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Antiviral activity of the MEK-inhibitor U0126 against pandemic H1N1v and highly pathogenic avian influenza virus *in vitro* and *in vivo*

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ABSTRACT

The emergence of the 2009 H1N1 pandemic swine influenza A virus is a good example of how this viral infection can impact health systems around the world in a very short time. The continuous zoonotic circulation and reassortment potential of influenza A viruses (IAV) in nature represents an enormous public health threat to humans. Beside vaccination antivirals are needed to efficiently control spreading of the disease. In the present work we investigated whether the MEK inhibitor U0126, targeting the intracellular Raf/MEK/ERK signaling pathway, is able to suppress propagation of the 2009 pandemic IV H1N1v (v = variant) as well as highly pathogenic avian influenza viruses (HPAIV) in cell culture and also *in vivo* in the mouse lung. U0126 showed antiviral activity in cell culture against all tested IAV strains including oseltamivir resistant variants. Furthermore, we were able to demonstrate that treatment of mice with U0126 via the aerosol route led to (i) inhibition of MEK activation in the lung (ii) reduction of progeny IAV titers compared to untreated controls (iii) protection of IAV infected mice against a 100× lethal viral challenge. Moreover, no adverse effects of U0126 were found in cell culture or in the mouse. Thus, we conclude that U0126, by inhibiting the cellular target MEK, has an antiviral potential not only *in vitro* in cell culture, but also *in vivo* in the mouse model.

1. Introduction

The Raf/MEK/ERK signaling pathway belongs to the mitogen-activated protein kinase (MAPK) cascade family. This cascade is a key intracellular signaling pathway that regulates essential cellular functions including cell proliferation, cell cycle regulation, cell survival cell migration and angiogenesis (Su and Karin, 1996). Activation of the canonical Raf/MEK/ERK signaling pathway is commonly initiated by receptor tyrosine kinases at the cell surface. Also a large variety of other receptors including cytokine receptors, T-cell receptors, G-proteins, integrins and serpentine receptors, have the ability to activate the cascade as well (Nakayama and Yamashita, 2010; Chang et al., 2003). Activation of these receptors leads – via different adaptor proteins and other mediators – to stepwise phosphorylation and activation of the serine threonine kinase Raf, the dual specificity kinase MEK (MAPK kinase/ERK kinase) and the MAPK ERK (extracellular signal-regulated kinase). Finally the signal is transmitted by ERK to phosphorylate multiple targets including Elk-1, c-Ets1, c-Ets2, p90RSK1, MNK1, MNK2, and TOB (reviewed by Pearson et al., 2001).

Infection of the host cell by influenza viruses results in the activation of various intracellular signaling pathways that are in part suppressed by the virus to ensure efficient replication (Ludwig et al., 2006). This dependency of the virus may be useful for the development of novel antiviral strategies. Two signaling pathways that are required for efficient influenza virus propagation have attracted considerable attention as suitable targets for an antiviral approach, the Raf/MEK/ERK mitogenic kinase cascade and the IKK/NF-κB module (Pleschka et al., 2001; Mazur et al., 2007). The Ras/Raf/MEK/ERK pathway has been a focus of intense investigation for therapeutic targeting in particular in the treatment of cancer because of its pivotal role in multiple cellular functions that underlines the importance of the cascade in oncogenesis and growth of transformed cells (Friday and Adjei, 2008).

We have previously demonstrated that specific blockade of the Raf/MEK/ERK pathway with the MEK-inhibitor U0126 strongly impaired growth of all influenza A and B-type viruses tested so far (Pleschka et al., 2001; Ludwig et al., 2004). Inhibition of the cascade

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led to nuclear retention of the viral ribonucleoprotein (RNP) complexes in late stages of the replication cycle. The timely activation is achieved by membrane accumulation of the viral haemagglutinin protein and subsequent protein kinase C α-dependent activation of the Raf/MEK/ERK cascade (Marjuki et al., 2007).

The 2009/2010 H1N1 influenza A virus pandemic situation clearly demonstrated that influenza is still a major threat for the public health worldwide. Although the pandemic swine origin influenza A virus (SOIV) caused mostly mild symptoms, the control of the outbreak still remains difficult (Tang et al., 2010; Girard et al., 2010). Even though a vaccine is available against this virus, the possibility of reassortment between the pandemic and a seasonal or avian A/H5N1 influenza virus strain is indeed a frightening, but possible scenario. This reassortant strain might be able to transmit easily between humans causing fatal infections and the current SOIV vaccine might no longer be sufficient to protect against the reassorted virus. In such a case, we can only rely on effective antiviral drugs. The frequency of reports describing the appearance of drug-resistant seasonal H1N1 and also H5N1 influenza A viruses increased in the recent past (Hurt et al., 2011; Memoli et al., 2011; Yi et al., 2010; Hayden, 2009). This increase of drug resistance highlights the urgent need for novel antiviral compounds with novel defense mechanisms.

Therefore, we investigated whether the MEK-inhibitor U0126 would be effective *in vitro* and *in vivo* against the 2009 pandemic H1N1 strain and two natural A/H5N1 strains, isolated in avian species during the 2006/2007 avian influenza outbreak in Germany. Moreover, the highly pathogenic avian influenza virus (HPAIV) laboratory strain FPV/H7N7 was included in this study.

2. Materials and methods

2.1. Viruses

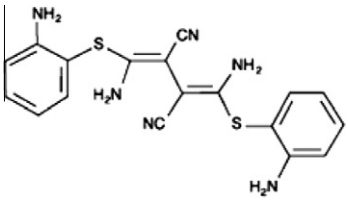
Mouse-adapted highly pathogenic avian influenza A/FPV/Bratislava/79 (H7N7; FPV) virus and swine origin human influenza A virus (SOIV) A/Regensburg/D6/2009 (H1N1v; RB1) were grown in Madin–Darby canine kidney cells (MDCK II; Matrosovich et al., 2006). Highly pathogenic H5N1 avian influenza virus A/mallard/Bavaria/1/2006 (H5N1; MB1) and A/goosander/Bavaria/20/2006 (H5N1; GSB; no H275Y mutation, access No. ABL63929.1) belonging to clade 2.2 were grown in embryonated chicken eggs and used throughout this study. Both isolates of the H5N1 subtype were originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany. The avian influenza A virus A/Bratislava/79 (FPV, H7N7) was originally provided from the strain collection at the Institute of Virology, Justus-Liebig University, Giessen, Germany. SOIV was obtained from the Robert Koch Institute, Berlin, Germany. All influenza A viruses were further propagated at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Tübingen, Germany.

2.2. 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, Tübingen, Germany and were used throughout all the experiments.

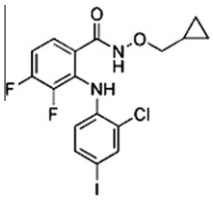
2.3. Antiviral compounds

Oseltamivir carboxylate was obtained from Toronto Research Chemicals, Inc. and dissolved in sterile PBS. For *in vitro* studies the MEK inhibitor U0126 (Taros GmbH, Germany) was

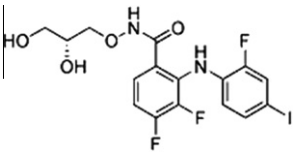


U0126; Formula: C18H16N6S2

dissolved at a stock-concentration of 10 mM in DMSO. From this solution a dilution series was prepared in DMSO to attain the desired U0126 concentration. Because DMSO affects the cell vitality only 1% U0126/DMSO solution was given to the medium. For *in vivo* application (4 ml per inhalation) U0126 was dissolved in 10% DMSO, 30% of Cremophor EL (Merck) and 60% PBS. PD184352 and PD0325901 were obtained from Selleck Chemicals LLC and disolved as recommended by the manufacture.



PD184352; Formula: C17H14ClF2IN2O2



PD0325901; Formula: C16H14F3IN2O4

2.4. Cell viability (cytotoxicity) analysis

In order to determine whether the concentration of U0126 used for experiments would affect cell viability, a compound toxicity analysis was performed. A549 and MDCK II cells were seeded in 96-well culture plates at a density of 8×10^4 cells per well in minimal essential medium (MEM) containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin. Cells were incubated at 37 °C with 5% CO_2 overnight. Thereafter, cells were washed twice with PBS. MEM containing different concentrations of U0126 (0.001–1000 μM) was added to the cells. After addition of U0126, cells were incubated further for 48 h at 37 °C and 5% CO_2. Then, cells were fixed by incubation for 30 min at 4 °C with 100 μl 4% paraformaldehyde (PFA). Adding 100 μl crystal violet for 30 min at room temperature stained viable cells. After staining, plates were washed and dried. For the extraction of crystal violet from viable cells 100 μl of 100% methanol was added to each well. After incubation for 30 min at room temperature, the extinction was measured with an enzyme-linked immunosorbent assay (ELISA) reader at OD = 490 nm. The percentage of cell viability after treatment with the antiviral compound was calculated as follows: Percent inhibition = 100/[(OD 490) cell-control sample x (OD 490) treated sample]. The CC_{50} value (i.e. the concentration of compound that reduces the cell viability by 50%) was determined with the GraphPad Prism 5 Software by plotting the percent cell viability as a function of compound concentration.

2.5. Viral CPE (cytopathological effect) inhibition screening for oseltamivir on MDCK II

MDCK II cells (Matrosovich et al., 2006) were seeded in 96-well plates as described for the cell viability (cytotoxicity) analysis. MDCK II cells with the same stage of growth were used for the toxicity and efficacy studies. After incubation at 37 °C with 5% CO_2 overnight, cells were washed twice with PBS and 50 μl of the different influenza A viruses at a MOI of 0.001 was added to all wells except for the wells with the cell control. Each microtiter plate included 8 wells with uninfected control cultures, virus-infected control wells and virus-infected cultures to which different oseltamivir concentrations (from 0.01 nM to 1 mM, 8 wells per concentration) were added. After incubation for 30 min at 37 °C, 150 μl of MEM (uninfected and virus-infected control) or MEM supplemented with oseltamivir in different dilutions was added to each well. During the incubation period (48 h) the medium was changed after 24 h. After incubation for 48 h at 37 °C and 5% CO_2, when a strong CPE was detectable in the virus-infected cell control, the plates were examined by photometric analysis as described above. The percent of cell viability after treatment with the antiviral compound was determined after correction for the background (virus-infected cell control) values as follows: Percent inhibition = 100/[(OD 490) cell-control sample x (OD 490) treated sample]. The EC_{50} value (i.e. the concentration of compound required to reduce the viral CPE on MDCK II cells to 50%) was determined with the GraphPad Prism 5 Software by plotting the percent cell viability as a function of oseltamivir concentration.

2.6. Influenza virus titration (AVICEL® plaque assay)

MDCK II and A549 cells were grown with MEM supplemented with 10% calf serum FCS and antibiotics (penicillin and streptomycin). For infection cells were grown overnight in 96-well plates (8×10^4 cells/well). Immediately before infection the cells were washed with PBS and subsequently incubated with the different influenza A viruses at a MOI of 0.001 for 30 min at 37 °C. After the 30 min incubation period the inoculum was aspirated and cells were incubated with either MEM or MEM containing different U0126 concentrations (10–100 μM). Supernatants were collected at 24 h past infection.

To assess the number of infectious particles (plaque/focus titers; pfu) in the collected cell culture supernatants and mice lung homogenates, a Avicel plaque assay was performed in 96-well plate format as described previously (Matrosovich et al., 2006). Virus-infected cells were immunostained by a 1 h incubation with a monoclonal antibody specific for the influenza A virus nucleoprotein (AbD Serotec) followed by 30 min incubation with peroxidase-labeled anti-mouse antibody (DIANOVA) and 10 min incubation with True Blue™ peroxidase substrate (KPL). After the reaction was stopped with tap water the plates were dried and scanned with a resolution of 1200 dpi using the CANONFCAN 8800F scanner (Canon). To define the virus titer of the supernatants the plaques/foci of infected cells for every sample in each lane of the 96 well plates were counted. The virus titer is given as the logarithm to the base 10 of the plaques/foci mean value (pfu). The detection limit for this test was <1.7 log 10 pfu/ml.

2.7. Treatment of C57Bl/6 mice with U0126

Treatment of mice with U0126 was performed in an inhalation chamber (ACTIVAERO, GmbH), to assure delivery directly into the lung since no data was available concerning pharmacokinetics and pharmacodynamics. Four mice were treated in a single inhalation tube. Four of those single inhalation tubes were connected via a flexible hose to a PARI® nebulizer (PARI® LC SPRINT). The flow rate in the inhalation chamber was set up to 8 l/min. U0126 (0.5, 1.0, 5.0 or 10 mM) or buffer solution with a pressure of 2.0 bars was given for 10 min (roughly 3.5 ml) to the chamber. For the survival experiment a similar device was used allowing treatment of 5 mice simultaneously. This experiment was performed twice with a total of ten mice. U0126 was dissolved in 10% DMSO, 30% Cremophor EL and 60% PBS. Sixty minutes after the treatment, mice were infected intranasally with the different influenza A viruses. Treatment of mice from the control groups were treated with solvent alone to assure the same moisture in the lungs of the animals. For the analysis of spleens and livers after U0126 treatment four animals per group were treated with the solvent or with 10 mM U0126 daily for 5 days.

2.8. 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 rate of 1:10 with PBS. Mice were infected intranasally with adequate virus doses diluted in 50 μl BSS by inoculating 25 μl into each nostril one hour after treatment. The MLD_{50} was determined for each virus strain and for each mouse strain (MB1 = 2×10^3 pfu; FPV = 1×10^2 pfu; GSB = 5×10^2 pfu; RB1 = 3×10^4 pfu). After infection with H5N1 viruses and SOIV the mice were kept in individually ventilated cages (Techniplast). All animal studies were approved by the Institutional Animal Care and Use Committee of Tübingen.

2.9. Virus titer determination in lungs

Mice were sacrificed 24 h post infection and lungs were weighed, transferred into a Lysing Matrix D tube (MP Bio) and BSS (buffered salt solution) in an amount of the 10-fold volume of the lung was applied to the samples. Organs were shredded using the FastPrep FP 120 (Savant). To remove the cell debris the homogenates were centrifuged for 15 min at 2000 rpm and the supernatant collected. The determination of virus titer in homogenates was performed using the AVICEL® plaque assay described above.

2.10. EC_{50} determination

For the determination of the $EC_{50,}$ viral titers of the cell culture supernatants or mice lungs were calculated in percent. The number of pfu of the untreated virus-infected control was set as 100% and the titers of U0126 treated samples were calculated as follows:

Percent inhibition = 100/(pfu virus-infected sample × U0126 treated sample].

The EC_{50} value (i.e. the concentration of U0126 required to reduce the virus titer to 50%) was determined with the GraphPad Prism 5 Software by plotting the percent virus titer as a function of U0126 concentration.

2.11. Statistical analysis

For investigation of the significance of the spleen and liver data, one way statistical analysis (ANOVA) followed by Bonferroni's post hoc comparison test was performed using the GraphPad Prism 5 Software. Statistical analysis of the survival experiment was done with the Wilcoxon Signed Rank Test using the GraphPad Prism 4 Software. Statistical analysis of the virus reduction of the PD inhibitors was done with the paired *t*-test using the GraphPad Prism 4 Software. Statistics with a value of $p < 0.05$ were considered significant.

3. Results

3.1. The MEK inhibitor U0126 suppresses replication of pandemic H1N1v and highly pathogenic avian influenza viruses in cell culture

In complementation to our earlier findings (Pleschka et al., 2001) and in relation to our additional investigation presented in this issue on the simultaneous reduction of virus titer and pro-inflammatory cytokine expression (Pinto et al., 2011), we now addressed the question whether U0126 is also effective against the current pandemic influenza isolates and highly pathogenic avian influenza viruses of the H5 and H7 subtypes. As representative strains we used the human pathogenic 2009 pandemic H1N1v A/Regensburg/D6/2009 strain, two H5N1-strains A/mallard/Bavaria/1/2006 (MB1) and A/goosander/Bavaria/20/2006 (GSB) and a mouse adapted H7N7 HPAIV strain A/FPV/Bratislava/79 (FPV). Treatment with the MEK inhibitor U0126 efficiently reduced progeny virus titers of all tested strains in A549 cells. While nM concentrations of U0126 were efficient to reduce H1N1v and H5N1 (MB1), μM concentrations of U0126 were required to reduce the virus titer of H5N1 (GSB) and H7N7. The EC_{50} values for U0126 against H1N1v were 1.2 ± 0.4 μM in A549 cells and 74.7 ± 1.0 μM in MDCKII cells (Table 1). U0126 was effective against both H5N1 viruses, while better inhibitory values were found for MB1 (4.4 ± 1.0 μM in A549 cells and 19.2 ± 1.3 μM in MDCKII cells) compared to GSB (36.6 ± 1.2 μM in A549 cells and 141.3 ± 18.4 μM in MDCKII cells). For the avian H7N7 strain FPV lower EC_{50} values were found in MDCKII cells than in A549 cells (82.8 ± 1.1 μM in A549 cells and 22.7 ± 1.1 μM in MDCKII cells). In addition the EC_{50} values for oseltamivir against these four influenza virus strains were determined, revealing high effectiveness for H1N1v (0.1 ± 0.004 μM in MDCKII cells) and against the H5N1 strain MB1 (0.3 ± 0.01 μM on MDCKII cells) while the H5N1 strain CGB showed resistance (356.9 ± 2.6 μM on MDCKII cells). Reduced effectiveness of oseltamivir was found against the H7N7 strain FPV (176.6 ± 1.1 μM in MDCKII cells). U0126 treatment with concentrations ranging from 1–100 μM had no influence on the cell viability of A549 (Fig. 3A) and MDCKII cells (Fig. 3B). The CC_{50} values for U0126 on A549 cells (6.5 ± 0,77 mM) and MDCKII cells (97.78 ± 1.26 mM) were in the mM range (Fig. 3A, B right panel).

3.2. Aerosol treatment of mice with the MEK inhibitor U0126 leads to reduction of ERK activity

U0126 shows no antiviral activity *in vivo* after oral application (data not shown). In order to investigate whether U0126 functions instead as an antiviral after local delivery into the lung, we first investigated the potency of U0126 to inhibit the Raf/MEK/ERK signaling pathway when administered to the mouse lung. Employing the experimental setup for local aerosol delivery into the lung (see Section 2) we were able to deposit 0.3 to 1% of the solution into the lung (data not shown). Three C57Bl/6 mice were treated twice with a 10 mM U0126 solution for 10 min. (roughly 3.5 ml) and two hours after the last treatment the animals were sacrificed. The lungs were analyzed for the presence of activated (phosphorylated) ERK (P-ERK). U0126 treatment resulted in reduced P-ERK levels in the lung of mice, compared to lungs of mice receiving the solvent (Fig. 1A). Quantification of the western blot data revealed that the reduction of both P-ERK isomers (p42 and p44) was around 50% (Fig. 1B).

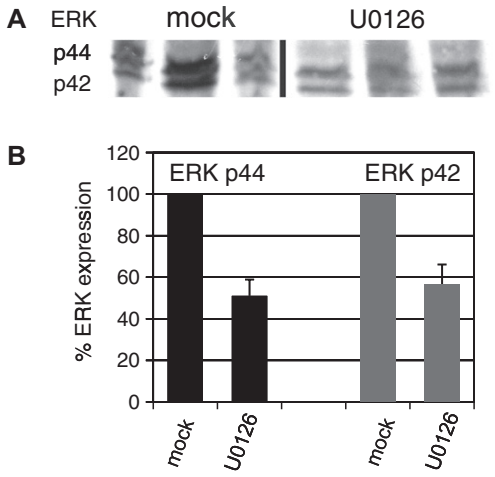


Fig. 1. Western blot analysis of ERK-phosphorylation in the lung after U0126 treatment. A: Western Blot analysis of three individual mouse lungs either treated with solvent or with U0126 using an anti-phospho ERK antibody that detects ERK in its phosphorylated active form. B: Quantitative analysis of the western blot analysis revealed a roughly 50% reduction of ERK-phosphorylation after U0126 treatment. The experiment was performed with three mice per group.

3.3. Single aerosol treatment of mice with the MEK inhibitor U0126 leads to reduction of influenza virus in the lung

Next we investigated whether U0126 would act as an antiviral against influenza virus *in vivo*. Viral titers were determined at 24 h p.i. to minimize the influence of the innate or adaptive immune response. U0126 treatment leads to reduced progeny virus titers of all 4 strains. After infection with the pandemic H1N1 strain, a titer of 4.2 ± 0.2 $\log_{10}$ pfu/ml was found at 24 h p.i. in solvent-treated controls (Fig. 2A, left panel black bar). In contrast, treatment of U0126 resulted in a virus titer of 3.4 ± 0.1 $\log_{10}$ pfu/ml (Fig. 2A, left panel white bar), which means a reduction of 84% (Fig. 2A right panel). H5N1 (MB1) influenza virus titers were reduced from 3.1 ± 0.1 $\log_{10}$ pfu/ml in controls (Fig. 2B left panel black bar) to 2.6 ± 0.1 $\log_{10}$ pfu/ml in U0126-treated mice (Fig. 2B left panel

Table 1
EC_{50} and S.I. values for U0126 and EC_{50} values for oseltamivir against different influenza A viruses.

	U0126 EC_{50} (μM)[a]			oseltamivir EC_{50} (μM)[b]	
	A549	S.I.[c]	MDCK II	S.I.[c]	MDCK II
RB1 (H1N1)	1.21 ± 0.39	5371	74.69 ± 1.02	1309	0.07 ± 0.004
MB1 (H5N1)	4.43 ± 0.98	1467	19.17 ± 1.25	5100	0.28 ± 0.01
GSB (H5N1)	36.61 ± 1.15	177	141.30 ± 18.34	692	356.92 ± 2.59
FPV (H7N7)	82.76 ± 1.10	79	22.74 ± 1.11	4299	176.60 ± 1.06

[a] determined by *in vitro* screening and represents the drug concentration required to reduce the virus titer to 50%. EC_{50} values were determined for a 48 h infection period (MOI 0.001) for each virus with the Graph Pad Prism 5 software by plotting the percent virus titer as a function of compound concentration. The experiment was performed in triplicates.

[b] determined by *in vitro* CPE inhibition screening on MDCKII cells and represent the drug concentration required to reduce the viral CPE on MDCKII cells to 50% compared with that of controls without drug. EC_{50} values were obtained for a 48 h infection period (MOI 0.001) with Graph Pad Prism 5 Software after crystal violet staining by plotting the OD450 as a function of compound concentration.

[c] CC_{50} values to determine selective indices (S.I.); A549: 6.5 ± 0.77 mM; MDCK II: 97.78 ± 1.26 mM.

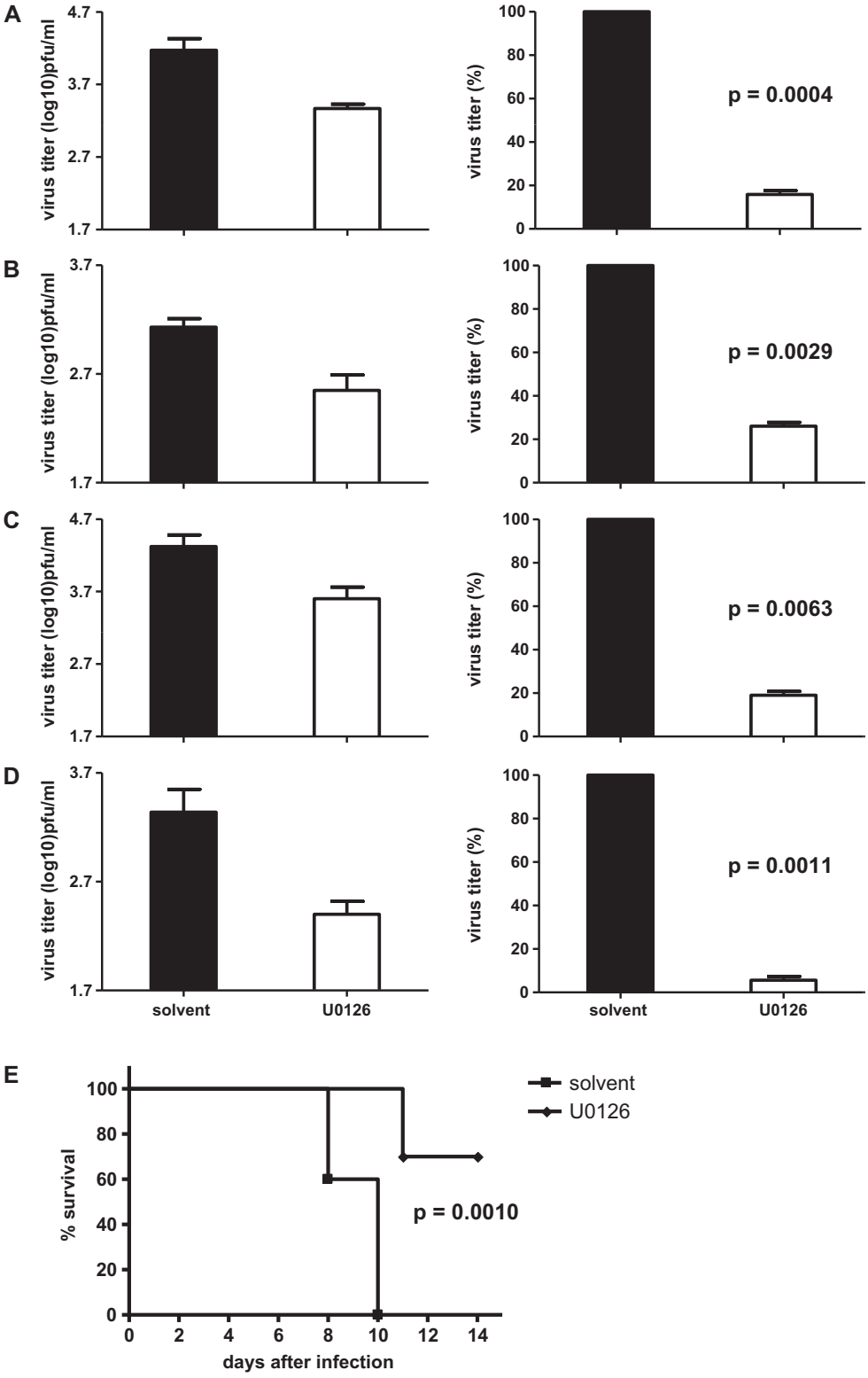


Fig. 2. Virus titer reduction in lungs after a single inhalation of U0126. One hour prior to infection animals were treated with 4 ml of 10 mM U0126. Viral titers were determined at 24 h p.i. Left panel virus titers are given in log10 pfu/ml; right panel virus titers in %. Virus titer reduction after A/Regensburg/D6/2009 (A), A/Mallard/Bavaria/1/2006 (B), A/goosander/Bavaria/20/2006 (C), A/FPV/Bratislava/79 (D) infection. Black columns: solvent-treated group. White columns: U0126-treated group. The graph represents the mean value of two independent experiments with four mice per group and virus strain. E: Survival of FPV-infected mice (100× MLD50) after treatment with U0126 (-♦-) compared to FPV infected mice treated with solvent (-■-) ($n = 10$).

white bar) representing a 74 % reduction (Fig. 2B right panel). H5N1 (GSB) titers were reduced from 4.3 ± 0.2 $\log_{10}$ pfu/ml in controls (Fig. 2C left panel black bar) to 3.6 ± 0.1 $\log_{10}$ pfu/ml in U0126 treated mice (Fig. 2C left panel white bar) representing a 81% reduction (Fig. 2C right panel). U0126 was most effective against the H7N7 strain FPV. Here, the amount of progeny virus was reduced from 3.3 ± 0.2 $\log_{10}$ pfu/ml in controls (Fig. 2D left panel black bar) to 2.4 ± 0.1 $\log_{10}$ pfu/ml in U0126 treated mice

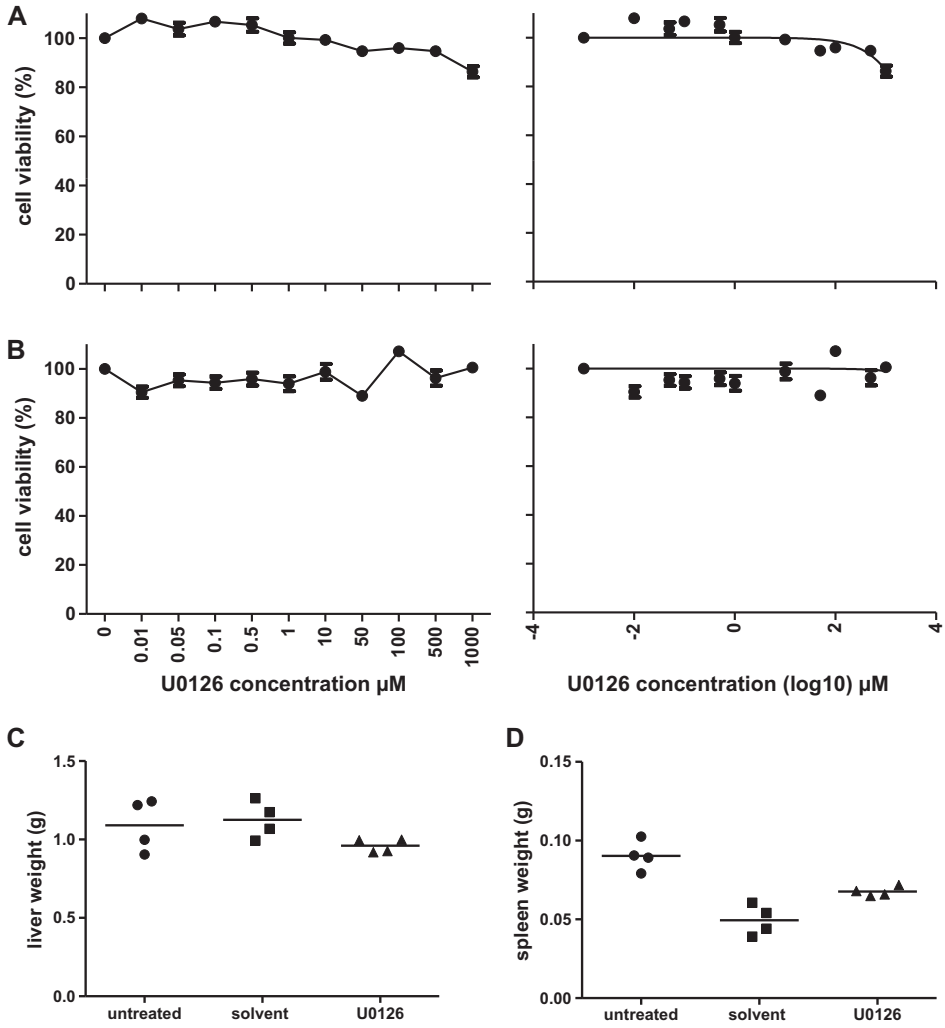


Fig. 3. Assessment of potential toxic effects of U0126 *in vitro* and *in vivo*. Left panel: Cell viability of A549 (A) and MDCK II (B) cells after treatment with different concentrations of U0126 (0.001–1000 µM). Right panel: CC50 determination curves from GraphPad Prism 5 Software. Weight of spleens (C) and livers (D) of uninfected untreated, solvent or U0126 treated mice after a 5-day application period. Significant differences were determined by Bonerroni's post hoc comparison test. Mice from the solvent and U0126 group had significantly ($p < 0.001$ and $p < 0.01$) smaller spleens when compared to untreated controls. No significant differences were detected between the liver sizes of the different animal groups.

(Fig. 2D left panel white bar) representing a 89% virus titer reduction (Fig. 2D right panel). We next questioned whether U0126 treatment would also result in prolonged survival. Therefore, mice were treated daily with 4 ml of 10 mM U0126 (10% DMSO/30% Cremophor/70% PBS) for five consecutive days beginning 1 h prior to infection with 10^4 pfu (100-fold MLD_{50}) H7N7 strain FPV. While all ten solvent-treated mice died between days 8–10 p.i. (Fig. 2E, black line), seven out of ten U0126-treated mice survived the H7N7 infection (Fig. 2E dotted line). As indicated in Figure3C and D treatment with 10 mM U0126 for five consecutive days did not result in adverse effects as indicated by measuring the weight of liver (Fig. 3C) and spleen (Fig. 3D). Nevertheless, the weight of the spleen of solvent-treated mice and U0126-treated mice were reduced significantly ($p < 0.01$ and <0.001) compared to untreated controls.

3.4. In vivo dose–response curve after infection of mice with H7N7 influenza virus

In order to determine a dose–response curve *in vivo*, four C57Bl/6 mice per group were treated with four different concentrations of U0126 (0.5, 1, 5 and 10 mM). As already demonstrated in the experiments before (Fig. 2A–D) treatment with 10 mM U0126 resulted in a strong reduction of the virus titers in the lung of FPV-infected mice 24 h after treatment. Nevertheless, even with 5 mM and 1 mM U0126 a titer reduction in the lung of U0126 treated mice was found, but this reduction was less than one log of magnitude. No obvious reduction of FPV was found when mice were treated with 0.5 mM U0126 (Fig. 4A: viral titers; Fig. 4B: % virus reduction). This set of data allowed us to draw an *in vivo* EC_{50} curve and to calculate the EC_{50} value, for U0126 against FPV in C57Bl/6 mice, which was 0.62 ± 0.16 mM. Since 4 ml were nebulized and only a maximum of 1% will reach the lung, one can roughly estimate that deposition of 9.4 µg into the lung is sufficient to reduce 50% of progeny influenza virus.

3.5. Antiviral activity of PD184352 and PD0325901 MEK-inhibitors in vitro

During this study two different MEK-inhibitors became available that were already used in clinical trails against cancer (Lorusso et al., 2005; Haura et al., 2010). Therefore, we investigated whether these inhibitors also function as antivirals against influenza virus in MDCKII cell culture.

PD 0325901 was able to reduce progeny virus (MB1) titer after infection of MDCKII cells by more than 1.5 log of magnitude, when

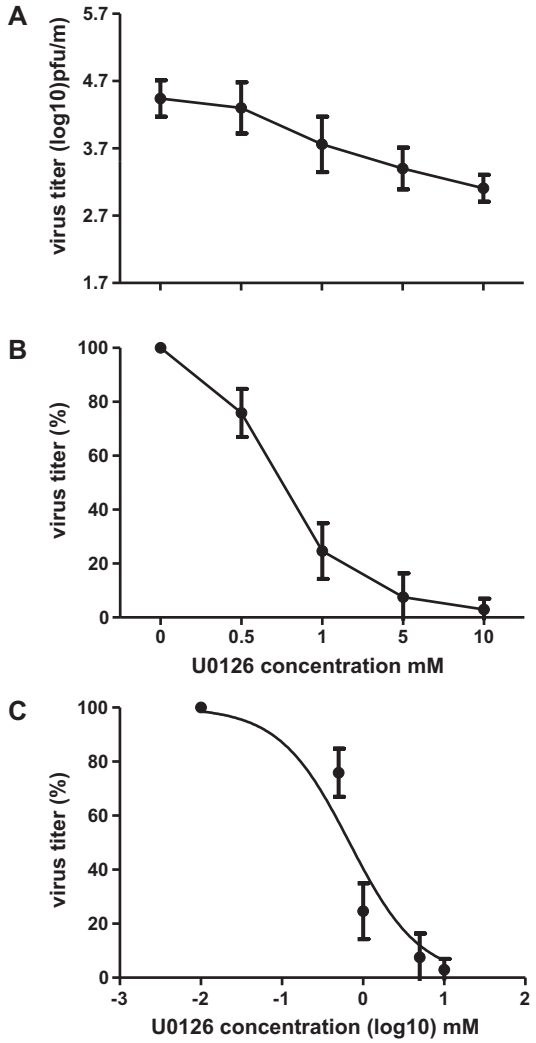


Fig. 4. In vivo dose response of U0126 against A/FPV/Bratislava/79. Reduction of virus titers in the lung after treatment with different U0126 concentrations (0, 0.5, 1, 5 and 10 mM). Four mice were used per group. A: Virus titer in log10 pfu/ml. B: Percentage virus titer. C: EC50 curve determined by GraphPad Prism 5 Software.

1 μM of the inhibitor was used. Higher concentrations like 10 and 100 μM did not further reduce virus titers (Fig. 5A). The PD 184352 Inhibitor was even more effective. While 1 μM also reduced virus titer by 1.5 log of magnitude (Fig. 5B left panel), which was equivalent to 95% (Fig. 5B right panel), a concentration of 10 μM resulted in a reduction of even more than 2.5 log of magnitude, while 100 μM PD184352 let to a further reduction of almost 3 logs of magnitude.

4. Discussion

Almost one decade ago we published that activation of the Raf/MEK/ERK signaling pathway is a prerequisite for efficient influenza virus replication and that inhibition of this intracellular pathway leads to reduced influenza virus production (Pleschka et al., 2001). This was the first time that this new antiviral strategy of targeting intracellular signaling pathways instead of the virus itself was discussed. In the last couple of years the number of intracellular pathways that were identified to be required for efficient influenza virus replication has steadily increased (reviewed by Ludwig, 2009). It could be demonstrated that in addition to influenza virus other viruses also need interaction with cellular signaling pathways in order to assure efficient replication (Planz et al., 2001; Seth et al., 2006; Ludwig and Planz, 2008; Pleschka, 2008; Ludwig et al., 2006). In this context, we were able to show that activation of the Raf/MEK/ERK signaling pathway is required by influenza virus for efficient export of RNPs from the nucleus into the cytoplasm (Pleschka et al., 2001; Ludwig et al., 2004; Marjuki et al., 2007). The strategy of using intracellular signaling pathway inhibitors as antivirals is most suitable for cellular pathways that are required by the virus to cross cellular barriers. These barriers/membranes need to be passed during entry or release of virus particles or when viral factors (RNPs) relocate between intracellular compartments (nucleus/cytoplasm) to initiate viral replication. Since ERK phosphorylation is required for efficient RNP export, the Raf/MEK/ERK signaling pathway seems to be a perfect target to interfere with influenza virus replication.

On one hand the great advantage of using blockers of intracellular signaling as antivirals is the extremely low tendency of these inhibitors to induce resistance, has been already proven for several compounds (Ludwig et al., 2004; Mazur et al., 2007). On the other hand one has to consider side effects, since these compounds interfere with the host cell machinery. In our studies, we focus on compounds that are already in clinical trials for different medical indications or even in clinical use for different purposes. In this regard we were previously able to demonstrate *in vitro* and in the mouse model that acetylsalicylic acid, which is known to inhibit the NF-kappaB signaling pathway (Yin et al., 1998) also functions as an antiviral against influenza virus, since they also need the NF-kappaB signaling pathway for their efficient replication (Wurzer et al., 2004; Mazur et al., 2007).

Inhibitors against the Raf/MEK/ERK signaling pathways were used in different anti-cancer trials. Various phase II studies were undertaken to assess the anti-tumour activity and safety of MEK-inhibitors including the orally available PD184352, ARRY-142886 and PD0325901. These compounds were generally well tolerated (e.g. during 28 days twice daily treatment), but demonstrated insufficient anti-tumor activity to warrant further development in anti-cancer treatment (Rinehart et al., 2004; Adjei et al., 2008). Moreover, it is known that the Raf/MEK/ERK signalling pathway is required for Th2 cell differentiation and that inhibiting this pathway supports a Th1 immune response, which is needed for an efficient control of pathogens (Nakayama and Yamashita, 2010). Thus, beside the antiviral activity of MEK-inhibitors, one might hypothesize that inhibiting the Th2 response leads to immunomodulation supporting antigen presentation, activation and clonal expansion to Th1 $CD4^+$ T-cells, efficient anti-influenza virus specific neutralizing antibody production that is independent of T-cell help and memory. Moreover, one also might argue that this immunomodulation could also support the immune response against a bacterial co-infection e.g. by S. pneumoniae.

In contrast to U0126 these MEK-inhibitors that were used in clinical trials against cancer became commercially available only recently. For this reason the present study was performed using U0126 even with the obvious disadvantage that the compound needs to be dissolved in DMSO/Cremophor. This resulted in certain adverse effects in mice as shown in the reduced weight of the spleens of mice treated with this solvent alone or with U0126 dissolved in DMSO/Cremophor. The fact that the weight loss of the spleens was less pronounced in the U0126 treated mice compared to the solvent treated animals clearly highlights the problem related to the solvent. Furthermore, U0126 is not available in an oral formulation. As shown by Pinto and colleagues, i.p. delivery of U0126 has only a slight antiviral effect (Pinto et al., 2011). For this reason we decided to deliver U0126 as an aerosol. With the device that we employed in the present study less than 1% U0126 reaches the lung. Consequently, of the 10 mM U0126 used in this study roughly 7.5 mg/kg U0126 was delivered into the lungs of mice

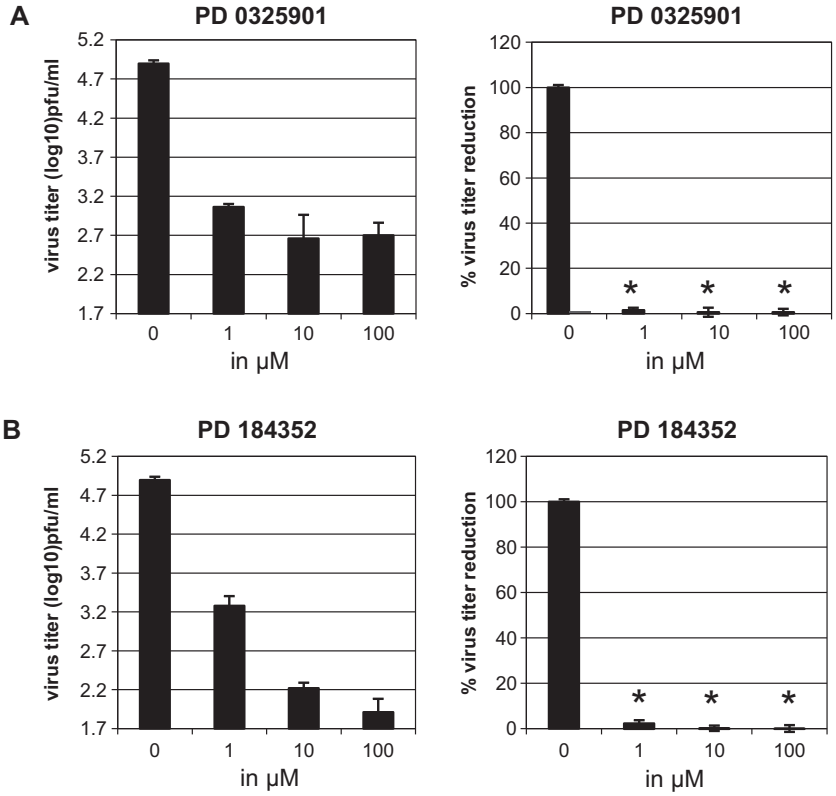


Fig. 5. Virus titer reduction assay after PD 184352 and PD 0325901 treatment. H5N1 influenza virus (MB1) infected MDCKII cells (MOI 0.001) were treated with different concentrations (0, 1, 10, 100 µM) of PD 0325901 (A) and PD 184352 (B). Virus titer was determined by plaque assay. Left panel virus titer reduction after treatment in log10 pfu/ml; right panel in %. The graphs represent the mean value from three experiments. Significant differences were determined by the paired *t*-test ($* = p < 0.001$).

and was responsible for the antiviral activity. In comparison, the MEK inhibitor PD0325901 demonstrated anti-cancer activity in mice when 20 mg/kg was given via the oral route. Furthermore, this inhibitor was given to humans 15 mg/kg twice daily (Haura et al., 2010). Thus the concentration of U0126 needed to demonstrate anti-viral activity in mice might demonstrate an achievable concentration in humans.

Because of these obvious limitations using U0126 in the present formulation, we additionally performed *in vitro* experiments using the clinically tested MEK-inhibitors PD184353 and PD0325901 to investigate their antiviral potential. The findings that both of these inhibitors were able to reduce progeny virus titers in cell culture by more than 90% not only serves as a proof-of-concept but also prompted us to speculate that these inhibitors might also have a high antiviral potential against influenza viruses *in vivo*. Despite the disadvantage of U0126 concerning the administration, our results clearly demonstrate the antiviral effectiveness of the MEK-inhibitor in cell culture and in the mouse model.

Moreover, the study by Pinto et al. (2011) further underlines that MEK-inhibition leads to reduced virus titers *in vitro*, *in vivo* and, in addition, demonstrates for the first time that the compound modulates pro-inflammatory cytokine expression simultaneously. This is another great advantage of MEK inhibition, as an unbalanced cytokine expression is often correlated with severe pneumonia caused by several IV strains, including H5N1-type HPAIV (Cheung et al., 2002; Beigel et al., 2005; Chan et al., 2005; de Jong et al., 2006).

5. Conclusions

Targeting the Raf/MEK/ERK pathway may have several advantages. Besides strong and broad antiviral activity, MEK inhibitors showed surprisingly little toxicity in cell culture, in an *in vivo* mouse model and in clinical trials for use as an anti-cancer agent. Several inhibitors of the Raf/MEK/ERK cascade from different pharmaceutical companies are under clinical investigation and these clinical trials for use as anti-cancer agents demonstrated that the cascade can indeed be effectively inhibited in humans without adverse side effects.

Our data clearly demonstrates that MEK-inhibitors might be very promising as a new class of antivirals against influenza virus that are also effective against oseltamivir-resistant strains. Although local administration was used here, an oral application can also be considered, since systemic treatment with MEK inhibitors shows almost no adverse effects. A promising approach should focus on MEK-inhibitors that already underwent with success phase I clinical trials in cancer studies for further development as new antivirals against influenza.

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