Antiviral Research 91 (2011) 304–313

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

# Antiviral activity of the proteasome inhibitor VL-01 against influenza A viruses

Emanuel Haasbach [a,b], Eva-Katharina Pauli [b], Robert Spranger [b], David Mitzner [b,c], Ulrich Schubert [b,c], Ralf Kircheis [b], Oliver Planz [a,d,*]

[a] Friedrich-Loeffler-Institut, Institute of Immunology, Paul-Ehrlich Str. 28, 72076 Tuebingen, Germany
[b] ViroLogik GmbH, Innovation Centre for Medical Technology and Pharmaceuticals, Henkestr. 91, 91052 Erlangen, Germany
[c] Clinical and Molecular Virology, Friedrich-Alexander-University of Erlangen-Nuernberg, Schloßgarten 4, 91054 Erlangen, Germany
[d] University of Tuebingen, Interfaculty Institute for Cell Biology, Department of Immunology, Auf der Morgenstelle 15, 72076 Tuebingen, Germany

ARTICLE INFO

*Article history:*
Received 28 January 2011
Revised 29 June 2011
Accepted 5 July 2011
Available online 13 July 2011

*Keywords:*
Influenza A virus
Proteasome inhibitor
Drug-resistance

ABSTRACT

The appearance of highly pathogenic avian influenza A viruses of the H5N1 subtype being able to infect humans and the 2009 H1N1 pandemic reveals the urgent need for new and efficient countermeasures against these viruses. The long-term efficacy of current antivirals is often limited, because of the emergence of drug-resistant virus mutants. A growing understanding of the virus–host interaction raises the possibility to explore alternative targets involved in the viral replication. In the present study we show that the proteasome inhibitor VL-01 leads to reduction of influenza virus replication in human lung adenocarcinoma epithelial cells (A549) as demonstrated with three different influenza virus strains, A/Puerto Rico/8/34 (H1N1) ($EC_{50}$ value of 1.7 μM), A/Regensburg/D6/09 (H1N1v) ($EC_{50}$ value of 2.4 μM) and A/Mallard/Bavaria/1/2006 (H5N1) ($EC_{50}$ value of 0.8 μM). In *in vivo* experiments we could demonstrate that VL-01-aerosol-treatment of BALB/c mice with 14.1 mg/kg results in no toxic side effects, reduced progeny virus titers in the lung ($1.1 \pm 0.3$ $\log_{10}$ pfu) and enhanced survival of mice after infection with a 5-fold $MLD_{50}$ of the human influenza A virus strain A/Puerto Rico/8/34 (H1N1) up to 50%. Furthermore, treatment of mice with VL-01 reduced the cytokine release of IL-α/β, IL-6, MIP-1β, RANTES and TNF-α induced by LPS or highly pathogen avian H5N1 influenza A virus. The present data demonstrates an antiviral effect of VL-01 *in vitro* and *in vivo* and the ability to reduce influenza virus induced cytokines and chemokines.



## 1. Introduction

H1N1 influenza viruses are a major topic of human health care, especially after the last H1N1 pandemic. Additionally, highly pathogenic avian influenza (HPAI) H5N1 virus infection leads to high lethality in humans, as a result of extensive alveolar immune inflammatory infiltrates, causing tissue damage that compromises lung function. The H5N1 virus infection results in high levels of inflammatory cytokines and chemokines, due to an immune dysregulation (hypercytokinemia) (Cheung et al., 2002; Chotpitayasunondh et al., 2005; Droebner et al., 2008; Tumpey et al., 2000; Wong and Yuen, 2006). Elevated levels of cytokines and chemokines, including IP10, MIG, MCP-1, IL-6, IL-8 and RANTES, have been observed in human cell lines, mice and macaques infected with H5N1 influenza virus (Chan et al., 2005; de Jong et al., 1997; Kobasa et al., 2007).

The pandemic situation of the last years clearly demonstrates that influenza A virus infection is still a major risk for the public health. The possibility of a new emerging pandemic influenza A virus strain or reassortment between the pandemic and seasonal or avian A/H5N1 influenza virus strain is indeed a frightening but not unlikely event. Since vaccines will not be available in the first 6 months after a pandemic outbreak, there is a strong need for effective antivirals. Today, neuraminidase-inhibitors such as oseltamivir represent the most common clinically approved medication against influenza A viruses. In the recent past the number of reports in which drug-resistant influenza A viruses were described increased (Hurt et al., 2009; Le et al., 2005; McKimm-Breschkin et al., 2007; Meijer et al., 2009; Rameix-Welti et al., 2008; Sheu et al., 2008). Drug resistance to the known antivirals highlights the urgent need to optimize the effectiveness of current and novel antiviral treatments through development of new formulations, delivery routes or novel defense mechanisms. Due to the high mutation rate of influenza A virus, the threat of fast resistance formation against these compounds exists. In contrast, the human genome possesses a million fold lower mutation rate. The inhibition of host cell factors, which the virus is depending on during its replication cycle, offers an interesting alternative target for the development of new therapies. Here, the virus cannot replace the missing cellular component by mutations. It is well known that influenza A virus recruits host cell factors for efficient replication

* Corresponding author at: University of Tuebingen, Interfaculty Institute for Cell Biology, Department of Immunology, Auf der Morgenstelle 15, D-72076 Tuebingen, Germany. Tel.: +49 7071 29 80995; fax: +49 7071 29 5653.
*E-mail address:* oliver.planz@uni-tuebingen.de (O. Planz).

0166-3542/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2011.07.006

(Ehrhardt et al., 2010; Konig et al., 2010; Watanabe et al., 2010). Therefore, it is well traceable that targeting such host cell factors would be a promising approach for the development of new antiviral drugs. We previously demonstrated that influenza virus replication is dependent on the NF-κB pathway and on the Raf/MEK/ERK mitogenic kinase cascade. Inhibition of the IKK/NF-κB by the use of acetylsalicylic acid (ASA), as well as inhibition of the Raf/MEK/ERK signaling pathways with the MEK inhibitor U0126, leads to strong reduction of influenza A virus infection (Ludwig and Planz, 2008; Ludwig et al., 2004; Mazur et al., 2007; Pleschka et al., 2001).

The 26S-proteasome – the central proteolytic component of the ubiquitin–proteasome system (UPS) – can be selectively inhibited by proteasome inhibitors. The ability of such substances to inhibit the proteasome and thereby to prevent NF-κB activation is well known (Breccia and Alimena, 2010; Vink et al., 2006; Yu and Kem, 2010). During influenza virus infection NF-κB acts via induction of proapoptotic factors, such as TNF-related apoptosis-inducing ligand (TRAIL) or FasL followed by activation of caspases. This caspase-activation resulted in an enhanced nuclear export of viral RNPs, presumably by specific cleavage of nuclear pore proteins, resulting in an enhanced diffusion limit through the pores (Ludwig, 2009). This function appears to be relevant for viral replication since a nuclear retention of viral ribonucleoprotein (RNP) complexes can be observed in the presence of both, caspase- and NF-κB-inhibitors (Mazur et al., 2007; Wurzer et al., 2003).

An antiviral effect of proteasome inhibitors on different RNA viruses has previously been shown (Ma et al., 2010; Ott et al., 2003; Schubert et al., 2000). Widjaja and colleagues showed that inhibition of proteasome activity affects influenza A virus infection at a post-fusion step (Widjaja et al., 2010). Furthermore, proteasome inhibitors such as PS-341 (Velcade) are currently in clinical trails against cancer e.g. against relapsed and refractory multiple myeloma (Richardson et al., 2003, 2005; San Miguel et al., 2008).

In the present study, we wanted to investigate the antiviral effect of the proteasome inhibitor VL-01 (ViroLogik GmbH, Germany) in cell culture and in the mouse model. Delivery of the substance *via* the aerosol route led to reduction of progeny virus titer in lungs of mice after human influenza A virus infection. Furthermore, we demonstrated that inhibiting the proteasome activity leads to an increased survival after lethal influenza A virus infection. The proteasome inhibitor VL-01 also reduced cytokine release induced by LPS stimulation or by infection with highly pathogenic avian H5N1 influenza A virus. Aerosol application of VL-01 at the concentrations required for antiviral activity did not lead to any adverse effects. Thus, we conclude that efficient influenza virus replication is dependent on proteasome activity and that temporarily inhibition of proteasome activity might be effective for the development of novel treatment strategies.

## 2. Materials and methods

### 2.1. Compound

The proteasome inhibitor VL-01 (ViroLogik GmbH, Germany) with a formula weight of 752.8 g/mol inhibits the 20S and 26S proteasome unit. The inhibitor was dissolved in 5% DMSO, 15% Cremophor in PBS before use in different concentrations.

### 2.2. Mice

Inbred BALB/c mice at the age of 6–8 weeks were obtained from the animal breeding facilities at the Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Tuebingen, Germany and were used throughout all the experiments.

### 2.3. Virus

The human influenza virus A/Puerto Rico/8/34 (H1N1, PR8) and the pandemic influenza virus strain A/Regensburg/D6/09 (H1N1, RB1) were propagated in Madin-Darby canine kidney (MDCK) cells. Additionally, we used the highly pathogenic avian H5N1 influenza A virus strain A/Mallard/Bavaria/1/2006 (H5N1, MB1) grown in embryonated chicken eggs, which was originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany.

### 2.4. Influenza virus titration (AVICEL® plaque assay)

To assess the number of infectious particles (plaque titers) in the samples, a plaque assay using AVICEL® was performed in 96-well plates (Vogel et al., 2010). Virus-infected cells were immunostained by incubating for 1 h with a monoclonal antibody specific for the influenza A virus nucleoprotein (Serotec, Duesseldorf, Germany), followed by 30 min incubation with peroxidase-labeled anti-mouse antibody (DIANOVA, Hamburg, Germany) and 10 min incubation with True Blue™ peroxidase substrate (KPL, Gaithersburg, USA). Stained plates were scanned on a flat bed scanner and the data were acquired by Corel DRAW 9.0 software. To define the titer of progeny virus, the foci of infected cells for every sample in each well of the 96-well plates were counted and multiplied with the dilution factor. The mean values were taken from the final number of foci in each well. The viral titers are shown as the logarithm to the base 10 of the mean values.

### 2.5. Measurement of pharmacological parameters in vitro

To determine the inhibitory effect ($IC_{50}$) of VL-01 *in vitro*, we treated human lung adenocarcinoma epithelial cells (A549) with different concentrations of VL-01 (0.1–50 μM) and measured the proteasome activity via P20-assay, based on the method of Adams and colleagues (1999). For determination of the effective concentration 50% ($EC_{50}$) A549 cells were infected with PR8 (MOI of 0.001) and subsequently VL-01 treatment was started by adding culture medium with different concentrations of VL-01 (0–64 μM). Progeny virus in the supernatant of infected and treated cells was measured by plaque assay as described in Section 2.4. For calculation, each experiment was repeated three times independently with each comprising triplicates.

The cytotoxic concentration ($CC_{50}$) of VL-01 was determined in A549 and MDCK cells as well in primary hepatocytes, tonsils and peripheral blood mononuclear cells. All cell types were incubated with different VL-01 concentrations (0.1–50 μM) for 24 h followed by a washing step and further incubation with VL-01 for 24, 48 or 72 h. After 48, 72 or 96 h of incubation with VL-01 in total, cytotoxic effects were measured. The effect in A549 cells and primary hepatocytes were examined by a water-soluble tetrazolium salt (WST-1) assay according to the manufactures protocol (Roche Diagnostics, Mannheim, Germany). MDCK cells were stained with crystal-violet as described by Lison and colleagues (2008). For the evaluation of cytotoxic effects in tonsils and PBMCs over a time of 72 h, cells were measured as apoptotic and necrotic cells by FACS analysis via annexin V/propidium iodide staining as described in the distributors protocol (Vybrant® Dead Cell Apoptosis Kit Invitrogen, Darmstadt, Germany). All experiments were done as triplicates. Results evaluated by GraphPad prism software showing a cytotoxic effect dependent on the VL-01 concentration (Nicole Studtrucker and Daniel Lüftenegger; unpublished data).

### 2.6. Virus inoculation of mice

Six- to eight-week-old BALB/c mice were obtained from the animal breeding facilities at the FLI. Before intranasal inoculation with

MB1 (H5N1) or PR8 (H1N1), mice were anaesthetized by intraperitoneal injection of 150 μl of a ketamine (Sanofi)-rompun (Bayer)-solution (equal amounts of a 2%-rompun-solution and a 10%-ketamin-solution were mixed at the rate of 1:10 with phosphate buffered saline (PBS)). All animal studies were approved by the Institutional Animal Care and Use Committee of Tuebingen.

### 2.7. VL-01-treatment of mice and virus infection

The aerosol-treatment of mice was performed in a self-made aerosol-application-device were four mice can be treated. For fumigation of dissolved VL-01 or solvent the PARI LC SPRINT® STAR nebulizer (PZN: 3870078 PARI GmbH, Starnberg, Germany) was used. The solutions were nebulized at 3 bar air pressure for 15 or 30 min. Discharged air was aspirated with 8 l/min via a vacuum-pump to ensure optimal air/aerosol flow. For prophylactic studies mice were treated via the aerosol route 1 h prior to viral inoculation. For infection, mice were anaesthetized by intraperitoneal injection of ketamine/rompun and infected intranasally with the 5-fold $MLD_{50}$ of different influenza A virus strains. After infection mice were treated at different time points, every 12 h, either with VL-01 or solvent. According to the experimental setup, body weight and visual clinical symptoms of mice were monitored for a 21-day observation period or lungs of VL-01-treated and solvent-treated control mice were collected 24, 48, 72 h after infection and viral titers were determined by plaque assay.

### 2.8. Cytokine analysis

All cytokine assays were performed using a multi Bio-Plex Protein Array System from BioRad (Bio-Rad Laboratories, Munich, Germany). To investigate the LPS induced cytokine response and the ability of VL-01 to modulate this response, four mice were 2 h prior to LPS treatment (lipopolysaccharides from *Escherichia coli* 055:B5, Sigma, Germany, 20 μg/mice) intravenously treated with 25 mg/kg VL-01 and four with diluent. At 1.5 and 3 h after LPS treatment serum samples were taken and the cytokine profile was measured. In a second experiment the avian H5N1 virus A/mallard/Bavaria/1/2006 (MB1) was used as a cytokine inducer. A group of seven mice were i.v. treated with 25 mg/kg VL-01 2 h prior to virus infection (MB1, 10-fold $MLD_{50}$), compared to seven mock-treated mice. Serum samples were collected at 0, 12, 30 and 72 h after infection and cytokines were measured.

### 2.9. Immunofluorescence

A549 or MDCK cells were infected with RB1 (H1N1), PR8 (H1N1) or MB1 (H5N1) with a MOI of 1 in the absence or presence of 2 μM VL-01. After 4, 6, 8 and 12 h p.i., cells were fixed for 30 min in 4% buffered paraformaldehyde at 4 °C. The fixed cells were washed twice with PBS and permeabilized. Cells were fluorescently labeled using primary mouse anti-Influenza A nucleoprotein antibodies (AbD Serotec) and secondary Alexa Fluor® 555 F(ab′)2 fragment of goat anti-mouse IgG (H + L) antibodies (Invitrogen). Nuclei were stained using DAPI (Fluka). Afterwards, cells were mounted with ProLong Gold Antifade Medium (Invitrogen, Darmstadt, Germany), and analyzed by the Apotome Care Invert Microscope Labovert FS (Leitz, Wetzlar, Germany) and the AxioVision Rel 4.5 (Carl Zeiss Imaging, Oberkochen, Germany).

### 2.10. Statistical analysis

Error bars are given as the SEM. For the calculation of the significance of differences, the paired *t*-test ($p \leqslant 0.05$) or the log-rank test was performed. All analyzes were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

## 3. Results

### 3.1. VL-01 inhibits proteasome activity and influenza virus replication in vitro

The pharmacological parameters, inhibition concentration 50% ($IC_{50}$), cytotoxic concentration ($CC_{50}$) and effective concentration 50% ($EC_{50}$) are most important to develop an antiviral drug. Therefore, we determined these parameters for the proteasome inhibitor VL-01. To examine the concentration of VL-01 to inhibit the proteasome activity up to 50% ($IC_{50}$), human lung adenocarcinoma epithelial cells (A549) were treated with different concentrations of VL-01 (0.1–50 μM). On different time points post infection (4, 8, 24 and 32 h) the proteasome activity was measured using the P20-assay (measurement of chymotrypsin-like activity of the 20S subunit from the 26S proteasome). The proteasome inhibition by VL-01 showed a time-dependent effect. After 4 and 8 h the $IC_{50}$ values ranged between 2.7 and 1.8 μM, whereas after 24 and 32 h the $IC_{50}$ was approximately 1 μM (Fig. 1A). The cytotoxic effect ($CC_{50}$) of VL-01 treatment was assayed on A549 and MDCK cells with different concentrations of VL-01 (0.1–50 μM). After incubation periods of 24, 48, 72 and 96 h the cell viability was measured by the WST-1 assay or crystal-violet staining. In terms of the $IC_{50}$ value of proteasome inhibition of 1 μM after 24 h, we could show that there was no cytotoxic effect of VL-01 after this treatment period. The $CC_{50}$ was found at approximately 4 μM after 24 h in A549 cells and there was no toxic effect on MDCK cells neither after 24 nor 48 h (Fig. 1B and C). A longer incubation of 48, 72 or 96 h shows a $CC_{50}$ in A549 cells between 2.8 and 3.6 μM (Fig. 1B and C). In addition to these cell lines, different primary human cell lines, e.g. primary hepatocytes, tonsils and peripheral blood mononuclear cells reveals similar $CC_{50}$ values (ViroLogik GmbH, unpublished data). Next, the effective concentration of 50% ($EC_{50}$), which demonstrates the ability to reduce influenza A virus titer up to 50%, was analyzed by measurement of progeny virus in presence of different VL-01 concentrations (0–64 μM) in infected A549 cells after 24 h. VL-01 showed an $EC_{50}$ value of approximately 1.7 μM in A/Puerto Rico/8/34 (H1N1, PR8) infected cells (Fig. 1D). The $EC_{50}$ of A/Regensburg/D6/09 (H1N1v, RB1) infected cells is about 2.4 μM (Fig. 1E) and for the A/Mallard/Bavaria/1/2006 (H5N1, MB1) virus 0.82 μM (Fig. 1F). The $IC_{50}$ and $EC_{50}$ – i.e. the concentrations which are effective to inhibit the proteasome (1 μM) or virus replication (1.7 μM), respectively, were found below the concentration where VL-01 showed cytotoxic effects (4 μM). The window between antiviral activity and cytotoxic effects was not very broad in these studies. However, this may not be surprising, as the A549 adenocarcinoma cell line represents highly proliferating tumor cells that are known to be inhibited by proteasome inhibitors.

### 3.2. Reduction of lung virus titer in mice after VL-01 treatment

To further analyze the anti-influenza A virus potential, *in vivo* experiments were performed in the mouse model. In this context we wanted to investigate the VL-01 concentration that is most effective in reducing influenza virus replication *in vivo*. Therefore, mice were aerosol-treated 1 h prior to human influenza A virus infection (PR8, H1N1, 5-fold $MLD_{50}$) with different concentrations of VL-01 (0–227.4 mg/kg). After 24 h mice were sacrificed and viral titers in the lungs were measured by plaque assay. As shown in Fig. 2A, an antiviral effect of VL-01 on progeny virus titer in the lung was found which increased with increasing

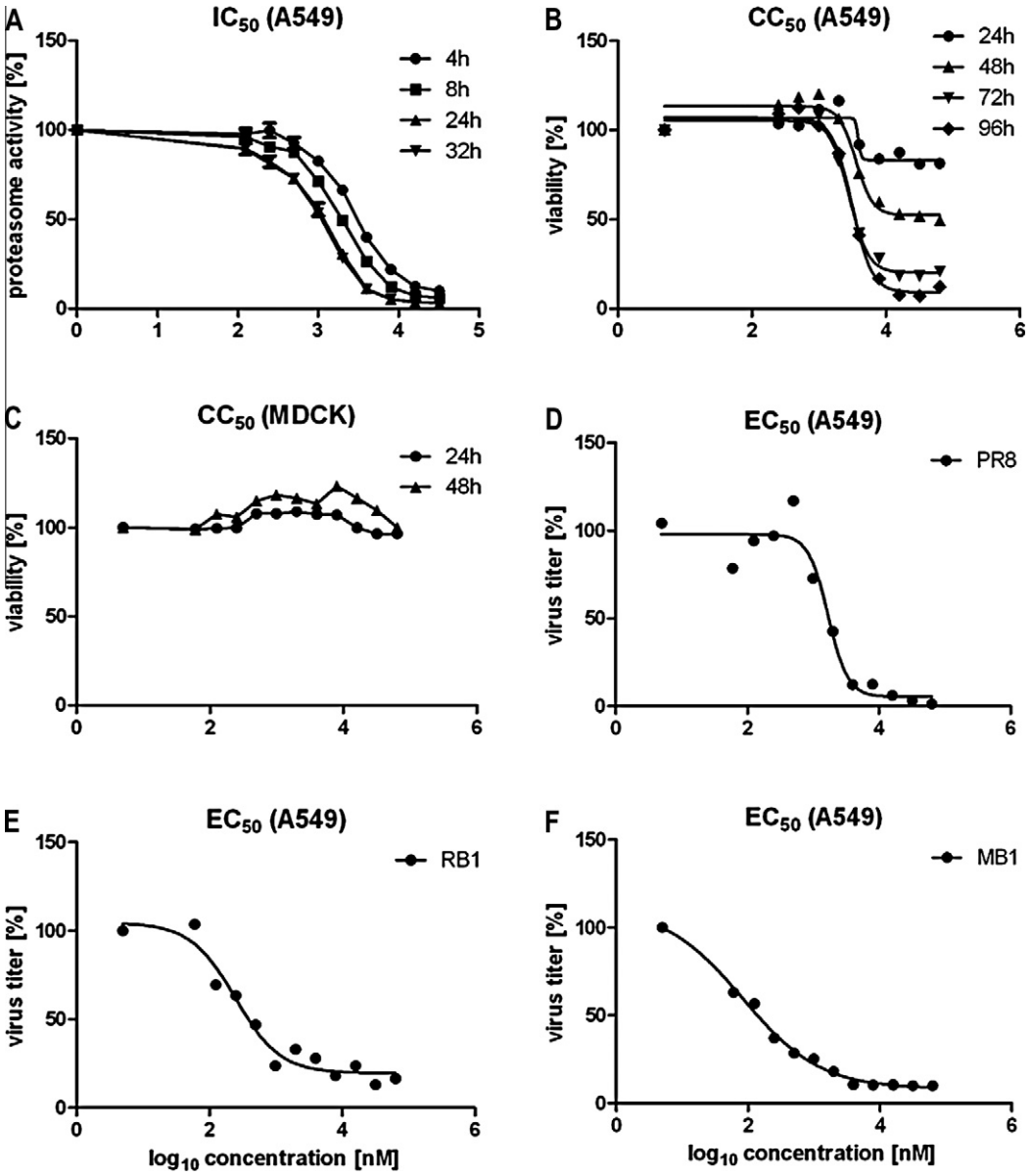


**Fig. 1.** Determination of the inhibition concentration 50% ($IC_{50}$) (A), cytotoxic concentration ($CC_{50}$) (B and C) and effective concentration 50% ($EC_{50}$) (D–F) of VL-01. A549 cells were treated with different concentrations of VL-01 (0.1–50 μM) and at different time points (4, 8, 24 and 32 h) the proteasome activity was measured by P20-assay (measurement of chymotrypsin activity of the 20S subunit from the 26S proteasome). Cytotoxic effects of VL-01 treatment were assayed on A549 and MDCK cells with different concentrations of VL-01 (0.1–50 μM). After the incubation period of 24, 48, 72 and 96 h the cell viability was measured by WST-1 assay or crystal-violet staining. The $EC_{50}$ was analyzed 24 h past infection by plaque assay. Therefore, A549 cells were infected with PR8 (D), RB1 (E) or MB1 (F) (MOI of 0.001) and subsequently VL-01 treatment was started by adding infection medium with different concentrations of VL-01 (0–64 μM). For calculation, each experiment was repeated three times independently with each comprising triplicates.

VL-01 doses up to 113.7 mg/kg. No adverse effects were seen, even at the highest doses of VL-01 used for single aerosol treatment (data not shown). The calculated *in vivo* $EC_{50}$ was 31.9 ± 7.5 mg/kg. Since a concentration of 14.1 mg/kg VL-01 was also sufficient to reproducible reduce progeny virus titer up to 1.1 ± 0.3 $\log_{10}$ pfu, this concentration was used for most of the further aerosol treatment experiments. It has to be noted that the aerosol device allows a drug deposition of approximately 1% of the evaporated drug in the lungs. Thus, the maximum amount of VL-01 that will reach the lung will be roughly 0.14 mg/kg (2.8 μg per mouse).

After finding the optimal VL-01 concentration *in vivo* we addressed the question, whether this concentration was able to induce adverse effects after multiple treatments. Therefore, we treated mice either with 14.1 mg/kg VL-01 or solvent by daily aerosol-treatment over a period of 10 days. The physical parameters and the body weight of the animals were monitored daily (Fig. 2B). There were no evidences for toxic side effects during the whole observation period, neither change in body weight nor in any physical parameters. In a second study to investigate toxicity of VL-01 in mice, the animals were treated intraperitoneal (i.p.) for 14 days using two different mouse strains. With this assay mouse toxic doses (MTD) of 25 mg/kg (DmVk-mice) and 150 mg/kg (CD1 nu/nu mice) VL-01 were determined. Study of the acute toxicity in mice of VL-01 revealed that $LD_{10}/LD_{50}$ value following single intravenous administration equals to 57.2 ($LD_{10}$) and 135.5 mg/kg ($LD_{50}$). Furthermore, effective doses were tolerated in animal models with repeated i.p. doses of VL-01. A i.p. dose of 150 mg/kg appears to be the suitable dose for the mice model (data not shown).

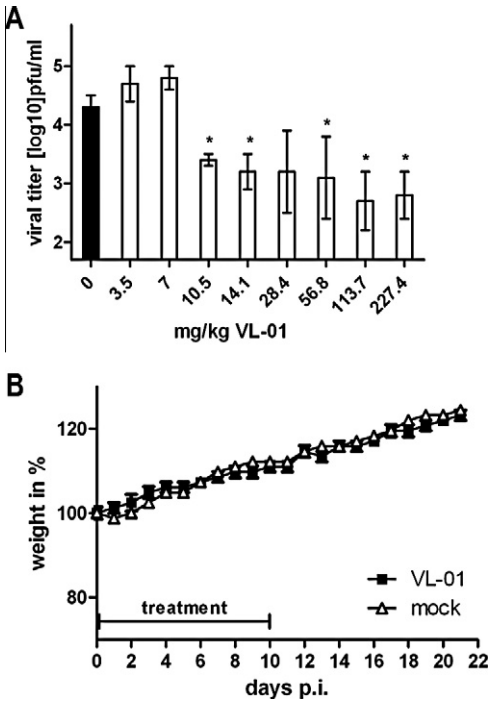


**Fig. 2.** Determination of the optimal VL-01 concentration for aerosol application *in vivo*. Four BALB/c mice were aerosol-treated 1 h prior to infection (PR8, H1N1, 5-fold $MLD_{50}$) with different concentrations of VL-01 (0–227.4 mg/kg). After 24 h mice were sacrificed and the viral lung titers were measured by plaque assay (A) ($n = 4$, $^*p < 0.05$). Investigation of toxic side effects after 10-day VL-01 treatment. Two groups of four BALB/c mice were treated either with 14.1 mg/kg VL-01 or solvent alone. The aerosol-treatment was applied once a day over a period of 10 days. The physical parameters and body weight of the animals were monitored daily (B) ($n = 4$).

### 3.3. Prolonged VL-01 aerosol-treatment leads to an increased antiviral effect

Optimization of frequency and duration of drug application is very important for efficient antiviral treatment. Thus, we raised the question whether multiple daily treatment or prolongation of drug-delivery time would improve antiviral activity. Therefore, we treated mice once with 14.1 mg/kg VL-01 1 h prior to infection (PR8, H1N1, 5-fold $MLD_{50}$) or twice (one prior and 11 h past infection). After 24 h mice were sacrificed and viral titers in the lungs were measured by plaque assay. A second treatment of mice with 14.1 mg/kg VL-01 resulted in an enhanced reduction of progeny virus titer in the lung ($3.4 \pm 0.06$ $\log_{10}$ pfu) compared to single treatment ($3.6 \pm 0.13$ $\log_{10}$ pfu). In comparison, in mock treated animals a viral titer of $4.6 \pm 0.15$ $\log_{10}$ pfu was measured (Fig. 3A and B).

The impact of the duration of aerosol-treatment was investigated by single treatment of mice for either 15 or 30 min resulting in a final VL-01 dose of 14.1 or 28.2 mg/kg, respectively. Less virus was found in the lung after 24 h and 30 min treatment ($2.3 \pm 0.08$ $\log_{10}$ pfu) compared to only 15 min treatment ($2.8 \pm 0.03$ $\log_{10}$ pfu) (Fig. 3C and D). Mock treated animals showed viral titers of $3.5 \pm 0.08$ $\log_{10}$ pfu. Taken together, the deposition of increased amounts of VL-01 was found to lead to an increased antiviral effect in mice.

### 3.4. Increased survival of human influenza A virus infected mice after VL-01 treatment

To examine the antiviral effect of VL-01 on survival, we used the optimized parameters, dose, frequency and duration to perform a prophylactic survival experiment. Thus, we treated mice with 14.1 mg/kg VL-01 or solvent one hour prior to infection (PR8, H1N1, 5-fold $MLD_{50}$) for 30 min. Mice were treated every 12 h for four days. The physical parameters, body weight, onset of disease and survival were monitored daily for 21 days. The body weight-curve shows a nearly similar course for mock and VL-01 treated mice until day 6 (Fig. 4A). All mice developed the first disease symptoms by day 3 post infection. Within a few days, the symptoms in the mock-treated group became severe and resulted in high disease scores and finally in death. In contrast, VL-01-treated mice showed a delayed development of severe symptoms (Fig. 4B). At day 7, three out of four mock-treated mice died, whereas only one out of four VL-01-treated mice died. The last mock-treated mouse died at day 8 past infection. At day 9, a second VL-01-treated mouse died. The two remaining VL-01-treated mice raised their body weight constantly until day 19 where they accomplished again their starting weight (Fig. 4C). This experiment was performed several times with similar results (data not shown). In summary, VL-01 treatment of mice starting prior to infection with human H1N1 influenza A virus leads to a survival rate of approximately 50% compared to 100% lethality found in mock-treated mice.

### 3.5. VL-01 treatment reduces cytokine release induced by LPS stimulation or by infection with highly pathogenic avian H5N1 influenza A virus

It is well known that highly pathogenic avian influenza A viruses induce release of a wide pattern of cytokines and chemokines called hypercytokinemia or cytokine storm. To investigate whether VL-01 treatment has an influence on the amount of cytokine and chemokine release, we used LPS as a positive control; a well-known cytokine- and chemokine inducer that acts as a common model to investigate anti-inflammatory effects (Carlsen et al., 2004; Errea et al., 2010). To examine the influence of VL-01 on influenza A virus mediated cytokine and chemokine release we used the highly pathogenic avian influenza A virus strain A/Mallard/Bavaria/1/2006 (MB1, H5N1). First, we investigated the LPS induced cytokine response and the ability of VL-01 to modulate this response. In order to test the effect of the proteasome inhibitor VL-01 on induced cytokine release, mice were treated intravenously (i.v.) 2 h prior to LPS (20 μg/mice) application with 25 mg/kg VL-01. As mentioned in Section 3.2 this concentration is not toxic for mice. At 1.5 and 3 h after LPS challenge serum samples were taken and the cytokine profile was measured. Mock-serum was taken 4 h prior to any treatment.

In a second experiment infection with the highly pathogenic avian influenza A virus, MB1 (H5N1) was used as a cytokine inducer. Mice were treated i.v. with 25 mg/kg VL-01 2 h prior to virus infection (MB1, 10-fold $MLD_{50}$). Serum samples were collected at 0, 12, 30 and 72 h after infection and cytokines were measured by using multiplex assay (BioRad). VL-01 treatment significantly reduced LPS induced cytokines IL-1β, IL-6, MIP-1β, RANTES and TNF-α, either on both time points 1.5 and 3 h (IL-1β, MIP-1β and RANTES) after induction or at one of the time point (1.5 h, IL-6; 3 h, TNF-α) (Fig. 5A–E).

Similar to LPS induction, the cytokines and chemokines induced by an avian H5N1 influenza A virus infection could also significantly be reduced by VL-01 treatment (Fig. 5 right panel, F–J). The IL-1α and TNF-α serum levels in VL-01 treated mice showed a significant reduction at 12 and 30 h after infection, compared to mock-treated mice. VL-01 treatment reduced the IL-6 serum level at 12 and 72 h after infection. The level of MIP-1β was only reduced at 72 h after infection, compared to untreated control group. In contrast, the expression of RANTES was only marginally altered by VL-01 treatment.

In summary, this experiment demonstrated that VL-01 reduced the systemic cytokine and chemokine release induced by avian H5N1 influenza A virus infection in mice.

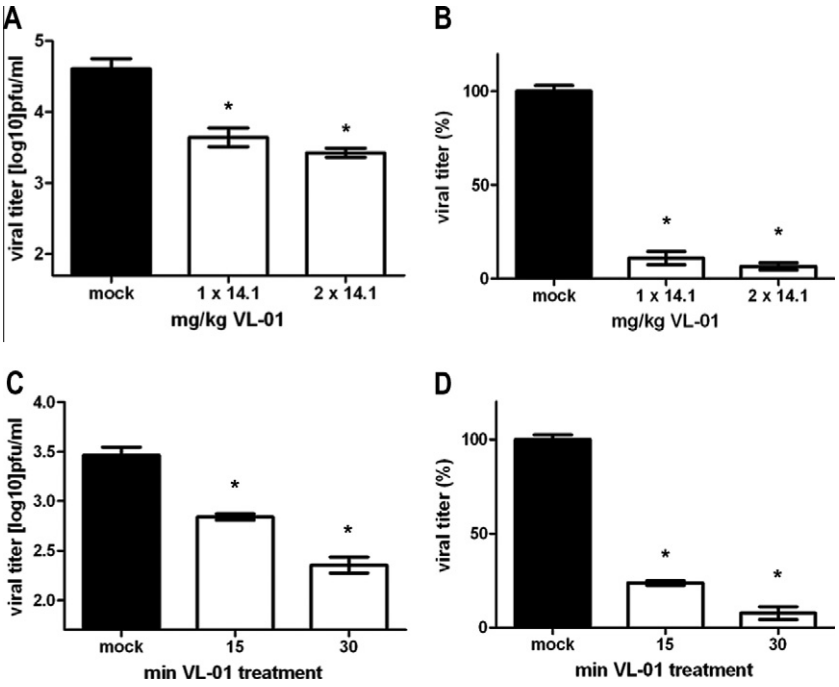


**Fig. 3.** Optimal frequency and duration of VL-01 treatment. BALB/c mice were either treated once with 14.1 mg/kg VL-01 for 1 h prior to infection (PR8, H1N1, 5-fold $MLD_{50}$) or twice (one prior and 11 h after infection). After 24 h mice were sacrificed and viral lung titers were measured by plaque assay (A in pfu/ml, B in %, $n = 4$, $^{*}p < 0.05$). Mice were treated once for 15 or 30 min with VL-01 resulting in a final dose of 14.1 or 28.2 mg/kg, respectively. After 24 h mice were sacrificed and viral lung titers were measured by plaque assay (C in pfu/ml, D in %, $n = 4$, $^{*}p < 0.05$).

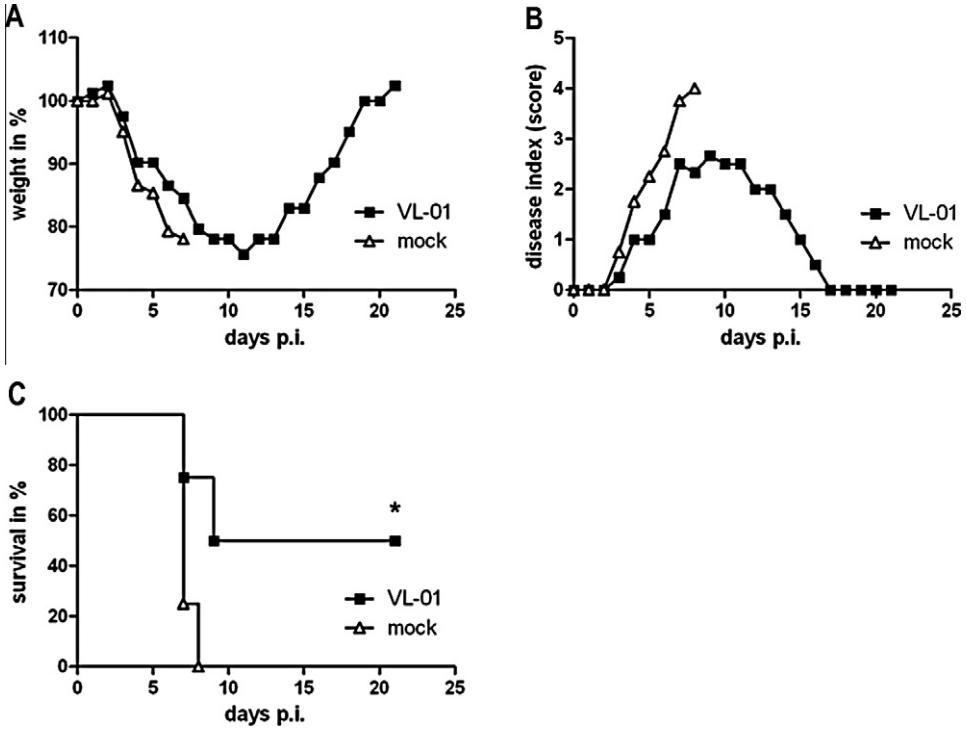


**Fig. 4.** BALB/c mice were treated with 14.1 mg/kg VL-01 or solvent one hour prior to infection (PR8, H1N1, 5-fold $MLD_{50}$) for 30 min. The mice were treated every 12 h for 4 days. The physical parameters, body weight (A), onset of disease (B) and survival of animals (C) were monitored daily. Several experiments show similar results ($n = 4$, $^{*}p < 0.05$, log-rank test).

### *3.6. VL-01 treatment leads to a delayed nuclear export of viral RNPs in vitro*

It is well known that NF-κB promotes influenza A virus production and enhances the nuclear export of viral RNPs (vRNP) via activation of caspase-3 (Planz, 2006; Wurzer et al., 2003). Next, we investigated whether the influenza virus vRNP-export is influenced by VL-01 treatment. Therefore, the nuclear export of the nucleoprotein (NP), the major part of the vRNP complexes, was analyzed by fluorescent microscopy in A549 and MDCK cells. Cells were

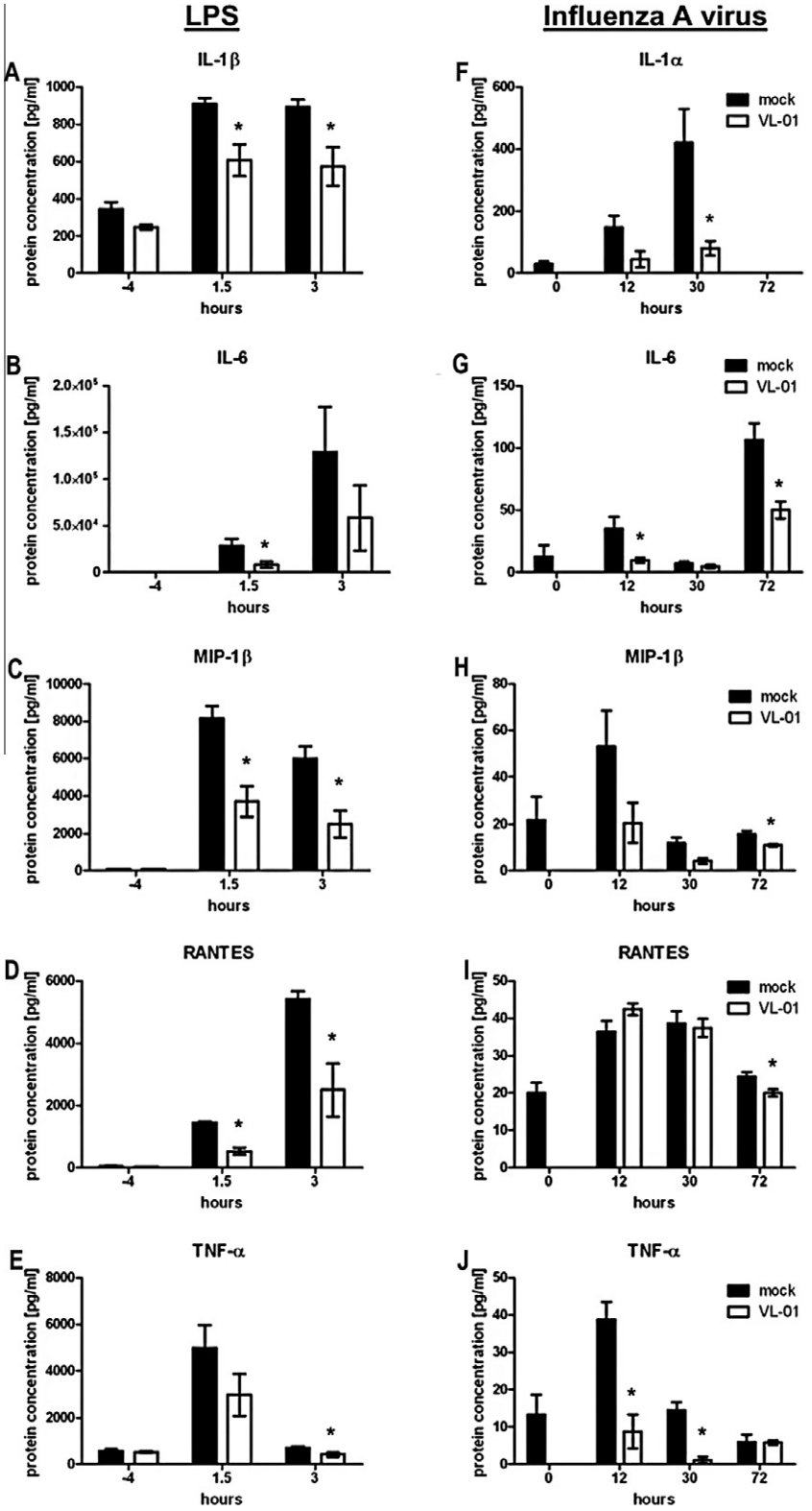


**Fig. 5.** Cytokine and chemokine expression after LPS treatment or avian H5N1 influenza A virus infection. Two-hour prior to LPS treatment (20 μg/mice) mice were i.v. treated with 25 mg/kg VL-01. Serum samples were taken 4 h before (control serum) and 1.5 and 3 h after LPS application and cytokine profile was measured by multiplex assay (left panel, A–E). In a second experiment, the avian H5N1 virus A/mallard/Bavaria/1/2006 (MB1) was used as a cytokine inducer. Mice were i.v. treated with 25 mg/kg VL-01 2 h prior to virus infection (MB1, 10-fold $MLD_{50}$). Serum samples were collected at 0, 12, 30 and 72 h after infection and cytokines were measured by multiplex assay (right panel, F–J) ($n = 4–7$, $^{*}p < 0.05$). Note, IL-1α was not reduced after LPS induction, as well as no reduction for IL-1β by influenza infection.

infected with human influenza virus PR8 (H1N1), pandemic influenza virus RB1 (H1N1v) and highly pathogenic avian influenza virus MB1 (H5N1) in the absence or presence of 2 μM VL-01. After 6 h, cells were fixed and stained against NP and visualized by

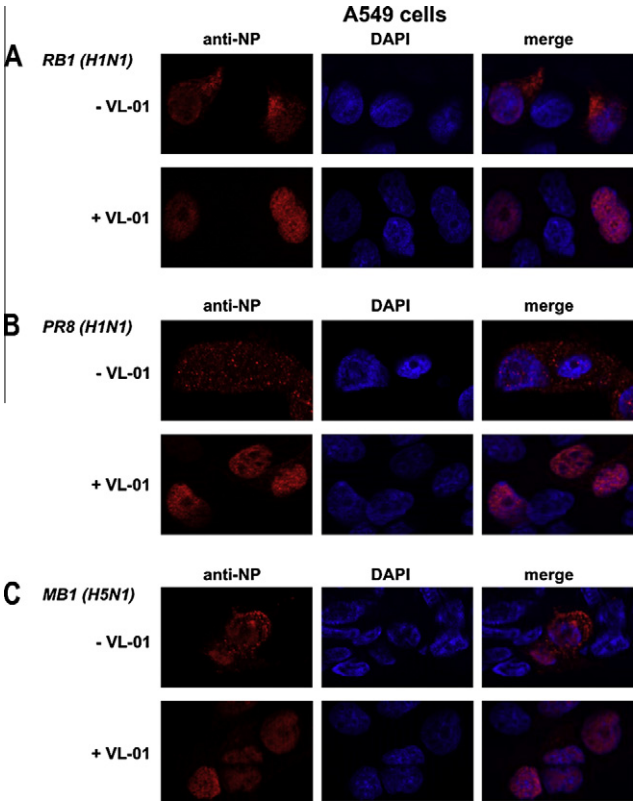


**Fig. 6.** VL-01 treatment of influenza A virus infected A549 cells results in nuclear retention of viral NP in the nucleus. A549 cells were infected with RB1 (H1N1) (A), PR8 (H1N1) (B) or MB1 (H5N1) (C) (MOI 1) in the absence or presence of 2 μM VL-01. Six hours p.i. cells were subjected to immunofluorescence analyzes using an anti-NP antibody to stain for viral RNP complexes; cell nuclei were counterstained with DAPI.

fluorescent microscopy. Studies in either RB1, PR8 or MB1 infected A549 cells showed that 6 h after infection NP is detectable in the cytoplasm of mock-treated cells (untreated) (Fig. 6A–C, upper panel) while in VL-01 treated cells NP is still located in the nucleus (Fig. 6A–C, lower panel). Similar results were found on MDCK cells (Supplementary Fig. S1). That demonstrates that vRNP export is efficiently impaired in the presence of VL-01 after influenza A virus infection.

## 4. Discussion

It is well known that influenza A virus recruits host cell factors for efficient replication (Ehrhardt et al., 2010; Konig et al., 2010; Ludwig et al., 1999; Watanabe et al., 2010). Therefore, it is traceable that targeting host cell factors could be a promising approach for new antiviral drugs. We were able to demonstrate that influenza virus infection of host cells leads to biphasic activation of the Raf/MEK/ERK signaling pathway and that inhibition of this pathway on the level of MEK leads to reduction in progeny virus (Ludwig et al., 2004; Pleschka et al., 2001). MEK inhibitors evaluated for cancer treatment in a phase I evaluation revealed that repeated treatment for 21 consecutive days was well tolerated (Lorusso et al., 2002). In another study we were able to demonstrate that acetylsalicylic acid (ASA) is highly effective against influenza virus (Mazur et al., 2007). ASA functions as an IKK/NF-κB inhibitor by interacting with IKK-β to reduce ATP binding activity (Yin et al., 1998). Under normal conditions, NF-κB is held in an inactive state in a cytoplasmic complex with its inhibitory protein (IκB). Phosphorylation of IκBα targets IκB for ubiquitination and subsequent proteasomal degradation. NF-κB is released for nuclear translocation and transcriptional activation, which is also a prerequisite for efficient influenza virus replication.

Thus, targeting host cell factors with clinically established compounds might be a promising strategy for the development of new antivirals against influenza viruses.

In this context, we wanted to investigate whether inhibition of the proteasome might be such a strategy, since efficient degradation of IκB by the proteasome is needed to activate the NF-κB pathway (Karin and Ben-Neriah, 2000). Inhibition of the proteasome-mediated degradation leads to retention of NF-κB in the cytoplasm that should finally lead to reduced virus replication. Because of the vital function of UPS, the usage of proteasome inhibitors in humans is not trivial, in order to avoid adverse events. Nevertheless, proteasome inhibitors are being used against cancer. The proteasome inhibitor PS-341 (Velcade) has got the market approval by the FDA for relapsed and refractory multiple myeloma (Richardson et al., 2003, 2005; San Miguel et al., 2008).

The goal of this study was to determine whether a newly designed proteasome inhibitor – VL-01 – would function as an antiviral agent against influenza A virus in mice. The $EC_{50}$ value of VL-01 against H1N1 influenza virus on A549 cells revealed activity in the low μM range. For delivery of the substance *in vivo*, we decided to use an aerosol route for local delivery instead of systemic application. An *in vivo* dose–response experiment (Fig. 2A) showed that highest reduction of virus titer in the lung could be achieved when 14.1 mg/kg VL-01 or more were administered to the animals. Interestingly, an increase of the amount of VL-01 did not lead to a significant further increase in reduction of viral titers in the lung. Therefore, a dose of 14.1 mg/kg VL-01 was used for further studies. This amount did not lead to any adverse side effects in the mouse. In this light, Ma and colleagues investigated the antiviral effect of proteasome inhibitors PS-341 and MG-132 in a murine model against SARS and described also no adverse effects (Ma et al., 2010). In various experiments we were able to show that delivery of VL-01 at a concentration of 14.1 mg/kg resulted in reduction of the viral titer in the mouse lung by more than one order of magnitude and a prolonged survival rate of 50%. Intriguingly, earlier studies have demonstrated that 1.2 μM of VL-01 is needed for inhibition of 50% of the NF-κB activity (data not shown). In this context, the amount of proinflammatory cytokines and chemokines were drastically reduced after i.v. VL-01 treatment of H5N1 infected mice or LPS treated mice (IL-1α/β, IL-6, MIP-1β, RANTES and TNF-α). Since the cytokine/chemokine gene expression is largely mediated and/or regulated by NF-κB (Homma et al., 2010; Lakshmanan and Porter, 2007; Pahl and Baeuerle, 1995), our data demonstrate that NF-κB function is impaired even when low concentrations of VL-01 were applied using i.v. application. However, fluorescent microscopy of the viral NP visualized that vRNP export is reduced in the presence of VL-01. This indicates an antiviral effect of VL-01 by inhibition of NF-κB via reduced proteasome-activity. In the study by Ma and colleagues, the authors also demonstrated a reduction of cytokine gene expression in mice after PS-341 treatment (Ma et al., 2010). This is in line with our observations. In this context, it has to be noted that VL-01 treatment of H5N1 infected mice led only to marginal reduction of virus titer in the lung and no differences regarding survival and severity of diseases. Since cytokines and chemokines were drastically reduced, this might be another example that the pathogenicity of HPAIV is more complex than currently known (Salomon et al., 2007). Using another model, i.e. H7N7 (FPV) infected mice, VL-01 induced a moderate reduction in viral titers in the lungs and a moderate increase in survival (25% in VL-01 treated mice vs. 0% in mock treated mice).

Expression of cytokines and chemokines (hypercytokinemia or cytokine storm) is a hallmark after influenza A virus infection, in particular with highly pathogenic avian influenza virus or pandemic

strains. This cytokine and chemokine deregulation of the immune system leads to higher virus loads as well as higher lethality (Cheng et al., 2010; de Jong et al., 2006). Thus, in addition to controlling the virus replication it is also necessary to control the expression of pro-inflammatory cytokines, particularly in severe cases of influenza virus infection. Glucocorticoid-mediated suppression of cytokine expression might be beneficial to treat for the development of influenza, but might also negatively influence the induction of the innate and adaptive immune response mediated by various cytokines and chemokines. Therefore, the use of glucocorticoids to treat severe influenza, accompanied with hypercytokinemia, is controversially discussed. VL-01 applied locally – directly into the lung – might have an advantage, since VL-01 has antiviral activity and antiinflammatory activity. Since the drug is given locally, accordingly the local inflammatory status at the application site, i.e. the lungs, rather than the systemic immune response will be affected.

In summary, this study presents evidence that proteasome inhibitors might be able to inhibit influenza virus replication *via* impaired vRNP export and induction of inflammatory cytokines. Nevertheless, the only marginal effect after H5N1 influenza A virus infection of mice indicate that a modification of the VL-01 formulation and/or of the application scheme may be required to assure a better up-take and deposition in the lung. Recently it was published that the proteasome inhibitor MG132 shows anti-influenza virus activity by targeting a post-fusion step (Widjaja et al., 2010). In our study, we demonstrate a new class of proteasome inhibitors (VL-01), which also might be used in combination with other antivirals in case of severe influenza A virus infection. In case of intensive care hospitalization due to influenza A virus infection, it might be of great advantage to counteract the disease pattern efficiency with antivirals having bivalent properties to fight against the virus and ameliorate the cytokine storm induced by influenza virus infection. These results suggest that proteasome inhibition by VL-01 is a novel therapeutic intervention that might be considered in case of severe influenza virus infection.

## Acknowledgments

We thank Nicole Studtrucker and Daniel Lüftenegger for providing $CC_{50}$-values in primary hepatocytes, tonsils and PBMCs, and also Carmen Mueller for her excellent technical assistance. This work was supported by Grants SFB 643-A1, SFB 796 A1, SCHU1125/3, and SCHU 1125/5, from the German Research Council to US. Furthermore, this research was partially supported by the Federal Government of Germany under the Influenza research program "FSI" and by the BMBF Zoonose Programm "FluResearchNet".

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.07.006.

## References

Adams, J., Palombella, V.J., Sausville, E.A., Johnson, J., Destree, A., Lazarus, D.D., Maas, J., Pien, C.S., Prakash, S., Elliott, P.J., 1999. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res. 59, 2615–2622.

Breccia, M., Alimena, G., 2010. NF-κB as a potential therapeutic target in myelodysplastic syndromes and acute myeloid leukemia. Expert Opin. Ther. Targets 14, 1157–1176.

Carlsen, H., Alexander, G., Austenaa, L.M., Ebihara, K., Blomhoff, R., 2004. Molecular imaging of the transcription factor NF-κB, a primary regulator of stress response. Mutat Res 551, 199–211.

Chan, M.C., Cheung, C.Y., Chui, W.H., Tsao, S.W., Nicholls, J.M., Chan, Y.O., Chan, R.W., Long, H.T., Poon, L.L., Guan, Y., Peiris, J.S., 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir. Res. 6, 135.

Cheng, X.W., Lu, J., Wu, C.L., Yi, L.N., Xie, X., Shi, X.D., Fang, S.S., Zan, H., Kung, H.F., He, M.L., 2010. Three fatal cases of pandemic 2009 influenza A virus infection in Shenzhen are associated with cytokine storm. Respir. Physiol. Neurobiol. 175, 185–187.

Cheung, C.Y., Poon, L.L., Lau, A.S., Luk, W., Lau, Y.L., Shortridge, K.F., Gordon, S., Guan, Y., Peiris, J.S., 2002. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 360, 1831–1837.

Chotpitayasunondh, T., Ungchusak, K., Hanshaoworakul, W., Chunsuthiwat, S., Sawanpanyalert, P., Kijphati, R., Lochindarat, S., Srisan, P., Suwan, P., Osotthanakorn, Y., Anantasetagoon, T., Kanjanawasri, S., Tanupattarachai, S., Weerakul, J., Chaiwirattana, R., Maneerattanaporn, M., Poolsavathitikool, R., Chokephaibulkit, K., Apisarnthanarak, A., Dowell, S.F., 2005. Human disease from influenza A (H5N1), Thailand, 2004. Emerg. Infect. Dis. 11, 201–209.

de Jong, J.C., Claas, E.C., Osterhaus, A.D., Webster, R.G., Lim, W.L., 1997. A pandemic warning? Nature 389, 554.

de Jong, M.D., Simmons, C.P., Thanh, T.T., Hien, V.M., Smith, G.J., Chau, T.N., Hoang, D.M., Chau, N.V., Khanh, T.H., Dong, V.C., Qui, P.T., Cam, B.V., Ha, D.Q., Guan, Y., Peiris, J.S., Chinh, N.T., Hien, T.T., Farrar, J., 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat. Med. 12, 1203–1207.

Droebner, K., Reiling, S.J., Planz, O., 2008. Role of hypercytokinemia in NF-κB p50-deficient mice after H5N1 influenza A virus infection. J Virol 82, 11461–11466.

Ehrhardt, C., Seyer, R., Hrincius, E.R., Eierhoff, T., Wolff, T., Ludwig, S., 2010. Interplay between influenza A virus and the innate immune signaling. Microbes Infect 12, 81–87.

Errea, A., Moreno, G., Sisti, F., Fernandez, J., Rumbo, M., Hozbor, D.F., 2010. Mucosal innate response stimulation induced by lipopolysaccharide protects against *Bordetella pertussis* colonization. Med. Microbiol. Immunol. 199, 103–108.

Homma, T., Matsukura, S., Hirose, T., Ohnishi, T., Kimura, T., Kurokawa, M., Ieki, K., Odaka, M., Suzuki, S., Watanabe, S., Sato, M., Kawaguchi, M., Schleimer, R.P., Adachi, M., 2010. Cooperative activation of CCL5 expression by TLR3 and tumor necrosis factor-alpha or interferon-gamma through nuclear factor-κB or STAT-1 in airway epithelial cells. Int. Arch. Allergy Immunol. 152 (Suppl. 1), 9–17.

Hurt, A.C., Ernest, J., Deng, Y.M., Iannello, P., Besselaar, T.G., Birch, C., Buchy, P., Chittaganpitch, M., Chiu, S.C., Dwyer, D., Guigon, A., Harrower, B., Kei, I.P., Kok, T., Lin, C., McPhie, K., Mohd, A., Olveda, R., Panayotou, T., Rawlinson, W., Scott, L., Smith, D., D'Souza, H., Komadina, N., Shaw, R., Kelso, A., Barr, I.G., 2009. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. Antiviral Res. 83, 90–93.

Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. 18, 621–663.

Kobasa, D., Jones, S.M., Shinya, K., Kash, J.C., Copps, J., Ebihara, H., Hatta, Y., Kim, J.H., Halfmann, P., Hatta, M., Feldmann, F., Alimonti, J.B., Fernando, L., Li, Y., Katze, M.G., Feldmann, H., Kawaoka, Y., 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 445, 319–323.

Konig, R., Stertz, S., Zhou, Y., Inoue, A., Hoffmann, H.H., Bhattacharyya, S., Alamares, J.G., Tscherne, D.M., Ortigoza, M.B., Liang, Y., Gao, Q., Andrews, S.E., Bandyopadhyay, S., De Jesus, P., Tu, B.P., Pache, L., Shih, C., Orth, A., Bonamy, G., Miraglia, L., Ideker, T., Garcia-Sastre, A., Young, J.A., Palese, P., Shaw, M.L., Chanda, S.K., 2010. Human host factors required for influenza virus replication. Nature 463, 813–817.

Lakshmanan, U., Porter, A.G., 2007. Caspase-4 interacts with TNF receptor-associated factor 6 and mediates lipopolysaccharide-induced NF-κB-dependent production of IL-8 and CC chemokine ligand 4 (macrophage-inflammatory protein-1). J. Immunol. 179, 8480–8490.

Le, Q.M., Kiso, M., Someya, K., Sakai, Y.T., Nguyen, T.H., Nguyen, K.H., Pham, N.D., Ngyen, H.H., Yamada, S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., Kawaoka, Y., 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437, 1108.

Lison, D., Thomassen, L.C., Rabolli, V., Gonzalez, L., Napierska, D., Seo, J.W., Kirsch-Volders, M., Hoet, P., Kirschhock, C.E., Martens, J.A., 2008. Nominal and effective dosimetry of silica nanoparticles in cytotoxicity assays. Toxicol. Sci. 104, 155–162.

Lorusso, V., Crucitta, E., Panza, N., Silvestris, N., Guida, M., Carpagnano, F., Mancarella, S., Sambiasi, D., De Lena, M., 2002. Phase I/II study of paclitaxel, gemcitabine and vinorelbine as first-line chemotherapy of non-small-cell lung cancer. Ann. Oncol. 13, 1862–1867.

Ludwig, S., 2009. Targeting cell signalling pathways to fight the flu: towards a paradigm change in anti-influenza therapy. J. Antimicrob. Chemother. 64, 1–4.

Ludwig, S., Planz, O., 2008. Influenza viruses and the NF-κB signaling pathway – towards a novel concept of antiviral therapy. Biol. Chem. 389, 1307–1312.

Ludwig, S., Pleschka, S., Wolff, T., 1999. A fatal relationship–influenza virus interactions with the host cell. Viral Immunol. 12, 175–196.

Ludwig, S., Wolff, T., Ehrhardt, C., Wurzer, W.J., Reinhardt, J., Planz, O., Pleschka, S., 2004. MEK inhibition impairs influenza B virus propagation without emergence of resistant variants. FEBS Lett. 561, 37–43.

Ma, X.Z., Bartczak, A., Zhang, J., Khattar, R., Chen, L., Liu, M.F., Edwards, A., Levy, G., McGilvray, I.D., 2010. Proteasome inhibition in vivo promotes survival in a lethal murine model of severe acute respiratory syndrome. J. Virol. 84, 12419–12428.

Mazur, I., Wurzer, W.J., Ehrhardt, C., Pleschka, S., Puthavathana, P., Silberzahn, T., Wolff, T., Planz, O., Ludwig, S., 2007. Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-κB-inhibiting activity. Cell. Microbiol. 9, 1683–1694.

McKimm-Breschkin, J.L., Selleck, P.W., Usman, T.B., Johnson, M.A., 2007. Reduced sensitivity of influenza A (H5N1) to oseltamivir. Emerg. Infect. Dis. 13, 1354–1357.

Meijer, A., Lackenby, A., Hungnes, O., Lina, B., van-der-Werf, S., Schweiger, B., Opp, M., Paget, J., van-de-Kassteele, J., Hay, A., Zambon, M., 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007–08 season. Emerg. Infect. Dis. 15, 552–560.

Ott, D.E., Coren, L.V., Chertova, E.N., Gagliardi, T.D., Nagashima, K., Sowder 2nd, R.C., Poon, D.T., Gorelick, R.J., 2003. Elimination of protease activity restores efficient virion production to a human immunodeficiency virus type 1 nucleocapsid deletion mutant. J. Virol. 77, 5547–5556.

Pahl, H.L., Baeuerle, P.A., 1995. Expression of influenza virus hemagglutinin activates transcription factor NF-κB. J. Virol. 69, 1480–1484.

Planz, O., 2006. Influenza viruses and intracellular signalling pathways. Berl. Munch Tierarztl. Wochenschr. 119, 101–111.

Pleschka, S., Wolff, T., Ehrhardt, C., Hobom, G., Planz, O., Rapp, U.R., Ludwig, S., 2001. Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. Nat. Cell Biol. 3, 301–305.

Rameix-Welti, M.A., Enouf, V., Cuvelier, F., Jeannin, P., van der, W.S., 2008. Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir. PLoS Pathog. 4, e1000103.

Richardson, P.G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S.V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orlowski, R.Z., Kuter, D., Limentani, S.A., Lee, S., Hideshima, T., Esseltine, D.L., Kauffman, M., Adams, J., Schenkein, D.P., Anderson, K.C., 2003. A phase 2 study of bortezomib in relapsed, refractory myeloma. N. Engl. J. Med. 348, 2609–2617.

Richardson, P.G., Sonneveld, P., Schuster, M.W., Irwin, D., Stadtmauer, E.A., Facon, T., Harousseau, J.L., Ben-Yehuda, D., Lonial, S., Goldschmidt, H., Reece, D., San-Miguel, J.F., Blade, J., Boccadoro, M., Cavenagh, J., Dalton, W.S., Boral, A.L., Esseltine, D.L., Porter, J.B., Schenkein, D., Anderson, K.C., 2005. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N. Engl. J. Med. 352, 2487–2498.

Salomon, R., Hoffmann, E., Webster, R.G., 2007. Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. Proc. Natl. Acad. Sci. USA 104, 12479–12481.

San Miguel, J.F., Schlag, R., Khuageva, N.K., Dimopoulos, M.A., Shpilberg, O., Kropff, M., Spicka, I., Petrucci, M.T., Palumbo, A., Samoilova, O.S., Dmoszynska, A., Abdulkadyrov, K.M., Schots, R., Jiang, B., Mateos, M.V., Anderson, K.C., Esseltine, D.L., Liu, K., Cakana, A., van de Velde, H., Richardson, P.G., 2008. Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. N. Engl. J. Med. 359, 906–917.

Schubert, U., Ott, D.E., Chertova, E.N., Welker, R., Tessmer, U., Princiotta, M.F., Bennink, J.R., Krausslich, H.G., Yewdell, J.W., 2000. Proteasome inhibition interferes with gag polyprotein processing, release, and maturation of HIV-1 and HIV-2. Proc. Natl. Acad. Sci. USA 97, 13057–13062.

Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., Gubareva, L.V., 2008. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob. Agents Chemother. 52, 3284–3292.

Tumpey, T.M., Lu, X., Morken, T., Zaki, S.R., Katz, J.M., 2000. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. J. Virol. 74, 6105–6116.

Vink, J., Cloos, J., Kaspers, G.J., 2006. Proteasome inhibition as novel treatment strategy in leukaemia. Br. J. Haematol. 134, 253–262.

Vogel, A.B., Haasbach, E., Reiling, S.J., Droebner, K., Klingel, K., Planz, O., 2010. Highly pathogenic influenza virus infection of the thymus interferes with T lymphocyte development. J. Immunol. 185, 4824–4834.

Watanabe, T., Watanabe, S., Kawaoka, Y., 2010. Cellular networks involved in the influenza virus life cycle. Cell Host Microbe 7, 427–439.

Widjaja, I., de Vries, E., Tscherne, D.M., Garcia-Sastre, A., Rottier, P.J., de Haan, C.A., 2010. Inhibition of the ubiquitin-proteasome system affects influenza A virus infection at a postfusion step. J. Virol. 84, 9625–9631.

Wong, S.S., Yuen, K.Y., 2006. Avian influenza virus infections in humans. Chest 129, 156–168.

Wurzer, W.J., Planz, O., Ehrhardt, C., Giner, M., Silberzahn, T., Pleschka, S., Ludwig, S., 2003. Caspase 3 activation is essential for efficient influenza virus propagation. EMBO J. 22, 2717–2728.

Yin, M.J., Yamamoto, Y., Gaynor, R.B., 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(κ)B kinase-beta. Nature 396, 77–80.

Yu, X., Kem, D.C., 2010. Proteasome inhibition during myocardial infarction. Cardiovasc. Res. 85, 312–320.