investigated for its antiviral effects. EPs® 7630 showed dose-dependent anti-influenza activity at non-toxic concentrations against pandemic H1N1, oseltamivir-sensitive and -resistant seasonal H1N1, seasonal H3N2 and the laboratory H1N1 strain A/Puerto Rico/8/34, while it had no antiviral activity against adenovirus or measles virus. The extract inhibited an early step of influenza infection and impaired viral hemagglutination as well as neuraminidase activity. However, EPs® 7630 did not exhibit a direct virucidal effect, as virus preincubation (unlike cell preincubation) with the extract did not influence infectivity. Importantly, EPs® 7630 showed no propensity to resistance development *in vitro*. Analysis of EPs® 7630 constituents revealed that prodelphinidins represent the active principle. Chain length influenced antiviral activity, as monomers and dimers were less effective than oligo- and polymers. Importantly, gallocatechin and its stereoisomer epigallocatechin exert antiviral activity also in their monomeric form. In addition, EPs® 7630 administered by inhalation significantly improved survival, body weight and body temperature of influenza-infected mice, without obvious toxicity, demonstrating the benefit of EPs® 7630 in treatment of influenza.

1. Introduction

Despite the availability of neuraminidase or matrix protein inhibitors, there is a great need for new anti-influenza drugs. Matrix protein inhibitors like amantadine or rimantadine are no longer recommended against pandemic and seasonal H1N1 because of widespread resistance (Fiore et al., 2011). While during the 2008/2009 season, up to 99% of seasonal H1N1 strains were resistant to oseltamivir (Centers for Disease Control and Prevention, 2009; Sheu et al., 2011), pandemic H1N1 shows a low percentage of oseltamivir-resistant strains so far (Hayden and de Jong, 2011), even though resistant clusters have emerged recently (Hurt et al., 2011). Although oseltamivir is generally well tolerated (Jackson et al., 2011; Matheson et al., 2007), side effects like abnormal behavior or delirium have been observed in children and adolescents (Jefferson et al., 2009) or adults (Nakamura et al., 2010). These problems highlight the need for new, effective and well tolerated antiviral drugs.

Pelargonium sidoides DC (Geraniaceae) has widely been used as a traditional indigenous medicine in South Africa against dysentery, fever and respiratory diseases (Brendler and van Wyk, 2008). In 2005, an ethanolic root extract (1:8–10), referred to as EPs® 7630 (Umckaloabo®), received full marketing authorization by the German drug regulatory agency (Conrad et al., 2007). To date, the extract is mainly used to treat acute bronchitis and has shown good tolerability in multiple clinical trials in both adults and children (Agbabiaka et al., 2008; Kamin et al., 2010; Matthys et al., 2003; Matthys and Heger, 2007).

EPs® 7630 mainly consists of polyphenolic compounds (Schötz and Nöldner, 2007; Schoetz et al., 2008). Oligo- and polymeric proanthocyanidins based on gallocatechin and epigallocatechin moieties account for about 40% of the dry extract. These prodelphinidins are present in an enormous structural variety (from monomers to at least 16-mers, A- and B-type bonding and different stereoisomers) (Schoetz et al., 2008), making it challenging to attribute activity to a single compound or even a group of compounds. Recently, proanthocyanidins have received some attention as antimicrobial compounds. Indeed, polyphenol-rich plant extracts have demonstrated antibacterial effects. For example cranberry extract inhibited adhesion of P-fimbriated *Escherichia coli* to uroepithelial cells (Howell et al., 1998) and EPs® 7630 and apple peel extract impaired the attachment of *Helicobacter pylori in vitro* (Beil and Kilian, 2007) and in a mouse model (Pastene et al., 2010). In addition,

Abbreviations: EC_{50}, half maximal effective concentration; IAV, influenza A virus; CC_{50}, half maximal cytotoxic concentration; MDCK, Madin-Darby canine kidney; MLD_{50}, half maximal mouse lethal dose; MOI, multiplicity of infection; $TCID_{50}$, half maximal tissue culture infectious dose.

* Corresponding author. Tel.: +352 490604 220; fax: +352 490686.
E-mail address: Claude.Muller@crp-sante.lu (C.P. Muller).

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efficacy of polyphenol-rich extracts or isolated polyphenols has been shown against various viruses, including influenza A virus (IAV) (Droebner et al., 2007; Haidari et al., 2009; Song et al., 2005), herpes simplex virus (Erdelmeier et al., 1996; Schnitzler et al., 2008; Shahat et al., 2002) or HIV (De Bruyne et al., 1999; Fink et al., 2009).

Here we investigated the antiviral efficacy of EPs® 7630 and its constituents against a variety of IAV strains, showing at which step of the virus life cycle it is active and demonstrate an antiviral effect of EPs® 7630 *in vivo*.

2. Materials and methods

2.1. EPs® 7630 extract, fractions and isolated compounds

The proprietary extract EPs® 7630 from *P. sidoides* roots, an oligo-/polymeric acetonic fraction eluted from a SephadexLH20 column (Vennat et al., 1992), isolated monomers (gallocatechin, epigallocatechin) and dimers (epigallocatechin-(4β→8)−gallocatechin, epigallocatechin-(4α→8)−epigallocatechin, gallocatechin-(4β→8)−gallocatechin, gallocatechin-(4α→8)−epigallocatechin) were kindly provided by Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, as dry powders. Monomers and dimers had a purity of >95% as verified by HPLC. EPs® 7630 dry extract was dissolved in PBS (1 h sonication at 25 kHz and 60 °C) at 2 mg/ml (*in vitro* experiments), in 10% ethanol at 20 mg/ml (CC_{50} and EC_{50} determination) or in water at 5 mg/ml (*in vivo* experiments). The oligo-/polymeric fraction was dissolved in DMSO (1 h sonication at 25 kHz and 60 °C), monomers and dimers in PBS (1 h sonication at 25 kHz). The tannin-free extract was prepared by stirring 0.5 g of skin powder (FILK, Freiberg) with 20 ml of EPs® 7630 (2 mg/ml in PBS, pH 7.2) for 1 h at room temperature, followed by filtration.

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) cells were grown in EMEM (Lonza) supplemented with 10% fetal bovine serum, 25 mM HEPES, 2 mg/ml bovine serum albumin, 100 U/ml penicillin and 100 U/ml streptomycin. A549, A549Slam and VeroSlam cells were maintained in DMEM (Lonza) supplemented with 10% fetal bovine serum, 2 mM ultraglutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were incubated at 37 °C and 5% CO_2. All IAVs were grown in MDCK cells (for producing virus stocks) in serum free virus growth medium containing 2 μg/ml L-1-tosylamido-2-phenylethyl chloromethylketone-(TPCK)trypsin (Sigma–Aldrich) or in A549 cells (*in vitro* efficacy experiments) in virus growth medium containing 0.2 μg/ml TPCK-trypsin. Half maximal tissue culture infectious dose ($TCID_{50}$) determinations of IAV were done on MDCK cells. Adenovirus was propagated and titered by $TCID_{50}$ on A549 cells, measles virus stocks on VeroSlam cells. For $TCID_{50}$, cells were incubated in quadruplicate for 3 days at 37 °C and 5% CO_2 with 3-fold serial dilutions of virus-containing supernatant. The cytopathic effect was scored and $TCID_{50}$ was calculated by the ID-50 5.0 program (http://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/html_ncbi/html/index/software.html#1).

2.3. In vitro cytotoxicity assay (XTT)

3×10^4 A549 cells per well of a 96-well plate (Greiner BioOne) were incubated with a 2-fold serial dilution of the EPs® 7630 stock solution or its components. XTT reagent (Cell proliferation kit II, Roche Diagnostics) was added after 24 h, the plate was incubated for 2 h at 37 °C and absorbance was read at 450 and 650 nm (subtracted background) on a SpectraMax plus plate reader (Molecular Devices).

2.4. Antiviral efficacy determination

A549 cells grown in μClear bottom plates (96-well, Greiner Bio-One) were washed, virus growth medium was added and the cells were infected with H1N1 GFP reporter virus (A/Puerto Rico/8/34-NS116-GFP) at a multiplicity of infection (MOI) of 0.01 (EPs® 7630) or 0.04 (oligo-/polymeric fraction and isolated compounds) for optimal fluorescent readout of the used virus batches. Serial dilutions of EPs® 7630 stock solution, monomers, dimers or oligo-/polymers were added immediately after virus inoculation. After 24 h, GFP fluorescence was read (ex 485 nm, em 535 nm) on a Tecan Genios plus Reader (Tecan, Austria). For wild type virus experiments, A549 (0.1 MOI IAV except if mentioned otherwise, 0.05 MOI adenovirus) or A549Slam (0.01 MOI measles virus) cells were used in triplicates. After infection, cultures were incubated for 24 or 48 h (A/Luxembourg/663/2008, A/Luxembourg/572/2008, measles). Supernatants were harvested and titered on MDCK (IAV), A549 (adenovirus) or VeroSlam (measles virus) cells.

2.5. Hemagglutination inhibition assay

Washed human erythrocytes (blood group O) were used at a concentration of 0.75%. The lowest virus (A/Puerto Rico/8/34) concentration that agglutinated erythrocytes was determined and used in the hemagglutination inhibition assay. Twenty microlitre EPs® 7630 stock solution (2 mg/ml) or PBS, 30 μl virus solution or PBS and 50 μl erythrocyte solution was added to round-bottom wells and scored after 60 min.

2.6. Neuraminidase inhibition assay

Fifty microlitre of serial dilutions of EPs® 7630 (in duplicates) were added to 50 μl of A/Puerto Rico/8/34 H1N1 virus (standard 1/10 dilution yielding 2.6×10^5 $TCID_{50}$ in assay buffer containing MES (2-(*N*-morpholino)ethanesulfonic acid), $CaCl_2$ and distilled water, pH 6.5) or to 50 μl assay buffer into a 96 well black μClear plate (Greiner BioOne). After 45 min at room temperature, 50 μl of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (0.3 mM, Sigma–Aldrich) was added to each well and incubated for 1 h at 37 °C. Stop solution (0.166 M NaOH in ethanol) was added and the plate was read on a Tecan Genios plus Reader (ex 360 nm, em 448 nm,). Background fluorescence (EPs® 7630 and substrate alone) was subtracted.

2.7. Resistance assay

To study the development of resistance, we used a multipassage protocol comparable to those used to monitor resistance to antiviral plant extracts and other drug candidates (Ehrhardt et al., 2007; Ludwig et al., 2004; Pleschka et al., 2009). Briefly, triplicates of A549 cells grown in 6-well plates (Greiner BioOne) were washed, virus growth medium was added and the cells were infected with 0.2 MOI H1N1 A/Puerto Rico/8/34 and incubated with 0 or 10 μg/ml EPs® 7630 for 24 h. Thereafter, fresh A549 cells were inoculated with 100 μl of supernatant and left untreated for 24 h to allow virus to re-expand, before 0 or 10 μg/ml EPs® 7630 were added again for 24 h. Four passages were performed. Supernatants were harvested and titered on MDCK cells after each passage.

2.8. Mice and viral infection

All experiments were done in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and national regulations on pathogen-free female 7 week old BALB/c mice (Harlan, NL). Peroral gavage of 5 mg/kg of an EPs® 7630 suspension in 0.2% agar (Merck, Darmstadt) or vehicle

only was performed three times a day over 10 days in mice intranasally infected with four half maximal mouse lethal doses (MLD_{50}) of A/Puerto Rico/8/34 in 50 μl PBS. A first gavage was performed 6 h before infection.

Inhalation treatment was performed in an inhalation chamber (volume 3.9 L) connected to two aerosol nebulizers (PARI BoySX, PARI, Starnberg, particle average mass median aerodynamic diameter 2.2 μm, total output rate 450 mg/min) on opposite sides of the chamber. Excess mist was allowed to escape from narrow gaps uniformly distributed on four sides of the cage, in a way that the whole cage volume was uniformly exposed to freshly nebulized drug under steady state conditions reached within 30 seconds. Inhalation chambers with PARI nebulizers have been used previously (Droebner et al., 2007). On day 1, 10 mice per group were pretreated by inhalation with EPs® 7630 (5 mg/ml) or water, anesthetized 10 min later with isoflurane (Forene, Abbott) and infected intranasally with 1 or 4 MLD_{50} of A/Puerto Rico/8/34 in 50 μl PBS. During the next 10 days, mice were treated for 10 min by inhalation with EPs® 7630 (5 mg/ml) or water at 6 a.m., 2 p.m. and 10 p.m. Rectal body temperature (TH-5 Thermalert Monitoring Thermometer, Phymep, France) and weight were monitored daily for 14 days. Animals were sacrificed when body weight loss exceeded 25% or on day 14. Lungs ($n = 25$) were explanted and homogenized (TissueLyserII, Qiagen) in virus growth medium for 12 min at 25 Hz, centrifuged for 10 min at 11000 rpm and $TCID_{50}$ in the supernatant was determined on MDCK cells.

2.9. Statistical methods

CC_{50} and EC_{50} values for the GFP virus assay and neuraminidase assay were calculated using SigmaPlot 9.0. Statistical analyses were done with SigmaPlot 9.0 using Student's *t*-test (statistical significance if $p < 0.05$). Values were calculated as means ± standard deviation. Survival analysis was done in SigmaPlot 9.0 using Gehan-Breslow test.

3. Results

3.1. Anti-IAV activity of EPs® 7630 is exerted at non-toxic concentrations in vitro

Cytotoxicity of EPs® 7630 was tested by adding quadruplicate serial dilutions of EPs® 7630 to A549 cells. After 24 h of incubation, cell viability was measured by XTT test. The half maximal cytotoxic concentration of EPs® 7630 (CC_{50}) was 557 μg/ml (Fig. 1A).

To assess its antiviral activity, serial dilutions of EPs® 7630 were added to A549 cells infected in quadruplicate with 0.01 MOI (giving an optimal fluorescent readout) of a reporter virus containing a NS1-GFP fusion protein (A/Puerto Rico/8/34-NS116-GFP) (Kittel et al., 2004). After 24 h, the half maximal antiviral concentration of EPs® 7630 (EC_{50}) was determined as 6.6 μg/ml (Fig. 1A), corresponding to a selectivity index (CC_{50}/EC_{50}) of 84.4. Concentrations above 50 μg/ml fully inhibited virus growth. Thus, the loss of GFP fluorescence was due to inhibition of virus proliferation and not to cytotoxic drug effects.

The anti-influenza viral activity of EPs® 7630 was further demonstrated for five wild type IAV strains, which all showed a dose-dependent titer reduction in the presence of EPs® 7630. $TCID_{50}$ determination of wild type pandemic H1N1 A/Luxembourg/46/2009 confirmed the fluorescent readout and with an EC_{50} of 5.4 μg/ml (Fig. 1B), corresponding to a selectivity index of 103.1. A/Puerto Rico/8/34 showed complete virus growth inhibition at 50 μg/ml, similarly after 8, 24 or 48 h post-infection (Fig. 1C). The concentrations required for complete virus clearance varied from 16 μg/ml (pandemic H1N1 A/Luxembourg/46/2009, Fig. 1B) up to 300 μg/ml for seasonal oseltamivir-resistant H1N1 A/Luxembourg/572/2008 (Fig. 1F). Intermediate values were found for A/Puerto Rico/8/34 (50 μg/ml, Fig. 1C), seasonal H3N2 A/Luxembourg/01/2005 (50 μg/ml, Fig. 1D) and seasonal oseltamivir-sensitive H1N1 A/Luxembourg/663/2008 (100 μg/ml; Fig. 1E). In contrast, EPs® 7630 had no antiviral activity against the unenveloped adenovirus type 5 (ATCC reference strain, Fig. 1G) or against the enveloped measles virus (Edmonston strain, Rimevax) at non-toxic concentrations (Fig. 1H).

3.2. EPs® 7630 affects an early step in the influenza virus life cycle

Next, we investigated at which step of the virus life cycle EPs® 7630 exerts its antiviral activity. A549 cells were infected with A/Puerto Rico/8/34 (MOI 0.1) and 50 μg/ml of EPs® 7630 was added at different time points (−2, 0, +2, +4, +6 h) before or after infection. $TCID_{50}$ in the supernatant was determined at 8 or 24 h post-infection (corresponding to approximately 1 or 3 virus life cycles).

Eight hours post-infection, the EPs® 7630 containing medium was replaced by EPs® 7630-free medium to allow proliferation of intracellular virus for another 24 h before titration. No virus was detectable in the supernatant when treatment with EPs® 7630 was started before or at the time of inoculation (Fig. 2A). However, when the plant extract was added at 2, 4 or 6 h post-infection, no effect on virus proliferation was observed (Fig. 2A), suggesting that the extract inhibited an early step of viral infection, presumably viral entry into the host cell.

When EPs® 7630 was allowed for 24 h (instead of 8 h) on the culture, no virus was detectable in the supernatant, irrespective of the start of the treatment (−2, 0, +2, +4, +6 h post-infection) (Fig. 2B). Thus, EPs® 7630 efficiently prevented virus released from host cells after the first life cycle (i.e. 8 h post-infection) to re-enter new host cells and complete its next life cycles.

3.3. EPs® 7630 inhibits hemagglutination and neuraminidase activity of influenza virus

The effect of EPs® 7630 on H1N1 A/Puerto Rico/8/34 virus binding to its receptor was tested by hemagglutination inhibition assay. EPs® 7630 prevented virus-mediated hemagglutination from 100 μg/ml (data not shown). In absence of virus, EPs® 7630 had no effect on hemagglutination. In a standard fluorescence based neuraminidase inhibition assay, 121.7 μg/ml EPs® 7630 reduced neuraminidase activity of A/Puerto Rico/8/34 by 50% (Fig. 2C). Thus, EPs® 7630 interferes with virus binding to its host cell receptors (attachment) as well as neuraminidase activity.

3.4. Effect of preincubation of cells or virus with EPs® 7630

A549 cells were preincubated for 2 h with 50 μg/ml of extract or PBS and then infected with 0.1 MOI of A/Puerto Rico/8/34. As already shown in Fig. 2A, continuous treatment with EPs® 7630 prevented virus growth. When EPs® 7630 was washed out before infection, the virus was inhibited by more than 2 log to an average titer of 6×10^2 $TCID_{50}$ compared to 3.7×10^5 $TCID_{50}$ in EPs® 7630 free cultures ($p < 0.05$) (Fig. 2D). Thus, the effect of EPs® 7630 on the host cells impairs viral infection. Washing out EPs® 7630 from the cells prior to infection allowed partial virus growth, suggesting that the extract's effect is partially reversible.

To assess a direct virucidal effect of EPs® 7630, virus stock was preincubated with up to 250 μg/ml of EPs® 7630 for 2 or 24 h before infection. At inoculation, virus was diluted 1:200-fold, corresponding to 0.1 MOI and a negligible final concentration of EPs® 7630 of <1.25 μg/ml. 24 h post-infection, supernatants were titered and no difference in virus growth was observed between cultures infected with EPs® 7630 treated and untreated virus (Fig. 2E

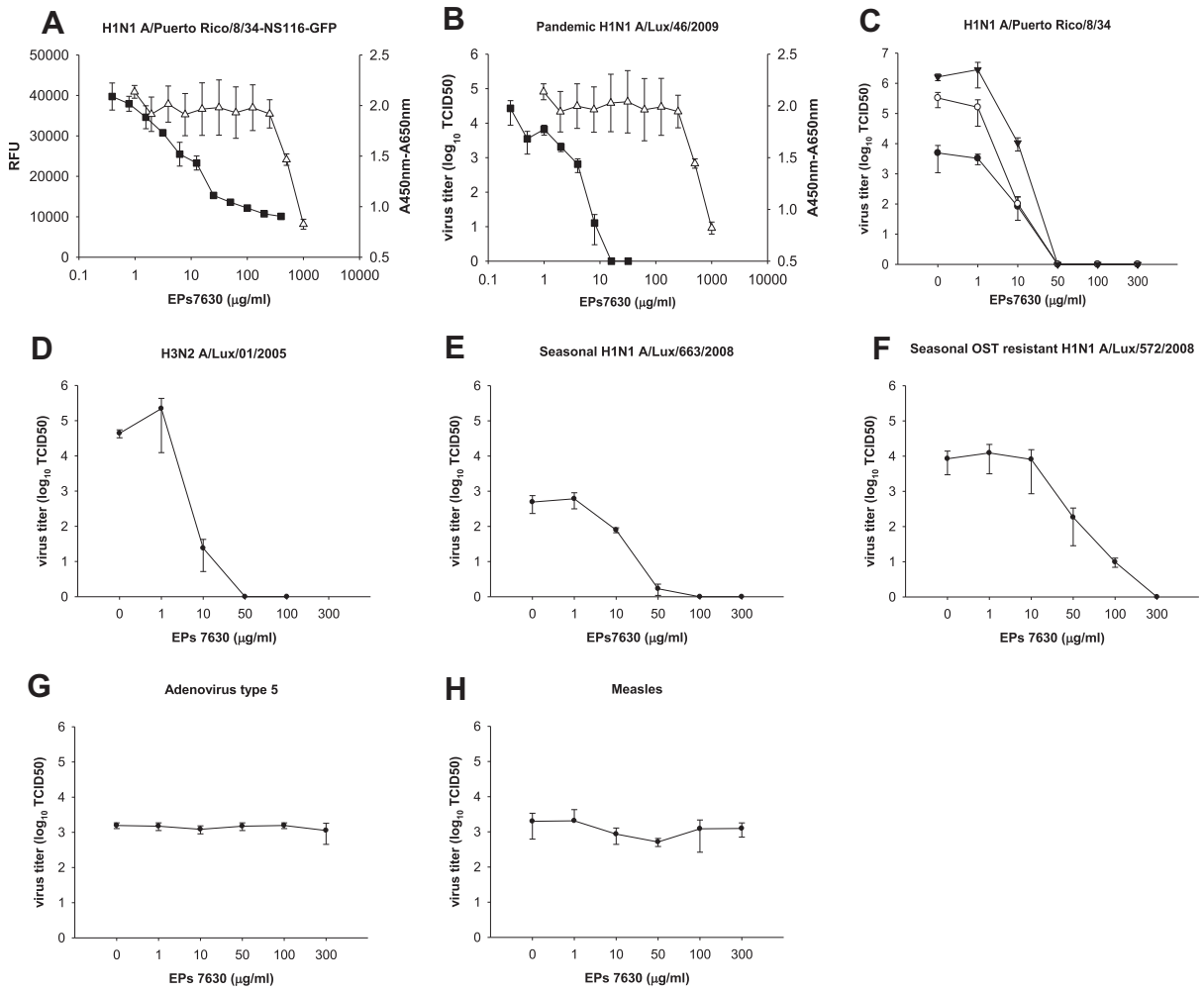

Fig. 1. Cytotoxicity and anti-IAV efficacy of EPs® 7630 on A549 cells. (A) A/Puerto Rico/8/34 expressing a GFP reporter gene. Virus-associated GFP fluorescence expressed as RFU (relative fluorescence units, closed squares) measured 24 h after infection of A549 cells with GFP-virus (MOI 0.01) in the presence of serial dilutions of EPs® 7630. RFU of untreated cells: 52,863 ± 2940. (B) Pandemic H1N1 A/Luxembourg(Lux)/46/2009. Virus titer measured at 24 h after infection of A549 cells (0.1 MOI, closed squares). Titer of untreated cells: $7.05 \pm 1.01 \times 10^4$ $TCID_{50}$. (A and B) Open triangles: A549 cell viability determined by XTT assay 24 h after addition of serial dilutions of EPs® 7630. Background absorbance (650 nm) was subtracted from reagent absorbance (450 nm). Absorbance of untreated cells: 2.14 ± 0.04. (C–H) EPs® 7630 activity against different virus strains. A549 cells were infected with 0.1 MOI of (C) A/Puerto Rico/8/34 (titered 8 h (closed circles), 24 h (open circles) or 48 h (closed triangles) post-infection), (D) seasonal H3N2 A/Luxembourg/01/2005, (E) oseltamivir (OST)-sensitive seasonal H1N1 A/Luxembourg/663/2008, (F) oseltamivir-resistant seasonal H1N1 A/Luxembourg/572/2008, (G) adenovirus type 5 (ATCC reference strain, MOI 0.05); or (H) A549Slam infected with measles virus (Schwarz vaccine strain, MOI 0.01) in presence of serial dilutions of EPs® 7630, titered 24 or 48 h (E, F and H) post-infection. $TCID_{50}$ determined in MDCK (IAV), A549 (adenovirus) or VeroSlam (measles).

and F). A direct virucidal activity of EPs® 7630, at least up to concentrations of 250 µg/ml, can therefore be excluded.

3.5. EPs® 7630 shows no propensity to generate resistant viruses

H1N1 A/Puerto Rico/8/34 was passaged four times in presence of 0 or 10 µg/ml EPs® 7630, a concentration reducing virus growth without completely inhibiting it (see Fig. 1C). In every passage, 10 µg/ml of EPs® 7630 constantly reduced the viral titer well below 1% of the untreated controls (P1 0.04%, P2 0.40%, P3 0.02%, P4 0.15%, Fig. 2G), showing that, at least over 4 passages, the susceptibility of the virus to EPs® 7630 did essentially not change. Thus, EPs® 7630 did not show propensity to induce resistant viruses.

3.6. Anti-influenza activity of EPs® 7630 is mediated by polyphenols

Since polyphenols in EPs® 7630 have been shown to impair adhesion of streptococci to epithelial cells (Janecki et al., 2010), we examined whether they were also involved in its antiviral activity. Therefore, polyphenols were removed from EPs® 7630 by precipitation with skin powder, a standardized procedure from the European Pharmacopoeia (Ph. Eur. 6.0). This treatment removes polyphenols with tanning properties (with molecular weights of roughly 500–3000 g/mol). While full EPs® 7630 extract abolished growth of 0.05 MOI of H1N1 A/Puerto Rico/8/34 above a concentration of 10 µg/ml, the tannin-free extract did not show any antiviral effect at least up to 100 µg/ml (Fig. 3A), indicating that polyphenols represent the active principle of EPs® 7630 against IAV.

3.7. Chain length of polyphenols influences antiviral activity

Gallocatechin and its stereoisomer epigallocatechin are the main moieties of prodelphinidins, the predominating constituents of EPs® 7630 (Schoetz et al., 2008). To assess the minimal chain length requirement for antiviral activity, we studied monomers (see Fig. 3B for structure), dimers of these two main compounds (Fig. 3C) and an oligo-/polymeric fraction (containing trimeric up to high molecular weight prodelphinidins) isolated from EPs® 7630 full extract by fractionation over a Sephadex LH20 column

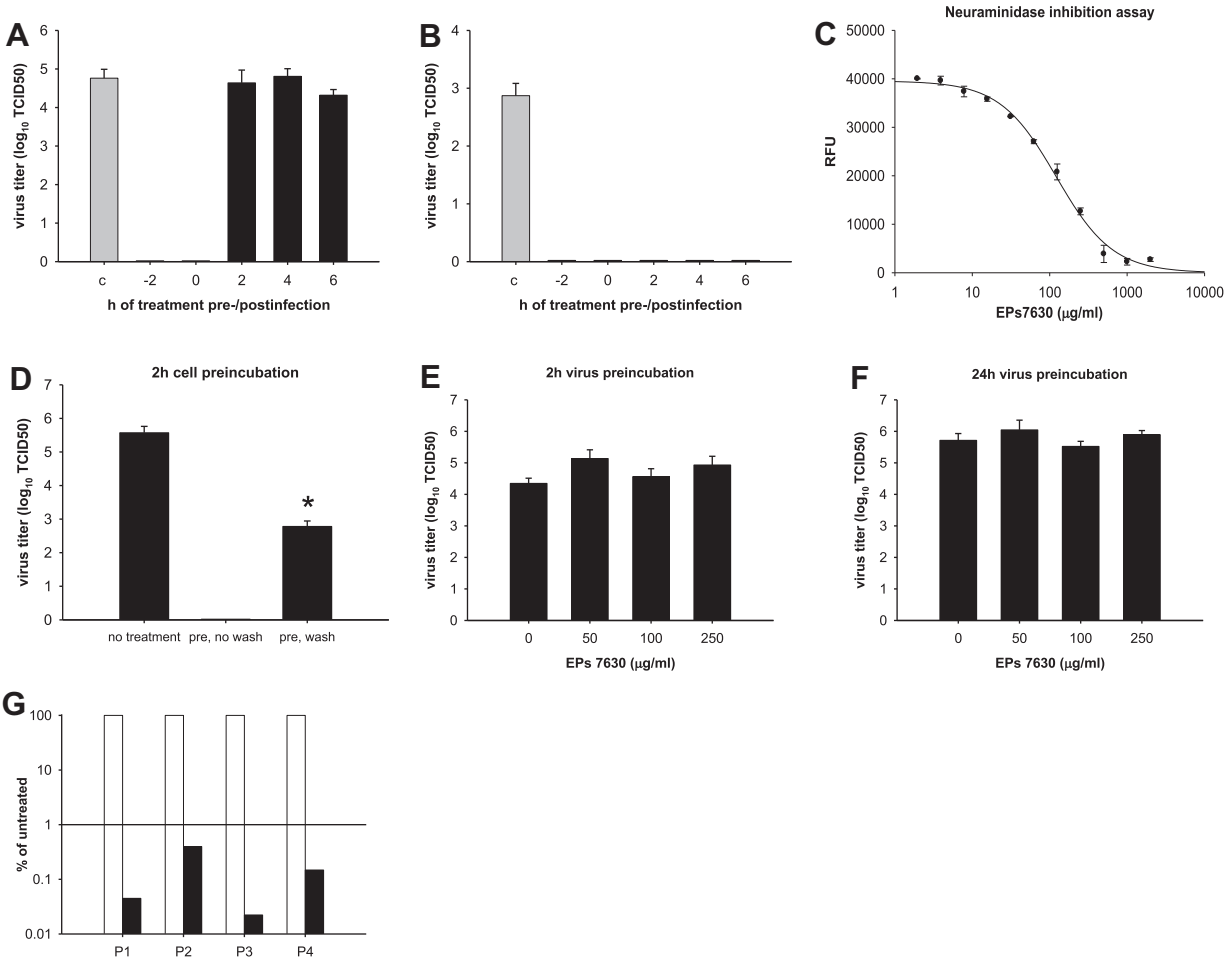

Fig. 2. Anti-influenza mechanism of EPs® 7630. (A and B) PBS (c, control) or 50 µg/ml of EPs® 7630 was added to A549 cells 2 h before infection or 0, 2, 4, or 6 h after infection with A/Puerto Rico/8/34 (MOI 0.1). (A) Supernatant replacement 8 h post-infection (1 virus life cycle) by EPs® 7630-free supernatant. $TCID_{50}$ determination 24 h after medium change. (B) $TCID_{50}$ determination after 24 h of incubation (3 virus life cycles). (C) Effect of EPs® 7630 dilutions (duplicates) on neuraminidase activity of A/Puerto Rico/8/34 expressed as relative fluorescence units (RFU) of a fluorogenic substrate of the neuraminidase, 2-(4-methylumbelliferyl)-a-D-*N*-acetylneuraminic acid. (D) Preincubation (pre) of A549 cells with EPs® 7630 (0 or 50 µg/ml) 2 h before infection, with and without washing step before infection with A/Puerto Rico/8/34. $TCID_{50}$ determination after 24 h. (E and F) Preincubation of A/Puerto Rico/8/34 for 2 h (E) or 24 h (F) with EPs® 7630 (0, 50, 100 or 250 µg/ml) before infection of A549 cells (MOI 0.1). $TCID_{50}$ determination 24 h after infection. (G) Resistance assay: infection of A549 cells with 0.2 MOI H1N1 A/Puerto Rico/8/34 cells in presence of 0 (open bars) or 10 µg/ml (closed bars) EPs® 7630. After 24 h, fresh A549 cells were inoculated with 100 µl of supernatant and left untreated for 24 h, before 0 or 10 µg/ml EPs® 7630 were added again for 24 h. Four passages (P1–P4) were performed. Supernatants were titered in MDCK cells and titers are expressed in % of titer of untreated cells, which was set to 100%. All experiments done in triplicates except otherwise indicated.

(Vennat et al., 1992) (acetonic fraction). Interestingly, both monomers had similar efficacy against H1N1 A/Puerto Rico/8/34-NS116-GFP (1.5-fold difference) (Table 1). The antiviral activity of the four homo- and heterodimers (epigallocatechin-(4β→8)–gallocatechin, epigallocatechin-(4α→8)–epigallocatechin, gallocatechin-(4β→8)–gallocatechin, gallocatechin-(4α→8)–epigallocatechin) was 2- to 7-fold higher than that of the monomers, as shown by their EC_{50} values and the oligo-/polymeric fraction was over 10-fold or 2- to 5-fold more active than the monomers or dimers, respectively, on a weight basis (Table 1, left column). Calculated on a molar basis, dimers were 4- to 13-fold more active than monomers (Table 1, right column). The oligo-/polymeric fraction had a CC_{50} of 13.4 µg/ml, while the monomers and dimers did not exhibit any cytotoxic effects on A549 cells at virus-inhibiting concentrations and up to 80 µg/ml, even after 5 days (data not shown). Thus, the anti-IAV efficacy depends on the polyphenolic chain length: gallocatechin and its stereoisomer have comparable efficacy and exert antiviral activity in their monomeric form, but dimeric and oligo-/polymeric (epi-)gallocatechins are more effective.

3.8. EPs® 7630 exerts anti-influenza activity in mice by inhalation

When mice were infected with 4 MLD_{50} of H1N1 A/Puerto Rico/8/34 and were treated by oral gavage with vehicle only or EPs® 7630 (5 mg/kg three times a day, corresponding to the human recommended dose recalculated for mice) (Reagan-Shaw et al., 2008), no difference in survival, body weight or temperature was observed (data not shown). The absence of antiviral effect after oral administration was in contrast to a clear anti-influenza effect observed in the following inhalation experiments. For the inhalation protocol, groups of 10 mice were infected with 1 or 4 MLD_{50} of H1N1 A/Puerto Rico/8/34 and treated with EPs® 7630 or water by inhalation three times a day. For both virus doses, EPs® 7630 significantly increased survival of virus-infected mice ($p < 0.003$). All 10 mock-treated animals infected with 4 MLD_{50} were euthanized between day 6 and 8 because of a >25% loss in body weight, while the first mouse of the EPs® 7630 treated group was euthanized only on day 8. Three of the 4 MLD_{50} infected and 9 of the 1 MLD_{50} infected EPs® 7630 treated animals did not show any sign of disease after day 10 and survived until the end of the monitoring

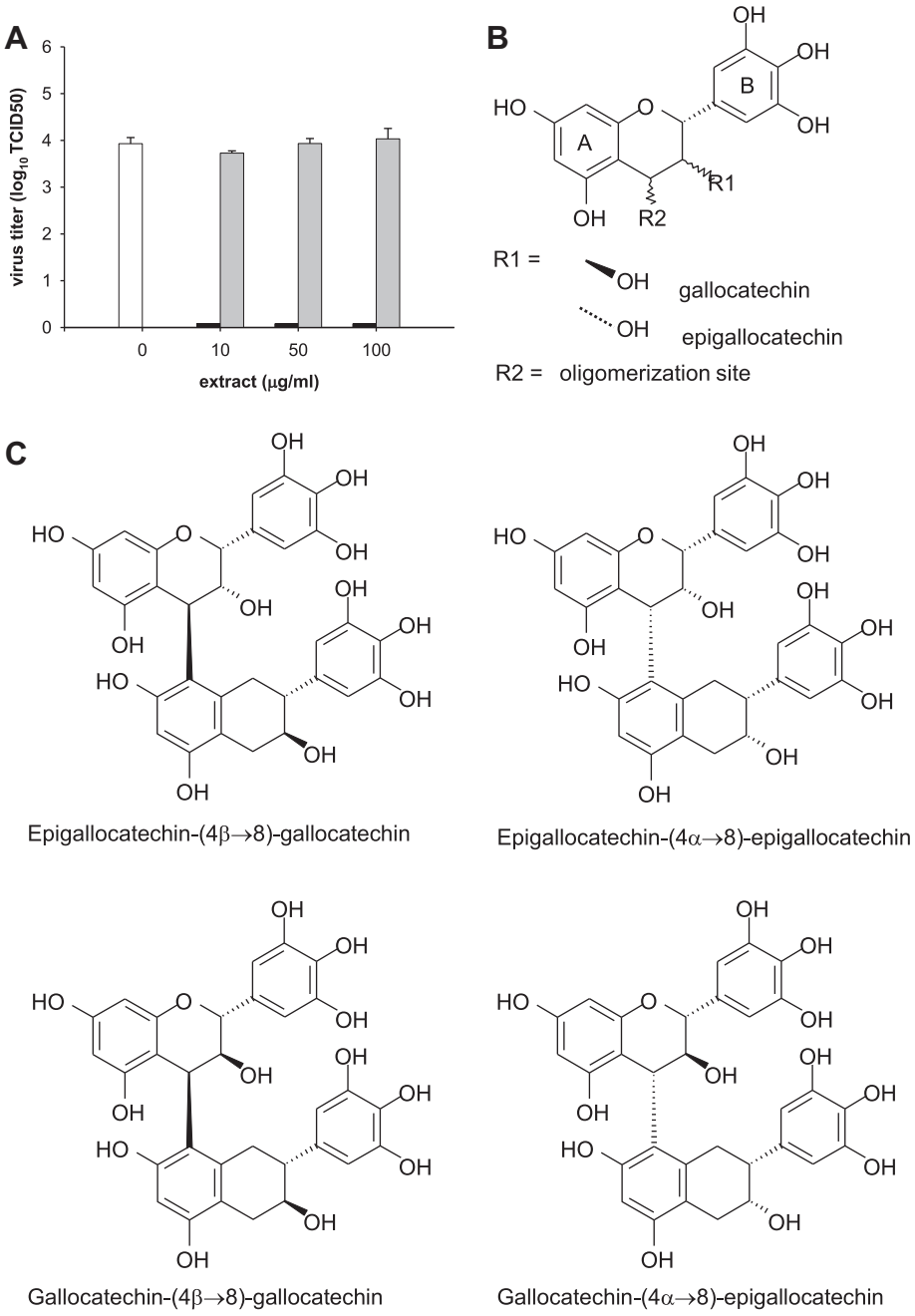

Fig. 3. EPs® 7630 constituents with anti-influenza activity. (A) Effect of polyphenols on H1N1 A/Puerto Rico/8/34 (0.05 MOI). Infection of A549 cells (triplicates) and incubation with dilutions of full extract (closed bars), polyphenol free extract (grey bars) or PBS (no treatment, open bar) for 24 h. (B and C) Structures of the main moieties of prodelphinidins gallocatechin and epigallocatechin (B), as well as isolated dimers tested for antiviral efficacy (C).

Table 1
EC_{50} of catechin monomers, dimers and oligomers present in EPs® 7630.

	EC_{50} (µg/ml)	EC_{50} (µM)
Epigallocatechin	42.5	138.8
Gallocatechin	28.4	92.7
Epigallocatechin-(4β→8)–gallocatechin	6.3	10.3
Epigallocatechin-(4α→8)–epigallocatechin	14.2	23.2
Gallocatechin-(4β→8)–gallocatechin	7.3	11.9
Gallocatechin-(4α→8)–epigallocatechin	13.0	21.2
Oligo-/polymeric fraction	2.8	Not applicable

period (day 14), while none (4 MLD_{50}) or only 2 (1 MLD_{50}) untreated mice survived (Fig. 4A and B).

Body weights (Fig. 4C) of EPs® 7630 treated mice were significantly higher as compared to the untreated group as of day 5, and body temperature as of day 6 (Fig. 4D). Surviving mice completely cleared the virus, while euthanized mock-treated mice had an average lung titer of 5.9×10^5 $TCID_{50}$ (4 MLD_{50}) or 3×10^3 $TCID_{50}$ (1 MLD_{50}) (Fig. 4E and F). Sacrificed EPs® 7630 treated mice showed a lower average lung titer (4×10^3 $TCID_{50}$ in the 4 MLD_{50} group, 7.41 $TCID_{50}$ in the 1 MLD_{50} group) as compared to the untreated group, but the difference was not significant (Fig. 4E and F). The only sacrificed treated mouse in the 1 MLD_{50} group may have recovered, as its lung titer was remarkably low. Thus, inhalation treatment with EPs® 7630 induced a robust improvement of survival, lower lung titers and fewer signs of

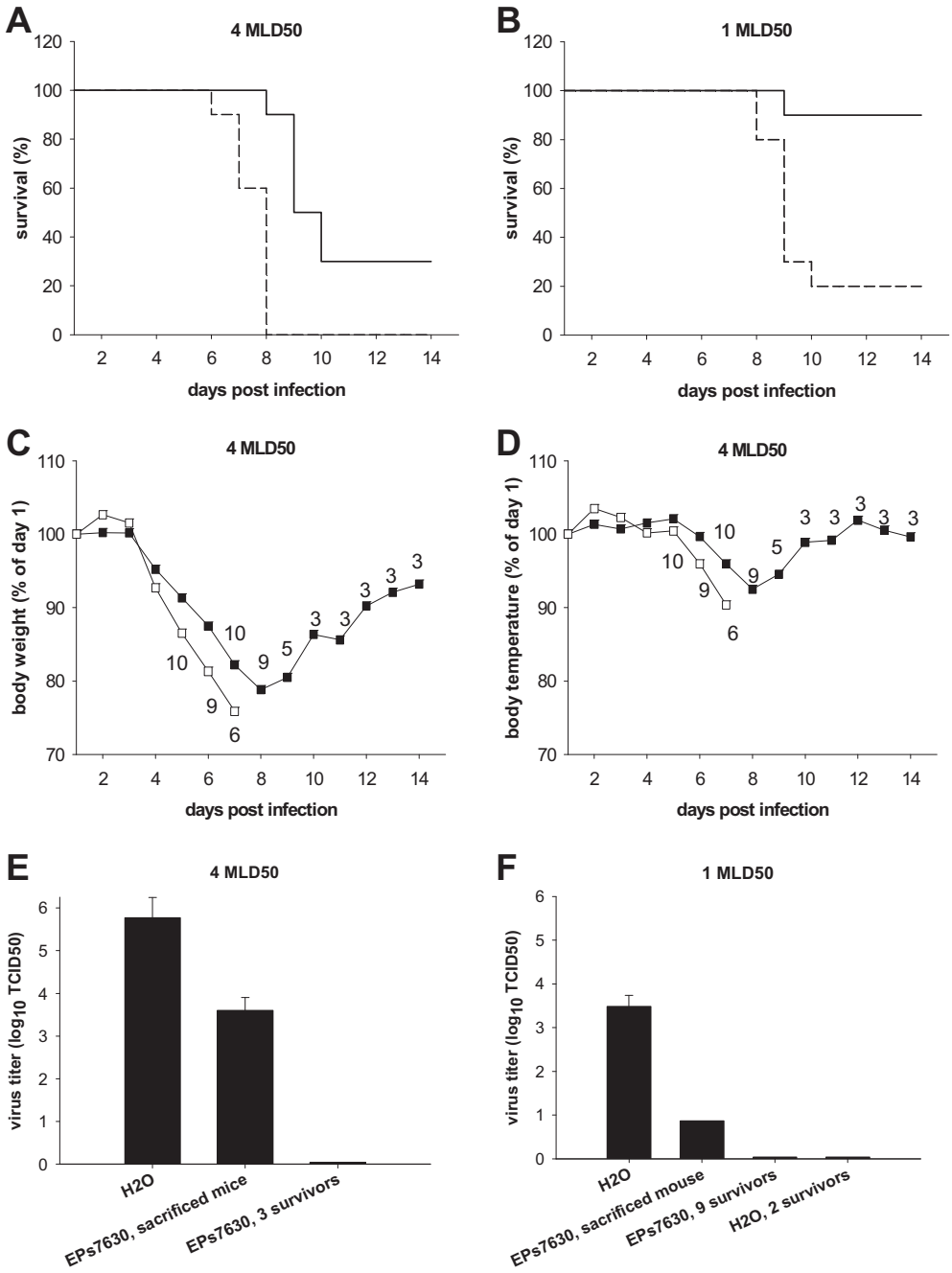

Fig. 4. Anti-influenza activity of EPs® 7630 in mice. (A–F) Infection of 10 mice per group with 4 MLD_{50} (A, C, D and E) or 1 MLD_{50} (B and F) of A/Puerto Rico/8/34, treatment with EPs® 7630 or water by inhalation three times a day for 10 days. (A and B) Survival analysis of EPs® 7630 (solid line) or water (dotted line) treated animals. (C and D) Evolution of body weight (C) or body temperature (D) of EPs® 7630 (closed squares) or water (open squares) treated animals. Numbers represent surviving animals from a group of 10. (E and F) Influence of EPs® 7630 treatment on virus lung titers. Lungs were removed on the day when less than 75% bodyweight was reached or on day 14 for surviving mice; homogenization and titration on MDCK cells. MLD_{50}, half maximal mouse lethal dose.

disease, demonstrating a clear benefit of the treatment of influenza with EPs® 7630 in the mouse model.

3.9. EPs® 7630 has no apparent toxic effect in mice

Groups of five mice were treated by the same regimen as the *in vivo* efficacy study. Daily monitoring of body weight (Fig. 5A) and body temperature (Fig. 5B) showed no significant toxicity of EPs® 7630 between both groups. Also, there was no significant difference in organ weight (lungs, liver, spleen, kidneys) between the two groups (Fig. 5C). Thus, EPs® 7630 did not produce obvious toxic effects in mice and had a good safety profile when used by inhalation.

4. Discussion

Our study confirmed the antiviral activity of prodelphinidin-rich plant extracts against several IAV strains, in contrast to non-enveloped adenovirus, as was reported before (Gescher et al., 2011b; Michaelis et al., 2010). However, EPs® 7630 does not exclusively inhibit enveloped viruses, as it was also effective against the non-enveloped Coxsackie virus (Michaelis et al., 2010). Also, the extract

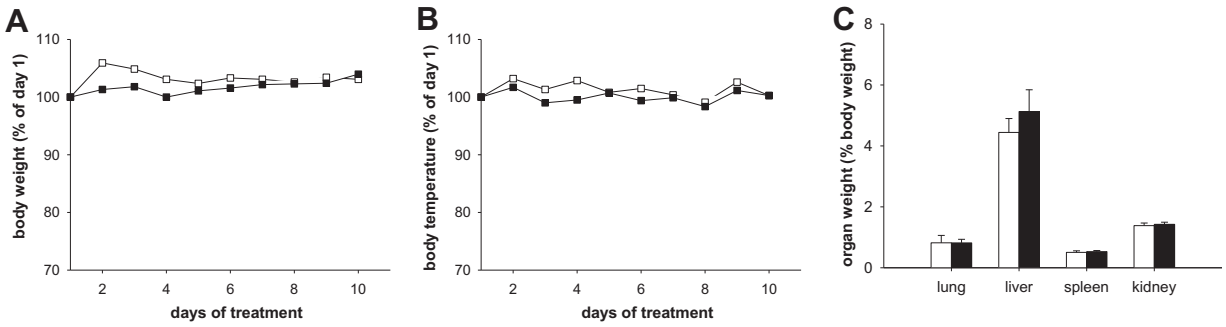

Fig. 5. EPs® 7630 in vivo toxicity. (A–C) Mock infection of five mice per group with 50 μl PBS and treatment with EPs® 7630 or water by inhalation three times a day for 10 days. Daily monitoring of body temperature and weight for 10 days. (A) Evolution of body weight for EPs® 7630 (open squares) or water (closed squares) treated animals. (B) Evolution of body temperature for EPs® 7630 (open squares) or water (closed squares) treated animals. (C) Influence of EPs® 7630 (open bars) or water (closed bars) on organ weight. Sacrificing of mice on day 10 and removal of lungs, spleens, kidneys and livers.

seemed to inhibit several paramyxoviridae (respiratory syncytial virus, parainfluenza virus) (Michaelis et al., 2010), but in our hands failed to inhibit measles virus proliferation. The differential efficacy of EPs® 7630 on paramyxoviridae as well as differential anti-IAV effects in A549 cells in comparison to MDCK cells (data not shown) is in line with some selectivity of polyphenols. While it was believed for a long time that polyphenol–protein interactions are a largely unspecific process, our observations are in line with previous findings that binding affinity correlated with protein size, structure and amino acid composition, and was pH-dependent (Hagerman and Butler, 1981). For instance, the specificity of the binding of a series of polyphenols was demonstrated, as epigallocatechin was shown to bind to the 5HT1-receptor while it had no affinity for the 5HT2 or adenosine 1 receptor (Zhu et al., 1997).

EPs® 7630 had no direct virucidal effect on the virus and had a partially reversible effect on the host cell upon preincubation. This is in line with a reversible effect of EPs® 7630 activity on hemagglutination and neuraminidase inhibition, suggesting that EPs® 7630 inhibits host cell infection by interfering with the action of these two surface glycoproteins. Interestingly, some IAV strains were inhibited at an up to 30-fold lower concentration than others. Differential sensitivity of IAV strains to polyphenols has been previously observed (Ehrhardt et al., 2007; Sundararajan et al., 2010). This suggests that the activity was due to a reversible astringent effect mediated by hydrogen bonds (Zhu et al., 1997), rather than a strong covalent tanning effect by proanthocyanidins, which are also referred to as condensed tannins. Therefore, we suggest a differential effect depending on the content or composition of surface proteins.

The concentrations at which EPs® 7630 interfered with receptor binding and neuraminidase activity of H1N1 A/Puerto Rico/8/34 (complete inhibition at 100 and 500 μg/ml, respectively) were 2- to 10-fold higher than those required for complete growth inhibition. This shows that, beyond its effect on viral hemagglutination and neuraminidase activity, the effect of EPs® 7630 on the host cell plays an important role. In addition to a surface effect on the host cell, EPs® 7630 has also been reported to activate the innate immune response, inducing macrophages and other cells to release interferons (Kolodziej et al., 2003; Kolodziej and Kiderlen, 2007) and intracellular nitric oxide (Thäle et al., 2011), which impairs influenza virus proliferation (Rimmelzwaan et al., 1999). In this context, the differential effect of EPs® 7630 on the distinct IAV strains may correspond to their differential sensitivity to antiviral host cell responses (Dittmann et al., 2008). We did not observe that EPs® 7630 increased induction of interferon ß in IAV-infected A549 cells by ELISA (data not shown). This may be due to the interferon inhibiting properties of the IAV NS1 protein (Hale et al., 2008). It also does not exclude an effect of EPs® 7630 on other cytokines or components of the immune system.

In addition, we showed that virus released from host cells after one life cycle is also efficiently prevented from entering new host cells to initiate its next life cycle. This suggests that in addition to a preventive effect, EPs® 7630 may have a therapeutic effect, limiting the spread of infection.

We have shown that over 4 passages in presence of EPs® 7630 no resistant virus mutants emerged, while resistance can develop already after 2–4 passages against oseltamivir or amantadine (Ehrhardt et al., 2007; Ludwig et al., 2004; Pleschka et al., 2009). As the extract inhibits IAV at an early stage of its life cycle and acts on both the virus and host cell, the development of resistance may be less likely, as has been shown also for other polyphenol-rich plant extracts (Ehrhardt et al., 2007; Pleschka et al., 2009).

EPs® 7630 consists of about 40% proanthocyanidins, more precisely of oligo- and polymeric prodelphinidins, which are composed of gallocatechin and epigallocatechin (Schoetz et al., 2008). When we depleted EPs® 7630 of polyphenols by precipitating them with skin powder, the antiviral effect was abolished, showing that polyphenols represent the active antiviral principle. However, this procedure does not remove polyphenols without tanning properties i.e. monomeric or >11-meric catechins, but their concentration in the tannin-depleted extract was probably too low to substantially interfere with viral growth. Our study showed that a group of polyphenols, prodelphinidins, especially gallocatechin and epigallocatechin in their monomeric, dimeric or oligo-/polymeric form, were active against IAV. These are the main components of EPs® 7630, suggesting that they are the active principle.

Lately, it has been shown that prodelphinidins from EPs® 7630 play a role in the prevention of bacterial infections (Wittschier et al., 2007). The extract inhibits, for instance, adhesion of group A streptococci to human epithelial cells (Janecki and Kolodziej, 2010). For this activity, a minimal structure of a trihydroxylated B-ring was required, as is present in epigallocatechin and gallocatechin. Polyphenols from *Rumex acetosa* L. have also been shown to have antiviral activity (Gescher et al., 2011a). In this case, proanthocyanidins galloylated in position 3 exhibited antiviral activity against herpes simplex-1 by blocking viral attachment to the host cell. Antiviral activity of prodelphinidin B2 3′-O-gallate was shown against herpes simplex-2 (Cheng et al., 2002), while epigallocatechin gallate showed good efficacy against enteroviruses (Ho et al., 2009). Compounds like gallocatechin, carrying only trihydroxylation at the B-ring, but no pyrogallol in position 3, showed a weaker antiviral effect against IAV than their galloylated homologs (Song et al., 2005). Nevertheless, since we demonstrated an antiviral effect of ungalloylated monomers and dimers, our study shows that galloylation in position 3 is not required for anti-IAV efficacy, although it is likely to potentiate an antiviral effect.

EPs® 7630 has shown good antiviral efficacy *in vivo* when administered as an aerosol of particle size 2.2 μm by inhalation.

Under these conditions, predicted deposition will be about 5% in the tracheobronchial system and 8–15% in the lungs (Oldham and Robinson, 2007; Raabe et al., 1988), but it is difficult to provide an estimate of the EPs® 7630 dose delivered. Mice treated by oral administration of 5 mg/kg three times a day had no advantage when compared to mock-treated controls. Our results showed that on a molar and on a weight basis, the monomers are less active than the dimers and oligo-/polymers, which is in line to previous data on herpes simplex virus (Takechi et al., 1985). After oral uptake, polyphenols are cleaved into their mono- or dimeric moieties (Spencer et al., 2000), before being absorbed through the gut (Deprez et al., 2001). As monomers and dimers retain antiviral activity (although less pronounced than oligo-/polymers), an antiviral effect after oral application is possible, although it could not be demonstrated in mice at the calculated human equivalent dose of EPs® 7630. It should be noted that uptake of at least low molecular weight catechins over the mucosa of the oral cavity (Yang et al., 1999) is possible when EPs® 7630 is taken orally, but this is not possible when the drug is delivered by gavage as in our experiments. Application by inhalation delivers the complete spectrum of EPs® 7630 polyphenols directly to the site of the respiratory infection and may therefore be more effective than peroral application. Thus, testing of EPs® 7630 in humans by inhalation would be of interest.

5. Conclusion

In this study, we investigated the anti-influenza mechanism of EPs® 7630 and demonstrate its efficacy *in vitro* and *in vivo*. The extract shows a robust effect against multiple different IAV strains *in vitro* and protection of mice if administered by inhalation against a lethal virus challenge at non-toxic concentrations, underlining the benefit of EPs® 7630 as a treatment for influenza virus infection.

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