18 Zheng BJ, Chan KW, Lin YP *et al.* Delayed antiviral plus immunomodulator treatment still reduces mortality in mice infected by high inoculum of influenza A/H5N1 virus. Proc Natl Acad Sci USA 2008; 105:8091–8096.
19 Shin T, Kuboki S, Huber N *et al.* Activaton of peroxisome proliferator-activated receptor-γ during hepatic ischemia is age-dependent. J Surg Res 2008; 147:200–205.
20 Vandermeer M, Thomas A, Kamimoto L *et al.* The role of statins in preventing death among patients hospitalized with lab-confirmed influenza infections (Abs. 706). Annual Meeting of the Infectious Diseases Society of America. October 30, 2009.
21 Bernard GR. Statins for acutely hospitalized patients: randomized controlled trials are long overdue. Crit Care 2010; 14:141.
22 InFACT Global H1N1 Collaboration. InFACT: a global critical care research response to H1N1. Lancet 2010; 375:11–13.
23 Adhikari NK, Fowler RA, Bhagwanjee S *et al.* Critical care and the global burden of critical illness in adults. Lancet 2010;376:1339–46. doi: 10.1016/S0140-6736(10)60446-1.

The NF-kappaB-inhibitor SC75741 efficiently blocks H5N1 influenza virus propagation *in vitro* and *in vivo* without the tendency to induce resistant virus variants

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Introduction

Influenza is still one of the major plagues worldwide. The appearance of highly pathogenic avian influenza (HPAI) H5N1 viruses in humans and the emergence of resistant H5N1 variants against neuraminidase inhibitors highlight the need for new and amply available antiviral drugs. We and others have demonstrated that influenza virus misuses the cellular IKK/NF-kappaB signalling pathway for efficient replication, suggesting that this module may be a suitable target for antiviral intervention.[1] Here, we show that the novel NF-kappaB inhibitor SC75741 efficiently blocks replication of influenza A viruses, including avian and human A/H5N1 isolates *in vitro* in concentrations that do not affect cell viability or metabolism. In a mouse infection model with HPAI A/H5N1 and A/H7N7 viruses, we were able to demonstrate reduced clinical symptoms and survival of SC75741 treated mice. Moreover, influenza virus was reduced in the lung of drug-treated animals. Besides this direct antiviral effect, the drug also suppresses H5N1-induced overproduction of cytokines and chemokines in the lung, suggesting that it might prevent hypercytokinemia we hypothesise to be associated with pathogenesis after infections with highly pathogenic influenza viruses, such as the A/H5N1 strains. Thus, a SC75741-based drug may serve as a broadly active nontoxic anti-influenza agent.

Material and methods

Antiviral compound

SC75741 was supplied by 4SC AG (Planeeg-Martinsried, Germany). SC75741 was dissolved in 10% DMSO/30% Cremophor (Merck).

Virus

Avian influenza A/mallard/Bavaria/1/2006 (H5N1) virus, grown in embryonated chicken eggs, was used throughout this study. The avian influenza A virus isolate was originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany and further propagated at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Tübingen, Germany. In addition the avian strain FPV (fowl plague virus) H7N7 was grown on MDCK cells. For cell culture experiments a multiplicity of infection (MOI) of 0·001 was used. For infection, the animals 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 PBS) and infected i.n. with 5×10^3 pfu MB1 (H5N1) in a volume of 50 μl PBS via the intranasal route. According to the German animal protection law, the mice were sacrificed as soon as they lost 25–30% of their weight. Five animals per group were monitored for 14 days after infection. The experiments were performed three times.

Influenza virus titration (AVICEL® plaque assay)

To assess the number of infectious particles (plaque titers) in organs a plaque assay using Avicel® was performed in 96-well plates as described by Mastrosovich and colleagues.[2] Virus-infected cells were immunostained by incubating for 1 hour with a monoclonal antibody specific for the influenza A virus nucleoprotein (Serotec) followed by 30 minutes incubation with peroxidase-labeled anti-mouse antibody (DIANOVA) and 10 minutes incubation with True Blue™ peroxidase substrate (KPL). Stained plates were scanned on a flat bed scanner and the data were acquired using Microsoft® Paint software. The virus titer is given as the logarithm to the basis 10 of the mean value. The detection limit for this test was $<1{\cdot}7$ $\log_{10}$ pfu/ml.

RNA isolation and reverse transcription real-time PCR

Organs of infected and control mice were homogenized and incubated over night in 1 ml TriZol® Reagent (Invitrogen) at 4°C. Total RNA isolation was performed as specified by the manufacturer (Invitrogen). RNA was solubilised in 50 μl RNase free water and diluted to a working concentration of 50 ng RNA/μl. Reverse transcription real-time PCR was performed using QuantiFast™ SYBR® Green RT-PCR Kit and QuantiTect Primer Assays (Qiagen). All samples were normalized to GAPDH and fold expression analyzed relative to uninfected controls.[3] Ct values were obtained with the SmartCycler® (Cepheid).

Results

To answer the question whether the NF-kappaB inhibitor SC75741 shows antiviral properties against influenza virus, H5N1 infected MDCK cells were treated with different concentrations of the inhibitor (Figure 1). Already treatment with 1 nm of SC75741 led to a reduction of viral CPE of more than 70%. Almost 100% protection of cells was achieved when cells were treated with 50 μm SC75741. The results indicated that SC75741 has antiviral properties at concentrations ranging from 1 to 5 nm.

We next tested whether SC75741 would also be effective in the mouse model of influenza virus infection. When H7N7 mice were treated i.v. once daily for 5 days with 5 mg/kg SC75741, survival rate of the animals increased significantly ($P < 0{\cdot}05$). The same results were found when H7N7 influenza virus infected mice were treated i.p. with 15 mg/kg SC75741 (data not shown). Moreover, SC75741 treatment was not only effective when the inhibitor was given prior to H5N1 influenza virus infection, but also in a therapeutic setup when SC75741 was applied to the animals 4 days after infection (data not shown).

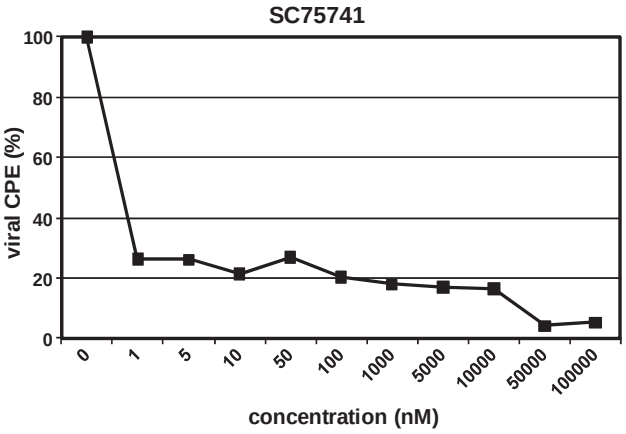

Figure 1. Virus reduction assay after SC75741 treatment of MDCK II cells. Virus infected cells (MOI 0·001) were treated with different SC75741 concentrations ranging from 0 to 100 000 nm). Virus titer was determined by plaque assay.

Since influenza virus infected mice showed increased survival after lethal infection, we next questioned whether the amount of influenza virus was reduced in the lung. Therefore, we performed quantitative real-time (qRT) PCR to detect viral mRNA. Mice were treated with either SC75741 or the solvent, and 48 hour later the lungs were prepared to perform qRT-PCR. As shown in Figure 2A the amount of viral mRNA was reduced by 90% in SC75741 treated mice compared to solvent treated controls, indicating that SC75741 leads to a reduced expression of H5N1 specific mRNA in the lung of infected mice. Since infection of mice with H5N1 leads to hypercytekinemia,[4] we also investigated the expression of cytokines in SC75741 treated mice. As shown in Figure 2B the amount of IL-6 specific mRNA was drastically reduced in SC75741 treated mice compared to solvent treated controls. Moreover, also the expression of IP-10 was altered in SC75741 treated H5N1 influenza virus infected mice. Here, roughly 90% reduction of specific

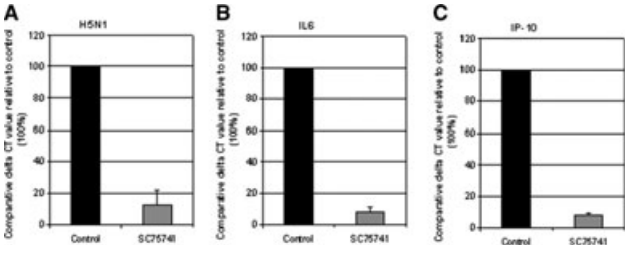

Figure 2. mRNA levels of cytokines and chemokines 2 days p.i. after infection of mice with 5×10^3 pfu H5N1 after a reverse-transcriptase real-time PCR according to manufacturer's protocol (QIAGEN). Solvent treated control (black bars) and SC75741 treated mice (grey bars) (A) H5N1, (B) IL-6, (C) IP-10. The bars represent the mRNA levels of three infected mice according to Boeuf.[3] This experiment was performed twice with similar results.

mRNA was detectable (Figure 2C). Thus, SC75741 leads to a reduced transcription of IL-6 and IP-10 in H5N1 infected mice.

Discussion

There is an urgent need for new concepts to develop antiviral drugs against influenza virus. Targeting cellular factors is a promising but challenging approach, and the concerns about side effects are obvious. However, it should be considered that drugs targeting viral factors, such as amantadine or oseltamivir, also exhibit a wide range of side effects in patients. Thus, drug safety has to be rigorously tested in clinical trials regardless whether a drug targets a cellular or a viral factor. Moreover, resistance against human H1N1 influenza viruses and highly pathogenic avian H5N1 virus strains to oseltamivir and amantadine have been reported.[5] In that respect, the strategy to target cellular factors [6,7] 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.

We were able to demonstrate that the NFkappaB inhibitor SC75741 is able to reduce influenza virus activity in cell culture. Moreover, the compound was also effective against highly pathogenic avian influenza viruses of the H5N1 and H7N7 subtypes in the mouse model. Next to the reduction of virus SC75741 was also able to reduce H5N1-induced overproduction of cytokines and chemokines in the lung in the lung of mice after infection with H5N1. Most importantly, the drug did not show any tendency to induce resistant virus variants (data not shown). Thus, a SC75741-based drug may serve as a broadly active non-toxic anti-influenza agent.

References

1 Mazur I, Wurzer WJ, Ehrhardt C *et al.* Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-kappaB-inhibiting activity. Cell Microbiol 2007; 9:1683–1694.

2 Matrosovich M, Matrosovich T, Garten W *et al.* New low-viscosity overlay medium for viral plaque assays. Virol J 2006; 3:63.

3 Boeuf P, Vigan-Womas I, Jublot D *et al.* CyProQuant-PCR: a real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use. BMC Immunol 2005; 6:5.

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

5 Hayden F. Developing new antiviral agents for influenza treatment: what does the future hold? Clin Infect Dis 2009; 1:48.

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

7 Pleschka S. RNA viruses and the mitogenic Raf/MEK/ERK signal transduction cascade. Biol Chem 2008; 389:1273–1282.