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The NF-kappaB inhibitor SC75741 protects mice against highly pathogenic avian influenza A virus

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ABSTRACT

The appearance of pandemic H1N1 and highly pathogenic avian H5N1 viruses in humans as well as the emergence of seasonal H1N1 variants resistant against neuraminidase inhibitors highlight the urgent need for new and amply available antiviral drugs. We and others have demonstrated that influenza virus misuses the cellular IKK/NF-kappaB signaling pathway for efficient replication suggesting that this module may be a suitable target for antiviral intervention. Here, we show that the novel NF-kappaB inhibitor SC75741 significantly protects mice against infection with highly pathogenic avian influenza A viruses of the H5N1 and H7N7 subtypes. Treatment was efficient when SC75741 was given intravenously in a concentration of 5 mg/kg/day. In addition, application of SC75741 *via* the intraperitoneal route resulted in a high bioavailability and was also efficient against influenza when given 15 mg/kg/day or 7.5 mg/kg/twice a day. Protection was achieved when SC75741 was given for seven consecutive days either prior to infection or as late as four days after infection. SC75741 treatment showed no adverse effects in the concentrations required to protect mice against influenza virus infection. Although more pre-clinical studies are needed SC75741 might be a promising candidate for a novel antiviral drug against influenza viruses that targets the host cell rather than the virus itself.

1. Introduction

Influenza is still one of the major plagues worldwide. The appearance of the H1N1 pandemic in 2009 clearly demonstrates that influenza A virus has a strong impact on global health systems (Mackey and Liang, 2012; Monto et al., 2011; Robertson and Inglis, 2011). Beside vaccination, antivirals are needed to efficiently control the infection. Indeed, in the early phase of a pandemic, when no vaccine is available yet, we have to rely on antiviral drugs. Moreover the appearance of influenza A viruses that are resistant against the currently licensed antivirals highlight the need for new and amply available antiviral drugs (Moss et al., 2010).

Beside direct targeting of the virus particle as shown for neuraminidase-inhibitors or M2 ion channel blockers a new strategy would be to interact with host cell factors required for replication in order to interfere with influenza virus propagation. Interaction of the virus with the host cell is mandatory, whenever the virus has to pass cellular barriers in order to get to another cellular compartment. We and others have demonstrated that influenza viruses misuse the cellular canonical IKK/NF-kappaB signaling pathway for efficient replication suggesting that this module may be a suitable target for antiviral intervention (Nimmerjahn et al., 2004; Wurzer et al., 2004). It has been demonstrated that inhibition of the NF-kappaB signaling pathway resulted in a strong reduction of influenza A virus replication *in vitro* and in a mouse infection model (Mazur et al., 2007). On a molecular level, the inhibition of the pathway results in a specific retention of the viral ribonucleoprotein (RNP) complexes in the nucleus which is most likely the mode of action leading to reduced progeny virus titers (Mazur et al., 2007; Wurzer et al., 2004).

Severe influenza virus disease, in particular after infection with highly pathogenic avian influenza virus strains from the H5N1 subtypes but also with the 2009 H1N1 pandemic strain, is accompanied by hypercytokinemia also known as cytokine storm (Cheng et al., 2011; de Jong et al., 2006). There is still an on-going debate on the influence of this virus mediated cytokine storm for the pathogenesis of influenza virus infections and whether targeting hypercytokinemia is beneficial for the outcome of disease (Salomon

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et al., 2007; Walsh et al., 2011). NF-kappaB is the main regulator of cytokine and chemokine production in general (Pahl, 1999) and during severe influenza in particular (Droebner et al., 2008b; Schmolke et al., 2009).

Here, we show that the novel NF-kappaB inhibitor SC75741 (Leban et al., 2007) significantly protects mice against lethal challenge with highly pathogenic avian influenza A viruses from the H7N7 and H5N1 subtypes. The *in vitro* data revealed a promising EC_{50} value for this compound. The bioavailability after i.v. and i.p. application allowed the use in the mouse model. The main aim of the present study was to get information of the route of application and dosage. We conclude that the present data is potentially useful for further pre-clinical development including validation of the animal experiments.

2. Materials and methods

2.1. Viruses

Mouse-adapted highly pathogenic avian influenza A/FPV/Bratislava/79 (H7N7; FPV) virus and avian influenza A/mallard/Bavaria/1/2006 (H5N1; MB1) were grown in embryonated chicken eggs and used throughout this study as described previously (Droebner et al., 2008a). The H5N1 subtype was originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany. The avian influenza A virus A/FPV/Bratislava/79 (FPV, H7N7) was originally provided from the strain collection at the Institute of Virology, Justus-Liebig University, Giessen, Germany. In addition, we used the human influenza A virus strain, A/Regensburg/D6/09 (H1N1, RB1). All influenza A viruses were further propagated at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tuebingen, Germany.

2.2. Determination of the 50% effective concentration (EC_{50})

To determine the 50% effective concentration of SC75741, human lung carcinoma cells (A549) were infected with the influenza A virus strain, A/Regensburg/D6/09 (H1N1, RB1) with a multiplicity of infection (MOI) of 0.001 for 30 min at 37 °C and 5% CO_2. After incubation the virus dilution was aspirated and the cells were treated with different concentrations of SC75741 (0–100 μM) for 24 h at 37 °C and 5% CO_2. Afterwards, the cell culture supernatants were taken to determine the progeny virus titer, as described previously (Haasbach et al., 2011). The EC_{50} value was calculated with the GraphPad Prism 5.0 software.

2.3. Determination of the 50% cytotoxic concentration (CC_{50})

The cytotoxic concentration (CC_{50}) of SC75741 was determined on A549 cells that were incubated with different concentrations of SC75741 (0–100 μM) for 24 h. The effect on A549 cells was examined by a water-soluble tetrazolium salt (WST-1) assay according to the manufactures protocol (Roche Diagnostics, Mannheim, Germany). All experiments were performed as triplicates. Results were evaluated using GraphPad prism software to determine the CC_{50}. Selective index is the 50% cytotoxic concentration of SC75741 divided by the 50% effective concentration.

2.4. Indirect immunofluorescence microscopy

A549 cells were seeded onto 15 mm glass-plates. Twenty-four hours later cells were infected with A/FPV/Bratislava/79 (H7N7; FPV) (MOI = 10) in the presence of either SC75741, DMSO or cells were left untreated as described before. Five hours post infection cells were washed twice with PBS and fixed for 20 min with 3.7% paraformaldehyde at RT. After washing with PBS, cells were permeabilized with acetone, washed again and blocked with 10% FBS in PBS for 20 min at RT. Cells were incubated with a 1:100 dilution of mouse antiserum against the viral NP (Serotec) for 30 min. After further washes, cells were incubated with Alexa Fluor 488 chicken anti-mouse IgG (Invitrogen) and DAPI (4′,6-Diamidino-2-phenylindol) for 1 h. Finally, cells were washed and mounted with MOVIOL. Fluorescence was visualized using a Zeiss Axiovert 135 fluorescence microscope.

2.5. Mice

Inbred female C57BL/6 mice at the age of 6–8 weeks were obtained from the animal breeding facilities at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tuebingen, Germany and were used throughout all of the experiments.

2.6. Infection of mice

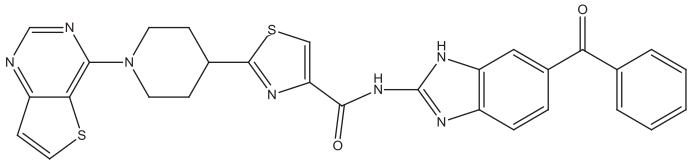
For infection, the animals were anesthetized by intraperitoneal injection of 200 μl ketamine/rompun. Equal amounts of a 2% rompun (Bayer) and a 10% ketamine (Sanofi) stock solution were mixed at a ratio of 1:10 with PBS. For survival experiments mice were infected intranasally with $2 \times MLD_{50}$ (H7N7 = 4×10^2 pfu; H5N1 = 4×10^3 pfu) diluted in 50 μl BSS by inoculating 25 μl into each nostril. For determination of lung virus titer reduction the same dose was used for H7N7 and H5N1 infection, while for H1N1pdm09 $5 \times MLD_{50}$ (RB1 = 1.5×10^5 pfu) was used. After infection with H5N1 or H7N7 viruses the mice were kept in individually ventilated cages (Techniplast). All animal studies were approved by the Institutional Animal Care and Use Committee of Tuebingen.

2.7. Determination of the clinical score

The following disease symptoms were found and defined in mice after influenza virus infection: ruffed fur, teeth crunching, ataxia, dyspneic, conjunctivitis. If mice showed one of these symptoms they got one score; 2 symptoms = score 2; 3 and more symptoms = score 3; death = score 4. Note, the score 4 was kept throughout the 14 days observation period. Score represents the mean value of the group.

2.8. Antiviral compound and treatment of C57BL/6 mice

SC75741 N-(6-benzoyl-1H-benzo[d]imidazol-2-yl)-2-(1-(thieno[3,2-d]pyrimidin-4-yl)piperidin-4-yl)thiazole-4-carboxamide (MW 565) was supplied by 4SC AG (Planeeg-Martinsried, Germany).



Structure of SC75741

SC75741 was dissolved in 10% DMSO, 30% Cremophor EL (Merck) and 60% PBS. SC75741 in a volume of 200 μl was applied to the mice by either intravenous (i.v.) injection to the tail-vein

or intraperitoneal (i.p.) injection. Control animals were treated with the formulation only, containing no compound (placebo).

2.9. Statistical analysis

Error bars are given as the SEM. For the calculation of the significance of differences, the paired *t*-test was used for virus titer reduction and the log-rank test was performed for survival experiments. All analyzes were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Determination of the EC_{50} value in vitro

First, the effective concentration 50% (EC_{50}) value of SC75741 against influenza virus was determined on human A549 cells. Therefore, seven concentrations of SC75741 were used ranging from 0 to 100 μM. As indicated in Fig. 1A the EC_{50} value of SC75741 was 0.3 ng/ml. Using the same concentrations SC75741 was not toxic to A549 cells indicating a cytotoxic concentration 50% (CC_{50}) value of >56.7 μg/ml (Fig. 1B). This results in a selective index (SI) of >189.000. Virustiter for EC_{50} determination were also given as $\log_{10}$ pfu/ml (Fig. 1C) and in % compared to MOCK (Fig. 1D).

3.2. SC75741 treatment of influenza virus infected cells results in retention of viral RNP complexes in the nucleus

Earlier findings clearly indicated that inhibition of NF-kappaB results in efficient retention of influenza virus RNP complexes in the nucleus of infected cells without affecting accumulation of viral proteins (Mazur et al., 2007; Wurzer et al., 2004). This mechanism involves inhibition of caspases that are responsible for the disintegration of the nuclear pore complex (Kramer et al., 2008; Wurzer et al., 2003). Immunofluorescence staining of the viral nucleoprotein (NP) that is the major constituent of the RNP complexes revealed a strong impairment of RNP export in the presence of SC75741. While in untreated or solvent treated cells RNP complexes can already be detected in the cytoplasm 5 h after infection (Fig. 2 upper and middle panel), NP remains in the nucleus in infected cells treated with SC75741 (Fig. 2 lower panel). Thus, our results confirmed a specific molecular action of SC75741, which blocks the NF-kappaB, thereby resulting in inhibition of viral RNP export and subsequent block of virus propagation.

3.3. Pharmacokinetic

In order to characterize the pharmacokinetic properties of SC74751, the compound was administered to mice intravenously at a dose of 5 mg/kg and intraperitoneally at a dose of 15 mg/kg, respectively. Pharmacokinetic data is summarized in Table 1. The area under curve (AUC) after i.v. administration was 3 μg h/ml compared to 13 μg h/ml after i.p. administration. Both the initial volume of distribution $V_{(5min)}$ (roughly 1100 ml/kg) and the clearance of SC74751 (Cl = 1450 ml/(kg h)) were relatively high, indicating rapid distribution and/or metabolism of the compound in mice. The plasma-levels of SC74751 after i.v. administration decreased mono-exponentially throughout the observation time of 4 h. The observed half-life in this time interval was roughly 40 min (r^2 = 0.97). After i.p. administration, elimination of SC75741 seemed to be limited by a slow uptake from the peritoneum. Consequently, the bioavailability after i.p. administration reached a calculated value of F = 132%. Here, a half-life of 55 min (r^2 = 0.98) was observed. C_{max} was reached 1 h after i.p. administration and amounted to roughly 7 μg/ml. No adverse effects were observed in the animals at these two dose levels (data not shown).

The *in vitro* IC_{50} for inhibition of NF-kappaB activity was 200 nM corresponding to a concentration of 113 ng/ml (data not shown).

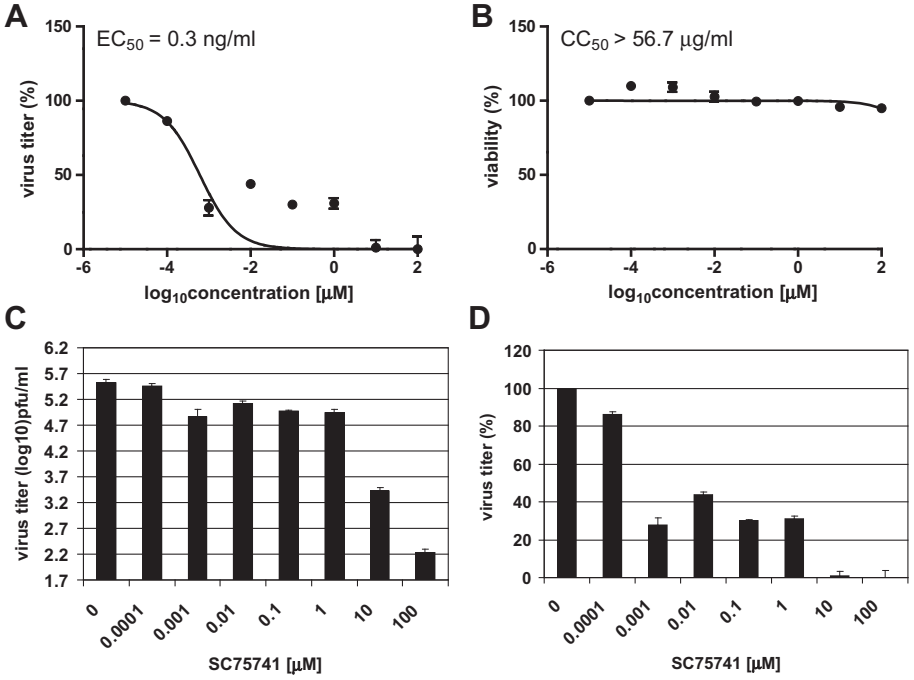


Fig. 1. Determination of the cytotoxic concentration 50% (CC_{50}); effective concentration 50% (EC_{50}) of SC75741 and virus titer reduction *in vitro*. The EC_{50} of SC75741 was measured by treatment of influenza A virus A/Regensburg/D6/09 (H1N1, RB1) infected A549 cells. Cells were infected with RB1 (MOI 0.001) and further treated with different concentrations of SC75741 (0–100 μM) for 24 h. Progeny virus titer was determined by plaque assay. (A) Mean of three independent experiments, calculated by GraphPad Prism software. (C) Virus titer in $\log_{10}$ pfu/ml and (D) virus titer in %. (B) The CC_{50} value of SC75741 was determined in A549 cells. Cells were incubated with SC75741 (0–100 μM) for 24 h. After incubation, cell viability was measured by WST-1 assay regarding manufactory guidelines. For calculation, each experiment was performed three times independently with each comprising triplicates.

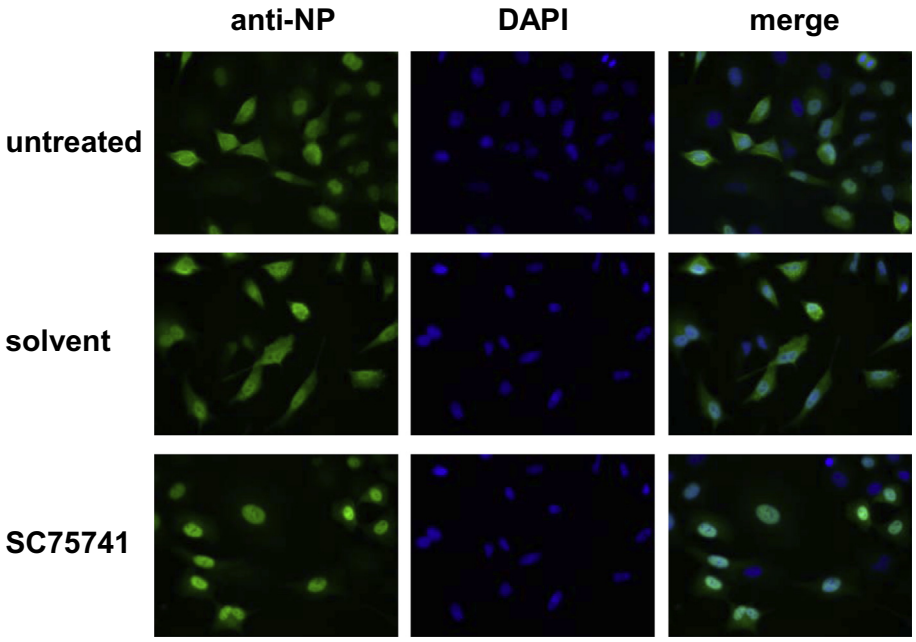


Fig. 2. Indirect immunofluorescence microscopy. A549 cells were infected with FPV (MOI = 10) and were treated with 5 μM SC75741 or 0.05% DMSO. Five hours post infection cells were fixed and RNP localization was determined by indirect immunofluorescence using a NP-specific mouse monoclonal antibody and an Alexa Fluor 488 chicken anti-mouse IgG. Localization of cell nuclei was visualized by DAPI stain.

Table 1
Pharmacokinetic data of SC75741 in the mouse.

AUC_{0-t} iv [ng*h /ml]	AUC_{∞} iv [ng*h /ml]	AUC_{extra} iv %	AUC_{0-t} ip [ng*h /ml]	AUC_{∞} ip [ng*h /ml]	AUC_{extra} ip %	Cl [ml/(h*kg)]	V_C (5 min) [ml/kg]	$t_{1/2}$ iv [min]	$t_{1/2}$ ip min]	C_{max} ip [ng/ml]	t_{max} ip min]	F ip [%]
3367	3438	1	13,304	13,480	2	1450	1104	39	54	7367	60	132

Note: No SD since *Serial Sacrifice Design* was used.

This concentration was sustained or exceeded for 3.5 and 6 h after the above described i.v. and i.p. administrations, respectively.

3.4. Treatment of mice with the NF-kappaB inhibitor SC75741 leads to reduction of H7N7, H5N1 and H1N1pan09 virus titer in the lung

Next we investigated whether SC75741 has antiviral properties against influenza virus in a complex organ system. Thus, C57BL/6 mice were treated with 15 mg/kg SC75741 1 h prior infection and 5 h after infection via the i.p. route. Viral titers were determined in the mouse lung 24 h p.i. (H7N7; H1N1pdm09) and 48 h p.i. (H5N1) to minimize the influence of the innate or adaptive immune response. After infection of mice with the H1N1pan09 strain, a titer of 5.6 ± 0.1 $\log_{10}$ pfu/ml was found 24 h p.i. in the lung of placebo treated controls (Fig. 3A, left panel black bar). In contrast, treatment with SC75741 resulted in a virus titer of 4.6 ± 0.4 $\log_{10}$ pfu/ml (Fig. 3A, left panel white bar), which is reduction of 90% (Fig. 3B right panel). Similar reduction after SC75741 treatment was observed when mice were infected with the H7N7 strain. Here, a titer of 4.1 ± 0.0 $\log_{10}$ pfu/ml was found 24 h p.i. in the lung of the controls (Fig. 3C, left panel black bar). In contrast, a virus titer of 3.4 ± 0.4 $\log_{10}$ pfu/ml (Fig. 3C, left panel white bar) was detectable in mice after SC75741 treatment, which is reduction of 77% (Fig. 3D right panel). After infection of placebo treated mice with the H5N1, a titer of 5.9 ± 0.2 $\log_{10}$ pfu/ml was found 48 h p.i. in the lung (Fig. 3E, left panel black bar), while treatment with SC75741 led to a virus titer of 4.9 ± 0.3 $\log_{10}$ pfu/ml (Fig. 3E, left panel white bar), which is reduction of 89% (Fig. 3F right panel).

3.5. Intravenous treatment of H7N7-influenza virus infected mice with SC75741

To answer the question whether the NF-kappaB inhibitor SC75741 shows antiviral properties against influenza virus *in vivo*, C57BL/6 mice were infected with 4×10^2 pfu, a 2 × mouse lethal dose 50% (MLD_{50}) of the highly pathogenic avian influenza virus A/FPV/Bratislava/79 (H7N7; FPV) and were treated i.v. with either 2 mg/kg (Fig. 4A), 5 mg/kg (Fig. 4B) or 10 mg/kg (Fig. 4C) SC75741 starting two hours prior to infection. The treatment was performed once daily for five consecutive days. Four out of five mice from the control group receiving placebo died between days six and seven post infection. The mouse that survived the infection developed severe disease but recovered. There was no significant difference ($p = 0.111$) in survival between control animals and mice that where treated with 2 mg/kg SC75741. Three out of five mice survived (Fig. 4A, left panel). The courses of the bodyweights of the two groups of mice were very similar (Fig. 4A, right panel) but disease symptoms were reduced in SC75741 treated mice (Fig. 4; solid grey line). When animals were treated with 5 mg/kg SC75741 a significant difference ($p = 0.035$) in survival compared to control animals was found. Here, only one mouse succumbed to the disease (Fig. 4B, left panel). No obvious loss of bodyweight was found in SC75741-treated animals for the first 6 days after infection. Thereafter, bodyweights also dropped until day eight p.i. and increased subsequently (Fig. 4B, right panel). SC75741-treated mice developed only slight influenza specific symptoms with a peak at day 9 (Fig. 4D; black dotted line). A similar result was found, when mice were treated with 10 mg/kg SC75741

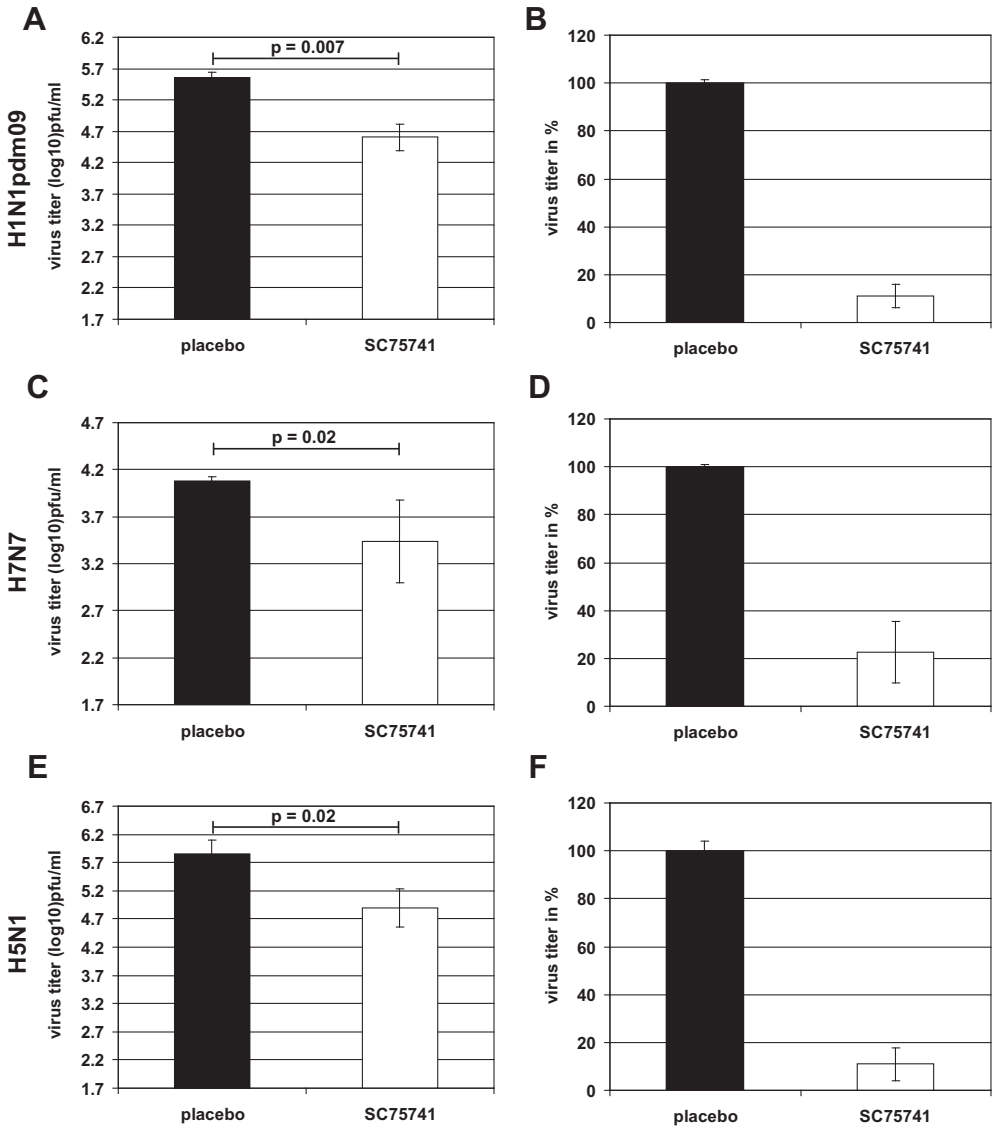


Fig. 3. Virus titer in the lung of influenza A virus infected placebo and SC75741-treated mice. Virus titer reduction in the lung of mice after SC75741 treatment was measured 24 h (H1N1pdm09; H7N7) or 48 h (H5N1) after infection and presented as (log_{10} pfu/ml) or as percentage where placebo control is set to 100%. SC75741 (15 mg/kg) treatment applied i.p. starting 1 h prior virus infection. The animals were infected either with 1.5×10^5 pfu H1N1pdm09 (A, B) 4×10^2 pfu H7N7 (C, D) or 4×10^3 pfu/ml H5N1 (E, F). Five hours after infection mice received a second treatment with the same dose. Titer represents the mean of four to seven animals per group.

(Fig. 4C). Only one mouse of the group that received the agent died. Comparing the bodyweight courses of mice that received 5 mg/kg SC75741 and 10 mg/kg SC75741, respectively, indicated that in the latter group the drop of bodyweight started already at day four post infection. Nevertheless, a similar recovery was found beginning at day nine post infection. In addition the clinical score was also comparable to mice treated with 5 mg/kg (Fig. 4D; grey dotted line). These data indicate that treatment of mice with 5 mg/kg of the NF-kappaB inhibitor SC75741 leads to protection against severe avian influenza virus infection.

3.6. Intraperitoneal treatment of H7N7-influenza virus infected mice with SC75741

The very good bioavailability ($F = 132\%$) after i.p. application prompted us to see whether protection of disease could also be achieved, when the agent would be applied *via* the i.p. route. According to the pharmacokinetic data either 15 mg/kg or 30 mg/kg were given to the animals for seven consecutive days starting with the day of infection; mice were infected with 4×10^2 pfu ($2 \times MLD_{50}$) FPV. Two out of four control mice survived the infection, while all five mice that received 15 mg/kg SC75741 i.p. survived (Fig. 5A left panel). Mice started to lose weight 5–6 days p.i. While bodyweight was below 80% in the control group, only 10% weight loss was found in the animals that received 15 mg/kg SC75741 (Fig. 5A right panel). Blood was taken from all mice that survived the infection and FPV-specific neutralizing antibodies were found in the range of 1:8–1:128 (data not shown) indicating that all mice were productively infected. When FPV-infected mice were treated with 30 mg/kg no significant protection from disease, no significant difference in bodyweight and no reduction of clinical symptoms were found after SC75741 treatment (Fig. 5B and C grey dotted line).

3.7. Prophylactic intraperitoneal treatment of H5N1-influenza virus infected mice with SC75741

The results in Fig. 4 demonstrate that i.p. treatment with 30 mg/kg did not protect mice against influenza infection with

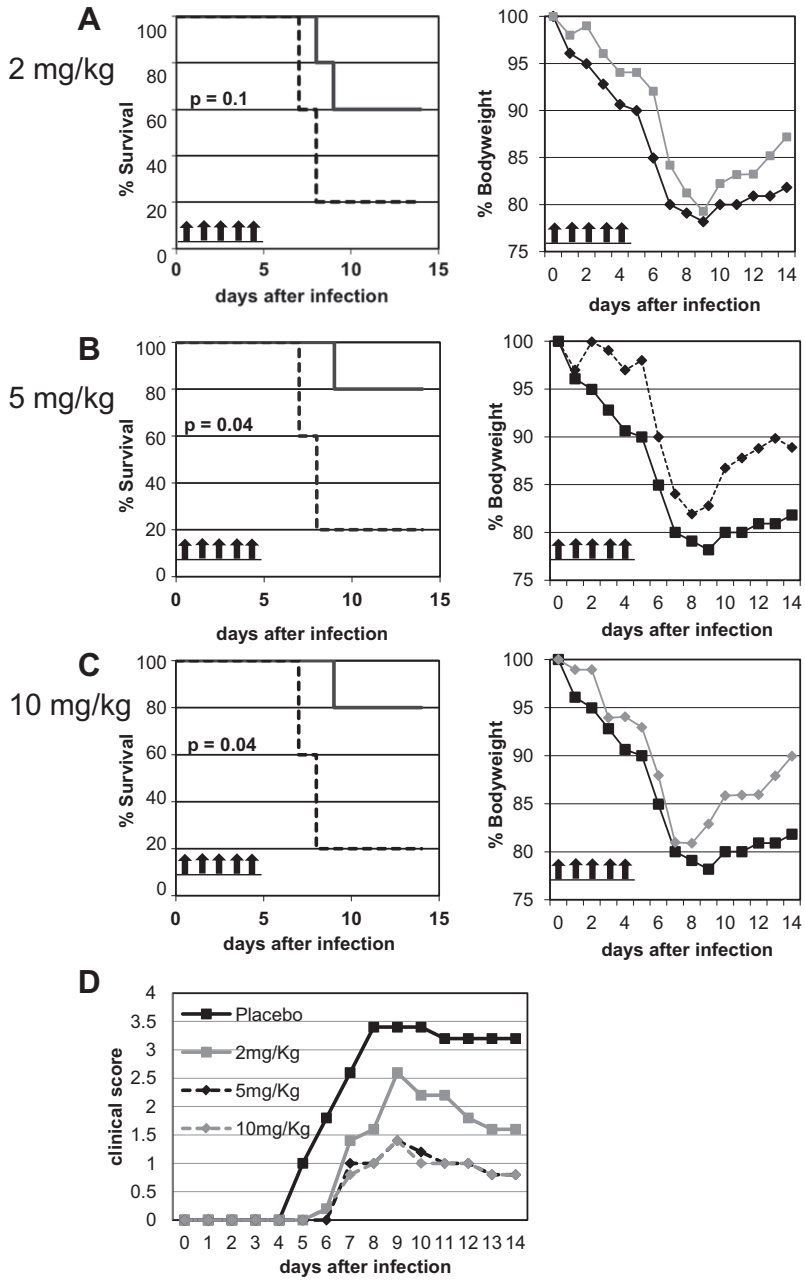


Fig. 4. Intravenous SC75741 treatment of H7N7, FPV infected (4×10^2 pfu = $2 \times MLD_{50}$) mice. Five mice per group were treated once daily i.v. with SC75741 or placebo for five consecutive days beginning 1–2 h prior to infection with FPV. (A) No significant difference ($p = 0.111$) in survival between control animals (dotted line) and mice treated with 2 mg/kg SC75741 (solid line) (left panel). (B) Signifikant difference ($p = 0.035$) in survival between control animals (dotted line) and mice treated with 5 mg/kg SC75741 (solid line) (left panel). (C) Significant difference ($p = 0.035$) in survival between control animals (dotted line) and mice treated with 10 mg/kg SC75741 (solid line) (left panel). The bodyweight (right panels) of all mice was checked daily. Bodyweight at the day of infection was set as 100% and the relative weight (%) was calculated for the following days. When animals died the last measured value was carried forward until the end of the observation period. According to Germany's animal protection law, mice have to be killed, if they lose more than 25% of their bodyweight. (D) Mean value of the clinical score of placebo and SC75741 treated mice.

H7N7. Unfortunately, using a $2 \times MLD_{50}$ (4×10^2 pfu) resulted in a 50% survival of the mice in the control group. Therefore in the following setup two changes were applied. In order to investigate whether the latter treatment schedule would increase protection against influenza, SC75741 was given at doses of 15 mg/kg once daily and 7.5 mg/kg twice daily, respectively. SC75741 treatment was performed for 7 days starting with the day of infection. Moreover, 4×10^3 pfu ($2 \times MLD_{50}$) of a H5N1 strain (MB1; A/Mallard/Bavaria/2005) was used for infection. MB1 is highly pathogenic for mice without adaptation to this mammalian host. Placebo treated control mice started to loose weight four days p.i. All mice developed severe disease and five out of six animals died (Fig. 6A left and right panel). In contrast, mice receiving 7.5 mg/kg SC75741 twice daily developed only slight disease (Fig. 6C grey solid line) and lost only less than 10% of the bodyweight (Fig 6A; grey solid line). All animals showed MB1-specific neutralizing antibodies in the blood indicating that these animals were productively infected (data not shown). Only two out of six animals developed influenza symptoms and died (Fig. 6A left and right panel).

When MB1-infected mice were treated with 15 mg/kg once daily for seven consecutive days, four out of six developed disease and three of them died (Fig. 6B left and right panel and Fig. 6C grey

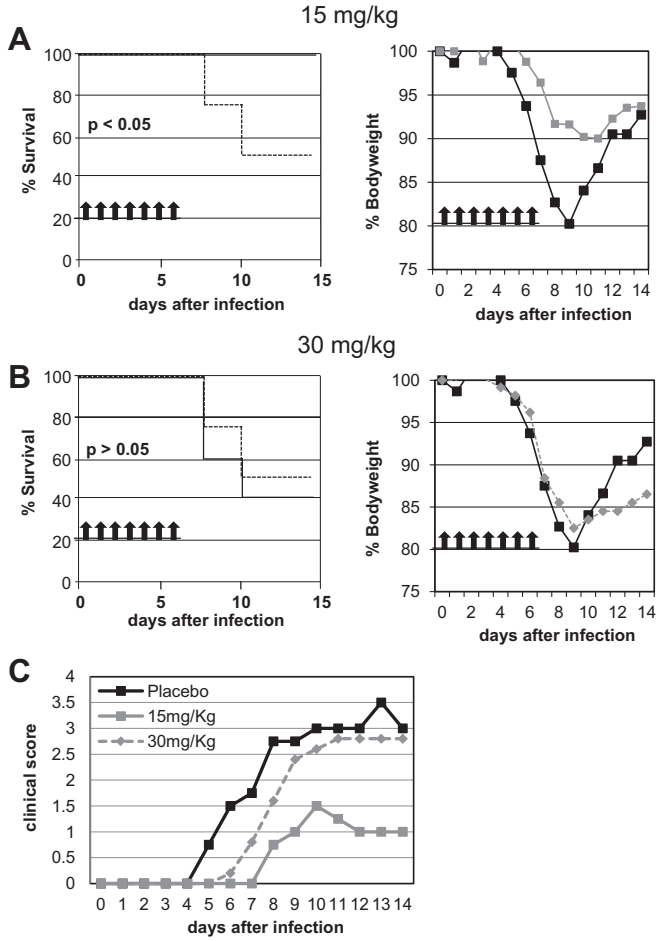


Fig. 5. Intraperitoneal SC75741 treatment of H7N7, FPV (4×10^2 pfu = $2 \times MLD_{50}$) infected mice. Five mice per dose group were treated once daily i.p. with SC75741 or placebo (group of four mice) for seven consecutive days beginning 2 h prior to infection with FPV. (A) Significant difference ($p < 0.05$) in survival between control animals (dotted line) and mice treated with 15 mg/kg SC75741(solid line) (left panel). (B) No significant difference ($p > 0.05$) in survival between control animals (dotted line) and mice treated with 30 mg/kg SC75741 (solid line) (left panel). Bodyweight (right panel) at the day of infection was set as 100% and the relative weight (%) was calculated for the following days. Inverted arrows denote administration of SC75741. (C) Mean value of the clinical score of placebo and SC75741 treated mice.

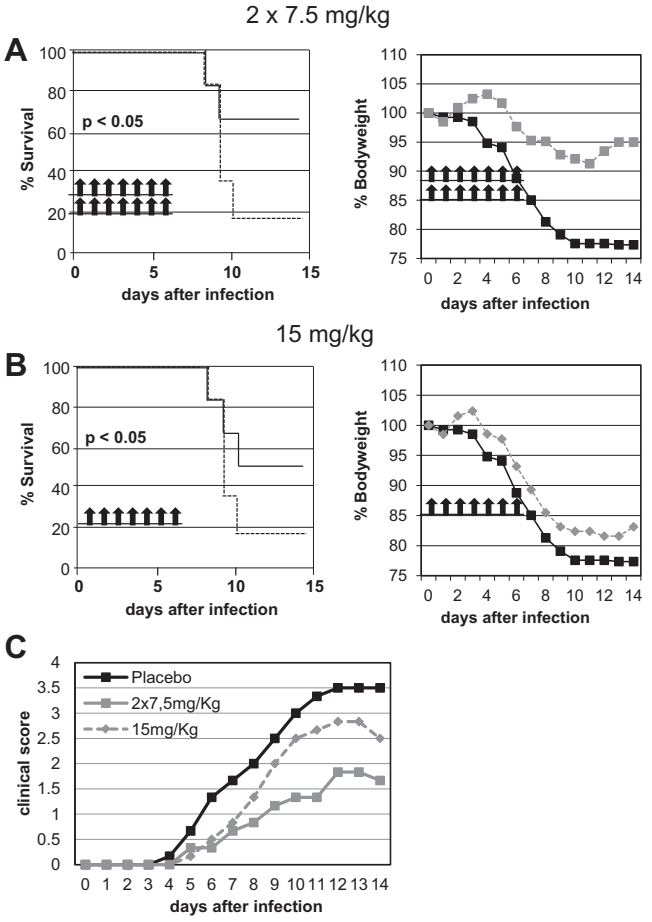


Fig. 6. Intraperitoneal SC75741 treatment of H5N1, MB1 (4×10^3 pfu = $2 \times MLD_{50}$) infected mice. Six mice per group were treated either (A) twice daily i.p. with 7.5 mg/kg SC75741 (solid line) or placebo (dotted line) for seven consecutive days beginning 2 h prior to infection with MB1 or (B) once daily i.p. with 15 mg/kg SC75741 (solid line) or placebo (dotted line) for seven consecutive days beginning 1 h prior to infection with MB1. A significant difference ($p < 0.05$) in survival between control animals and both groups of SC75741 treated mice was found. Bodyweight (right panel) at the day of infection was set as 100% and the relative weight (%) was calculated for the following days. Inverted arrows denote administration of SC75741. (C) Mean value of the clinical score of placebo and SC75741 treated mice.

dotted line). This indicates that twice daily treatment might be more efficient in protecting mice against influenza than once daily treatment with effectively the same dose per day.

3.8. Therapeutic intraperitoneal treatment of H5N1-influenza virus infected mice with SC75741

Since H5N1-infected mice were significantly protected against disease when SC75741 treatment started prior to infection, we next raised the question whether a therapeutically treatment starting 4 days after infection would also protect mice. Therefore, six mice were infected with 4×10^3 pfu ($2 \times MLD_{50}$) of the H5N1 strain MB1 and where treated with placebo (controls) or were either treated with 15 mg/kg once daily or 7.5 mg/kg twice daily. Treatment started at day four p.i. for seven consecutive days.

Mice started to loose weight already at day two after infection. This weight loss was more severe after day four p.i. (Fig. 7A, B right panel). At onset of treatment with 15 mg/kg SC 75741 this weight loss and clinical symptoms were drastically reduced (Fig. 7B, C grey dotted line) and only one out of six mice developed clinical symptoms and died. Mice treated twice daily with 7.5 mg/kg showed no difference in loss of bodyweight compared to controls until day 10 after infection. Interestingly, only one out of six mice developed disease and died. Thus, in this experiment loss of bodyweight and clinical score does not seem to be correlated with survival (Fig. 7A and C).

4. Discussion

There is an urgent need for new concepts to develop antiviral drugs against influenza virus. Targeting cellular factors is a promising but also a challenging approach and the concerns about side effects are obvious. However, it should be noted that this is not a problem that exclusively exists for drugs targeting cellular factors; for approved anti-influenza drugs which target viral factors, a wide range of side effects in patients have been already reported (Toovey et al., 2008). Thus, drug safety has to be rigorously tested in clinical trials regardless whether a drug targets a cellular or a viral factor. Moreover, increasing resistance development against human H1N1 influenza viruses and highly pathogenic avian H5N1 virus strains to oseltamivir and amantadine were reported. In this respect, the strategy to target cellular factors (Ludwig,

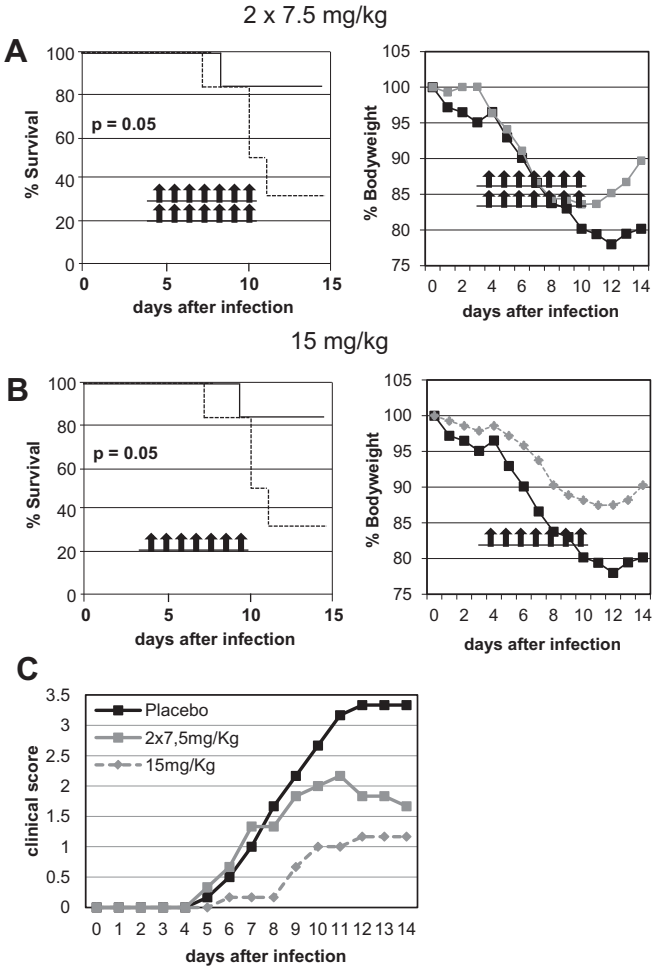


Fig. 7. Intraperitoneal SC75741 treatment of H5N1, MB1 (4×10^3 pfu = $2 \times MLD_{50}$) infected mice. Six mice per group were treated either (A) twice daily i.p. with 7.5 mg/kg SC75741 (solid line) or placebo (dotted line) for seven consecutive days beginning 4 days after infection with MB1 or (B) once daily i.p. with 15 mg/kg SC75741 (solid line) or placebo (dotted line) for seven consecutive days beginning 1 h prior to infection with MB1. A significant difference ($p = 0.05$) in survival between control animals and both groups of SC75741 treated mice was found. Bodyweight (right panel) at the day of infection was set as 100% and the relative weight (%) was calculated for the following days. Inverted arrows denote administration of SC75741. (C) Mean value of the clinical score of placebo and SC75741 treated mice.

2009, 2011; Ludwig and Planz, 2008) might be one way to ensure that new drugs against influenza virus will be useful and effective for a long time without causing the development of resistant virus variants.

The present work should get an answer for the best route of application and whether the NF-kappaB inhibitor SC75741 is effective against different influenza virus strains. Therefore, we have used H1N1pan09 in cell culture experiments. In the mouse infection model H1N1pan09, H5N1 and H7N7 highly pathogenic avian influenza viruses were used. The *in vitro* EC_{50} value was 0.3 ng/ml, which is roughly 2.000 times less than the C_{max} (7 μg/ml) after i.p. application. Even tough a direct correlation of *in vitro* data with *in vivo* data is not suitable; it shows at least evidence that the antiviral effective concentration is in an appropriate range. Immunofluorescence studies revealed reduced export of the viral RNP complexes in the presence of the drug. With regard to the mechanism it has been shown that caspases promote diffusion of larger protein complexes due to an increase of the diffusion limit of nuclear pores (Faleiro and Lazebnik, 2000). We already proposed earlier, that by such a mechanism RNPs may be exported by passive diffusion (Wurzer et al., 2003). Data of a more recent collaborative study using atomic-force microscopy supported this assumption by demonstrating that caspase action leads to a very ordered degradation of the nuclear pore complexes (Kramer et al., 2008). This allowed diffusion of particles of the size of RNP complexes through the widened pores without disrupting general barrier function of the nuclear membrane (Kramer et al., 2008).

The pharmacokinetic data of the compound revealed sufficient plasma exposure allowing antiviral efficacy when the drug was applied either intravenously or intraperitoneally. Using i.v. application we were able to demonstrate a significant protection ($p = 0.035$) of mice that were treated with 5 and 10 mg/kg. A significant protection could be achieved as well when SC75741 was given via the i.p. route. Here, we were able to show that the drug (7.5 mg/kg) administered twice daily was more efficient than 15 mg/kg once daily. Interestingly, SC75741 was effective when given shortly prior to infection for five to seven consecutive days demonstrating a prophylactic potential. Moreover, when SC75741 treatment started as late as 4 days after infection, the drug was still efficacious in significantly protecting mice against lethal H5N1 influenza virus infection. In this case, an advantage of a twice-daily treatment over a single treatment per day could not be observed. It is noteworthy that SC75741 was able to protect mice, when given as late as 4 days after infection, a time-point too late for neuraminidase-inhibitors to protect mice against lethal influenza virus infection (Govorkova et al., 2009; Sidwell et al., 1998; Smee et al., 2009).

Using 2 mg/kg was not effective against influenza virus infection of mice. Nevertheless, this could also be due to the number of mice used in the present study. As shown in Fig. 4, the protection is still substantial using 2 mg/kg. One would expect a significant result using more mice per group, which could be of interest for the next step of the drug development. As the purpose of the study was indeed to determine a dose-dependent effect of the drug, we do not see a need to perform additional experiments. From the present data one might argue that treatment with 30 mg/kg is toxic to the animal. From a earlier study we know that treatment of uninfected mice with 30 mg/kg for 2 weeks did not result in adverse effects (4SC unpublished data).

It is well documented that in severe influenza an overproduction of cytokines and chemokines is found, the so-called "cytokine storm", a term which gained popularity during the H5N1 pandemic threat. Although the cytokine storm represents a pathogenicity factor for influenza, its exact role and influence for influenza pathogenesis is still not known (Tisoncik et al., 2012). In previous studies, we and others were able to demonstrate expression of IP-10 and IL-6 in the mouse lung after H5N1 infection (Droebner et al., 2008b; Salomon et al., 2007; Wareing et al., 2004). Therefore, it might be speculated that besides targeting the virus it is also worth to target the cytokine storm, e.g. by the use of glucocorticoids. There is no doubt that cytokine inhibition does not protect against severe influenza or even death, but therapies addressing both, virus and cytokines, may be preferable. Such a dual mode of action is displayed by SC75741 which takes advantage of the fact that influenza virus needs to activate the NF-kappaB signaling pathway for efficient replication (Ludwig et al., 2006; Nimmerjahn et al., 2004; Wurzer et al., 2004). Recently, we were able to show that inhibition of either NF-kappaB or of the proteasome, leading to IκB accumulation, also inhibits influenza virus replication *in vitro* and *in vivo* (Dudek et al., 2010; Haasbach et al., 2011; Mazur et al., 2007). Moreover it is known that NF-kappaB is a major regulator of cytokine and chemokine expression (Pahl, 1999). Overexpression of cytokines and chemokines induced by influenza virus infection is depended on NF-kappaB signaling pathway (Droebner et al., 2008b; Schmolke et al., 2009). Thus, by targeting NF-kappaB both, virus replication and overexpression of cytokines and chemokines can be addressed at the same time. Along this line

we wondered whether SC75741 treatment has an influence on cytokine expression in the mouse lung after H5N1 infection. Our recent results by Ehrhardt and coworkers demonstrate that apart from the reduction of influenza virus titers, SC75741 was also able to reduce H5N1-induced IL-6 and IP-10 production in the lung (Ehrhardt et al., 2013). A previous work demonstrated that delayed treatment with zanamivir decreased the survival rate of infected mice. Zanamivir alone reduced viral load but not inflammation and mortality. A combination of zanamivir with immunomodulators that reduced cytokine storm and prevented apoptosis was beneficial for the survival of mice (Zheng et al., 2008). From these data one might argue that in the present study in the early phase after infection a twice daily delivery is beneficial to target the virus while late after infection a once daily treatment might by sufficient to target the pro-inflammatory response modulating the severe cytokine storm and therefore the outcome of disease. Thus, the effect of SC75741 on cytokine expression or production in influenza virus infected mice is essential and might contribute directly to the survival.

In conclusion we demonstrate that SC75741, a small molecule compound with inhibitory activity on NF-kappaB, is able to protect mice from lethal influenza virus infection with highly pathogenic avian strains. This protection is comparable to data obtained with oseltamivir treated mice. The concentrations required to inhibit virus replication did not cause adverse effects. These data represent a proof of principle that inhibiting cellular targets can be a successful approach for treatment of influenza virus infections and lay the foundation for further investigations whether SC75741 or a derivate can be developed into an antiviral drug against influenza.

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