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Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-κB-inhibiting activity

Igor Mazur,[1] Walter J. Wurzer,[1†] Christina Ehrhardt,[1] Stephan Pleschka,[2] Pilaipan Puthavathana,[3] Tobias Silberzahn,[4] Thorsten Wolff,[5] Oliver Planz[4*‡] and Stephan Ludwig[1*‡]

[1]*Institute of Molecular Virology (IMV), ZMBE, Westfaelische-Wilhelms-University, Von-Esmarch-Street 56, D-48149 Muenster, Germany.*
[2]*Institute of Medical Virology, Justus-Liebig-University, Frankfurter Strasse 107, D-35392 Giessen, Germany.*
[3]*Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*
[4]*Friedrich-Loeffler-Institute (FLI), Paul-Ehrlich-Strasse 28, D-72076 Tübingen, Germany.*
[5]*Robert-Koch-Institute (RKI), Nordufer 20, D-13353 Berlin, Germany.*

Summary

Influenza is still one of the major plagues worldwide. The statistical likeliness of a new pandemic outbreak highlights the urgent need for new and amply available antiviral drugs. We and others have shown that influenza virus misuses the cellular IKK/NF-κB signalling pathway for efficient replication suggesting that this module may be a suitable target for antiviral intervention. Here we examined acetylsalicylic acid (ASA), also known as aspirin, a widely used drug with a well-known capacity to inhibit NF-κB. We show that the drug efficiently blocks influenza virus replication *in vitro* and *in vivo* in a mechanism involving impaired expression of proapoptotic factors, subsequent inhibition of caspase activation as well as block of caspase-mediated nuclear export of viral ribonucleoproteins. As ASA showed no toxic side-effects or the tendency to induce resistant virus variants, existing salicylate-based aerosolic drugs may be suitable as anti-influenza agents. This is the first demonstration that specific targeting of a cellular factor is a suitable approach for anti-influenza virus intervention.

Received 20 November, 2006; revised 10 January, 2007; accepted 18 January, 2007. *For correspondence. E-mail ludwigs@uni-muenster.de; Tel. (+49) 251 83 57791; Fax (+49) 251 83 57793; E-mail oliver.planz@fli.bund.de; Tel. (+49) 7071 967 254; Fax (+49) 7071 967 105. †Present address: Aventis Pharma, Saturn Tower, Leonard-Bernstein Strasse 10, A-1220 Vienna, Austria. ‡These two authors have equally contributed to the study.

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Introduction

Activation of the transcription factor NF-κB is one of the hallmark cell responses to infection with various pathogens. As this transcription factor controls expression of a variety of antiviral cytokines and is also a regulator of apoptotic gene expression, the concept emerged that NF-κB is a main mediator of the antiviral response to infection (Chu *et al.*, 1999). Influenza viruses are negative-strand RNA viruses that induce NF-κB activity upon productive virus infection (Ludwig *et al.*, 2003). Activation of the transcription factor can be induced by expression of some viral proteins (Flory *et al.*, 2000) or by either double-stranded or single-stranded RNA (most likely in its 5′triphosphate form), mimicking viral RNA replication intermediates (Alexopoulou *et al.*, 2001; Lund *et al.*, 2004; Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). The sensory mechanisms involve the Toll-like receptor family members 3 and 7/8 as well as the RNA helicase proteins RIG-I and mda-5 depending on the cell type affected (Alexopoulou *et al.*, 2001; Andrejeva *et al.*, 2004; Diebold *et al.*, 2004; Heil *et al.*, 2004; Kato *et al.*, 2005).

The most crucial step in the canonical NF-κB pathway is the activation of the inhibitor of κB (IκB) kinase (IKK) that in turn phosphorylates IκB leading to its ubiquitinylation. IκB is then rapidly degraded and releases the transcriptionally active NF-κB factors, such as p50 or p65/A-Rel to migrate to the nucleus (Karin, 1999a). Among the three isozymes in the IKK complex IKKβ, also named IKK2, is the enzymatically most important subunit towards phosphorylation of IκB (Karin, 1999b).

In a recent study we examined the role of IKK2 and NF-κB in influenza virus infection (Wurzer *et al.*, 2004). We analysed viral replication in host cell lines that expressed either a dominant-negative or a co-activating mutant of IKK2, as well as a non-degradable mutant of IκBα that blocks the signal by retention of p50/p65 in the cytoplasm. When yields of progeny virus grown in these different cell lines were compared with wild-type or with vector-transduced cells we made a surprising observation. In contrast to the common view that NF-κB is a main regulator of the antiviral response, virus titers were reduced in cells expressing dominant-negative mutants and enhanced in cells expressing a co-activating mutant of IKK2 (Wurzer *et al.*, 2004). Thus, IKK2 and NF-κB appeared to be required for efficient influenza virus

propagation. These challenging findings were also confirmed by others in a different and independent approach (Nimmerjahn *et al*., 2004). Towards the underlying mechanism we found that in the context of an influenza virus infection the IKK/NF-κB module mediates the expression of proapoptotic factors such as TRAIL and FasL/CD95L (Wurzer *et al*., 2004). These factors induce activation of caspases in an auto- and paracrine fashion. From another study it was known that active caspases appear to mediate export of viral ribonucleoprotein (RNP) complexes from the nucleus in late stages of infection (Wurzer *et al*., 2003). Thus, it was intriguing to propose a virus-supportive signalling event involving NF-κB-dependent regulation of proviral factors that induce caspases which subsequently support replication by enhancing RNP export, a hypothesis that was verified in the present study. While NF-κB was also a regulator of the type I antiviral IFN in response to influenza virus infection, this virus-supportive effects appeared to be dominant and independent of IFN (Wurzer *et al*., 2004).

This unexpected dependence of influenza virus multiplication on the activity of the IKK/NF-κB module (Ludwig, 2007) offers the intriguing possibility to target this signalling pathway for an antiviral intervention. One of the advantages to target a cellular rather than a viral factor would be that the virus cannot easily adapt to the missing cellular function. Thus, as indicated by earlier studies, the formation of resistant variants should not frequently occur (Scholtissek and Muller, 1991). However, other important prerequisites for such a cell-directed antiviral drug would be that the compound is safe to use and well tolerated.

Results

Acetylsalicylic acid and other pharmacological inhibitors of IKK and NF-κB block influenza virus propagation

A variety of small organic molecules have been described to inhibit the NF-κB signalling pathway (Karin *et al*., 2004). Here we focused on a NF-κB-inhibiting compound that is already in frequent medical use. Acetylsalicylic acid (ASA) and other salicylates are well-known blockers of NF-κB activation (Kopp and Ghosh, 1994; Grilli *et al*., 1996) via a quite specific inhibition of IKK2 in a low millimolar range (Yin *et al*., 1998). ASA does not inhibit the kinase activity of the close homologue IKK1 and its action involves specific interference with the IKK2 helix–loop–helix domain (Kwak *et al*., 2000). Confirming these earlier results we found that ASA inhibits NF-κB-dependent gene expression in a concentration range around 1–5 mM in the host cell lines used (Fig. 1A). Interestingly the compound also efficiently blocked influenza virus replication in the same concentration range, leading to a significant titer reduction of up to three orders of a magnitude. This was observed in different host cell types and with different virus strains including a human isolate of the highly pathogenic avian H5N1 viruses (Fig. 1B–E). We also tested other inhibitors that are known to block NF-κB activation such as the radical scavenger pyrrolidine dithiocarbamate (PDTC) (Piette *et al*., 1997), the proteasome inhibitor MG132 (Fiedler *et al*., 1998) or the specific NF-κB inhibitor BAY11-7085 (Fig. 2B) (Pierce *et al*., 1997). Although at least the first two of these inhibitors might have different secondary effects, they all overlap in the effective inhibition of NF-κB. With all these compounds virus titers were strongly impaired (data not shown) in part confirming earlier results (Uchide *et al*., 2002; Nimmerjahn *et al*., 2004). Moreover, the drug amounts required to block viral propagation directly correlated with the concentration needed to inhibit NF-κB (data not shown).

Acetylsalicylic acids and salicylates are also known as blockers of cyclooxygenases (COX). However, when we applied a pure COX inhibitor, indometacin (Tegeder *et al*., 2001), no inhibition of virus propagation was observed, concomitant with the ineffectiveness of the drug to inhibit NF-κB (Fig. 1A and B).

The antiviral activity of ASA is due to its NF-κB-inhibiting activity

Acetylsalicylic acid might be a multitarget compound and although the data so far suggest that its anti-influenza virus activity directly correlates with its inhibiting effect on NF-κB, other cellular signalling mediators may also be targeted. Influenza virus infection is known to result in the activation of several mitogen-activated protein kinase (MAPK) cascades one of which, namely the classical mitogenic Raf/MEK/ERK (extracellular signal regulated kinase) cascade, supports virus replication by regulating RNP nuclear export (Pleschka *et al*., 2001). However, when we analysed the effect of ASA on the virus-induced activation of the MAPKs ERK, p38 and c-Jun NH2-terminal kinase (JNK), no inhibition was observed (Fig. 2A). In the same samples virus-induced degradation of IκBα as a hallmark of NF-κB activation is clearly inhibited (Fig. 2A, lower panel). To further test whether the antiviral activity of ASA can really be attributed to NF-κB inhibition we examined virus titers from cells where NF-κB activation was already pre-inhibited by means of the specific NF-κB inhibitor BAY11-7085. While viral titers were strongly reduced by either treatment with ASA or BAY11-7085 alone, the combined treatment with both inhibitors did not show any synergistic effects (Fig. 2B), suggesting that both inhibitors are targeting the same cellular pathway. Together, the data so far indicated that the anti-influenza virus activity of ASA is predominantly caused via the NF-κB-inhibiting activity of the compound.

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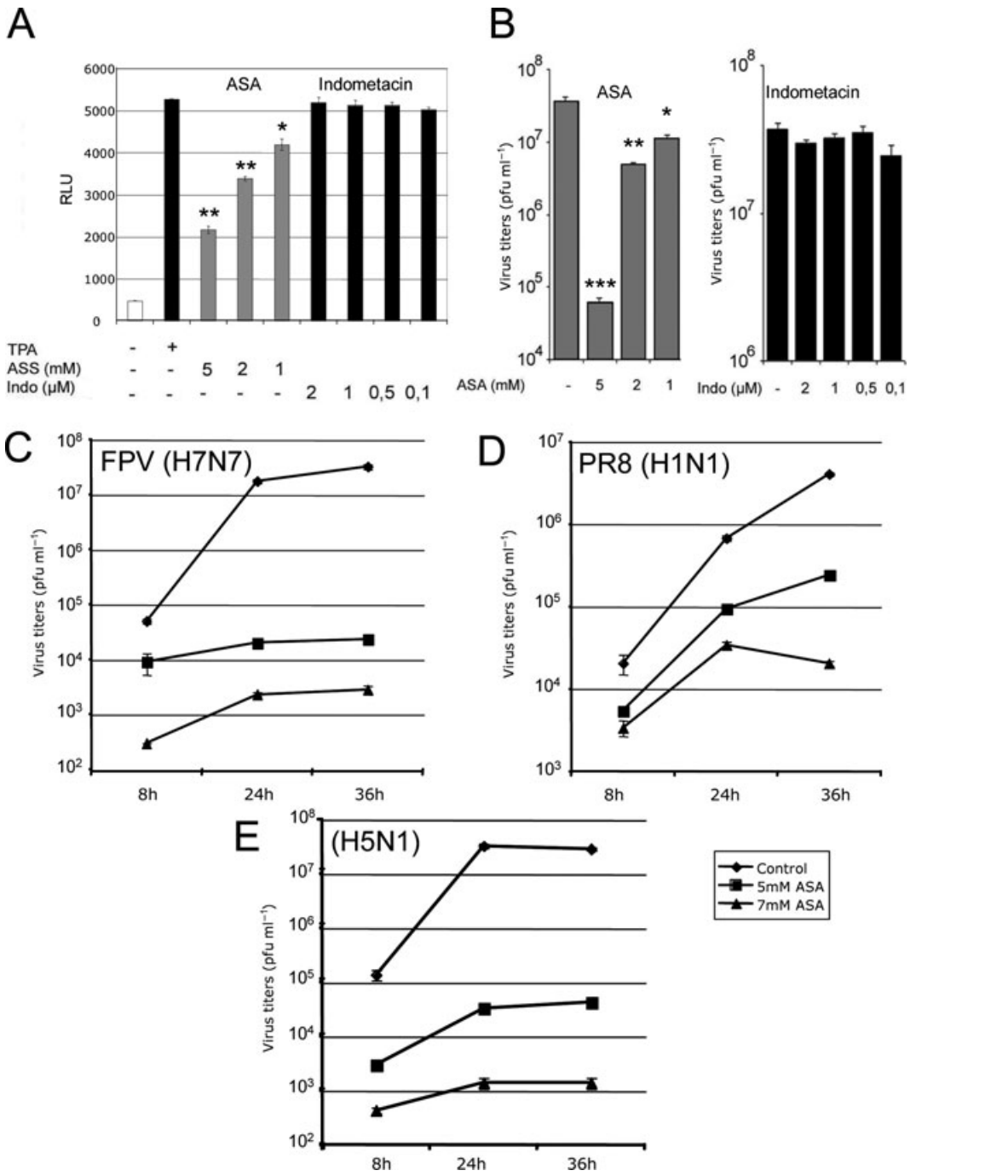

Fig. 1. NF-κB-inhibiting concentrations of ASA efficiently block influenza virus propagation.
A. A549 cells were transfected with a 3xNF-κB binding site promoter-luciferase plasmid. Twenty-four hours post transfection cells were stimulated with the NF-κB activator 12-O-tetradecanoyl phorbol-13-acetate (TPA) (100 ng ml^{-1}) in the presence or absence of different concentrations of ASA or the COX inhibitor indometacin. Fourteen hours later cells were harvested and assayed for luciferase activity. *P = 0.0002, **P = 0.0001, TPA stimulated control versus TPA stimulated and aspirin treated as calculated by Student's t-test.
B. A549 cells were infected with FPV (moi = 0.1). ASA or the COX inhibitor indometacin were added to the medium at different concentrations. Twenty-four hours p.i. supernatants were collected and virus titers were determined by plaque assay. *P = 0.0003, **P = 0.0002, ***P = 0.0001, control (infected, non-treated) versus infected and ASA-treated.
C–E. A549 cells (C, E) or MDCK cells (D) were infected with the HPAIV A/FPV/Bratislava/79 (H7N7) (FPV, moi = 0.01) (C), the human influenza virus strain A/Puerto-Rico/8/34 (H1N1) (PR8, moi = 0.01) (D) or the human isolate A/Thailand/1(KAN-1)/2004 (H5N1) (moi = 0.1) of the Asia type H5N1 HPAIV (E) in the presence or absence of different concentrations of ASA as indicated. Supernatants were harvested 8 h, 24 h and 36 h p.i. to be assayed for titers of progeny virus as in B.

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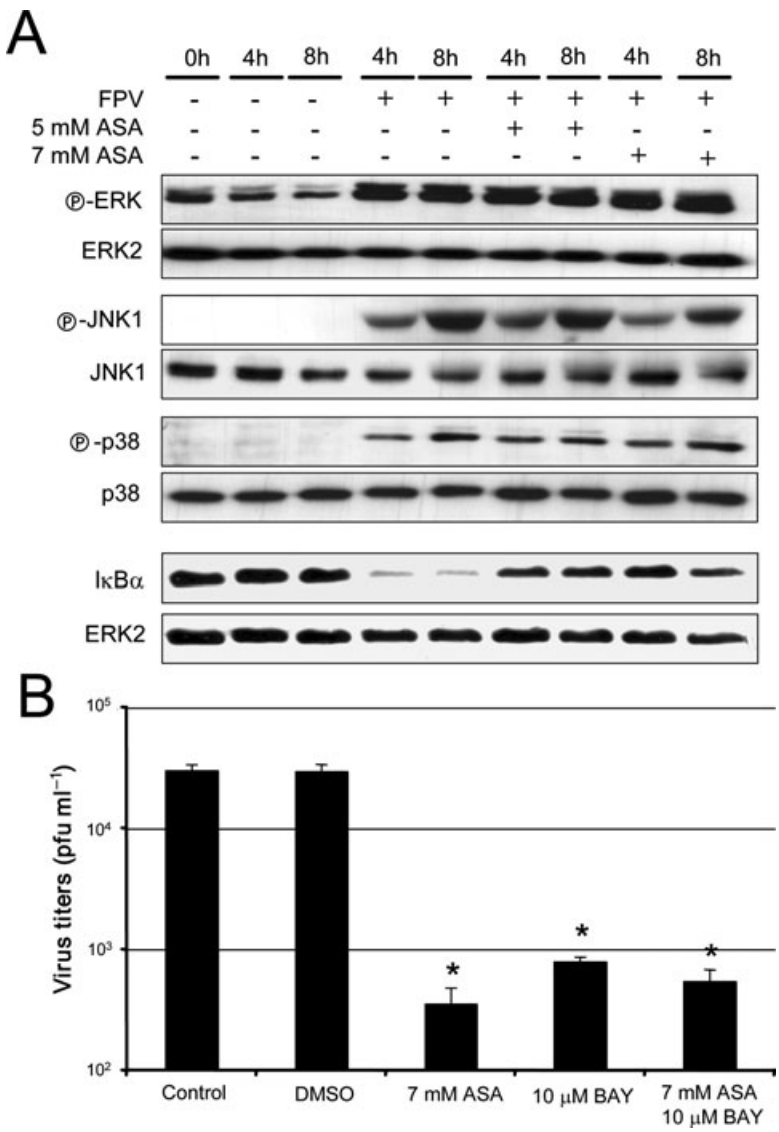

Fig. 2. The anti-influenza activity of ASA is due to a block of NF-κB activation but does not affect virus-induced activation of MAPKs ERK, JNK or p38.
A. A549 cells were infected with FPV (moi = 5) in the presence of 5 mM or 7 mM ASA and lysed after different time points as indicated. Cell lysates were subjected to Western blot analyses with activation state-specific antibodies against phosphorylated forms of ERK, JNK and p38 MAPKs. Equal protein load was verified using pan-antisera to ERK2, JNK1 and p38. As a measure for the activation state of NF-κB the degradation of IκBα was determined using an IκBα-specific antiserum (lower panel). An ERK2 blot served as a loading control.
B. Cells were infected with FPV at a moi = 0.01 in the absence or presence of either ASA (7 mM) or the specific NF-κB inhibitor BAY 11–7085 (10 μM) alone or in combination. Eight hours p.i. supernatants were assayed to determine virus titers as described. $^*P < 0.0001$ control (infected, DMSO treated) versus infected and ASA, BAY or ASA/BAY treated as calculated by Student's *t*-test.

Acetylsalicylic acid blocks virus-induced expression of TRAIL and FasL as well as subsequent caspase activation and apoptosis induction

In our recent studies we showed that NF-κB regulates the expression of TRAIL and Fas/FasL, which in turn activate caspases that subsequently regulate viral RNP export (Wurzer *et al*., 2003; 2004). This has been identified as one chain of events by which NF-κB regulates influenza virus propagation in cell culture. If ASA acts via inhibition of NF-κB the compound should block all the different steps of this proposed mechanism. Indeed we observed that ASA blocks virus-induced expression of FasL and TRAIL (Fig. 3A). Impaired expression of these proapoptotic factors was also achieved by virus-inhibiting concentrations of PDTC or MG132 but not by the COX-2 inhibitor indometacin (data not shown). In accordance with the inhibition of proapoptotic factors we also found that ASA blocks virus-induced cleavage of a major caspase substrate, poly-(ADP-ribose)-polymerase (PARP) (Fig. 3B), as well as the activating cleavage of caspase 3 (Fig. 3C). This results in a decreased onset of apoptosis in the presence of ASA as indicated by reduced levels of hypodiploid apoptotic nuclei measured in Nicoletti assays (Fig. 3D).

Acetylsalicylic acid results in retention of viral RNP complexes in the nucleus of the infected cell

Earlier findings demonstrated that inhibition of caspases, especially caspase 3, resulted in efficient retention of influenza virus RNP complexes in the nucleus of the infected cell without affecting accumulation of viral proteins (Wurzer *et al*., 2003). The same was observed when virus-inhibiting concentrations of ASA were applied

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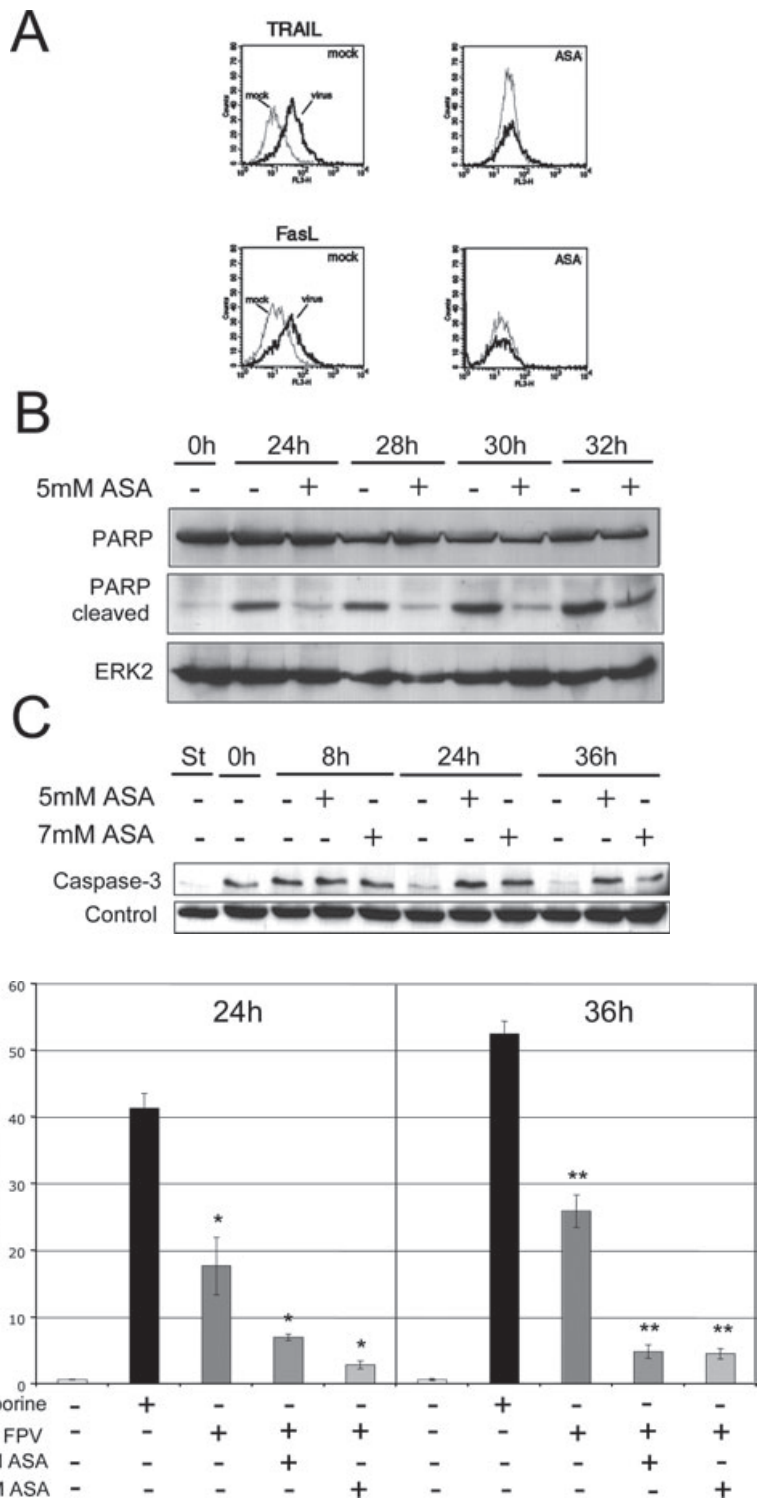

Fig. 3. ASA treatment results in block of virus-induced expression of TRAIL and FasL as well as subsequent inhibition of caspase activation.
A. A549 cells were infected with FPV (moi = 0.1) and incubated with ASA (5 mM). Approximately 24 h p.i. cells were fixed with 4% paraformaldehyde and stained for surface expression of TRAIL and CD95L. Subsequently FACS analysis was performed.
B and C. A549 cells were infected with FPV (moi = 0.01) in the absence or presence of either 5 mM or 7 mM ASA and lysed after different time points as indicated. Cell lysates were subjected to Western blot analyses with an anti-PARP antiserum (B) or an antiserum to the uncleaved form of caspase 3 (C). The apoptosis inducer staurosporine (St, 2.5 μM) was used as a positive control. Equal protein load was verified with an ERK2 blot (B) or an unspecific loading control (C).
D. A549 cells were infected in the presence and absence of 5 or 7 mM ASA. Apoptotic hypodiploid nuclei were measured in Nicoletti assays 24 and 36 h p.i. as described in *Experimental procedures*. Staurosporine (0.5 μM) was used as a positive control. *P*-values were calculated from six independent experiments to be $*P = 0.0062$ or $**P = 0.0001$, control (infected) versus infected and ASA-treated, respectively, as assessed by Student's *t*-test.

(Fig. 4). Accumulation of the viral matrix protein (M1), the non-structural protein (NS1), the viral nucleoprotein (NP) and the PB1 polymerase (PB1) was not significantly affected in the presence of ASA (Fig. 4A). However, immunofluorescence stainings of the viral NP, the major constituent of the RNP complexes, show that RNP export is efficiently impaired in the presence of the drug (Fig. 4B). This indicates that the antiviral action of ASA indeed involves the pathway proposed earlier to inhibit viral RNP export and subsequent virus multiplication.

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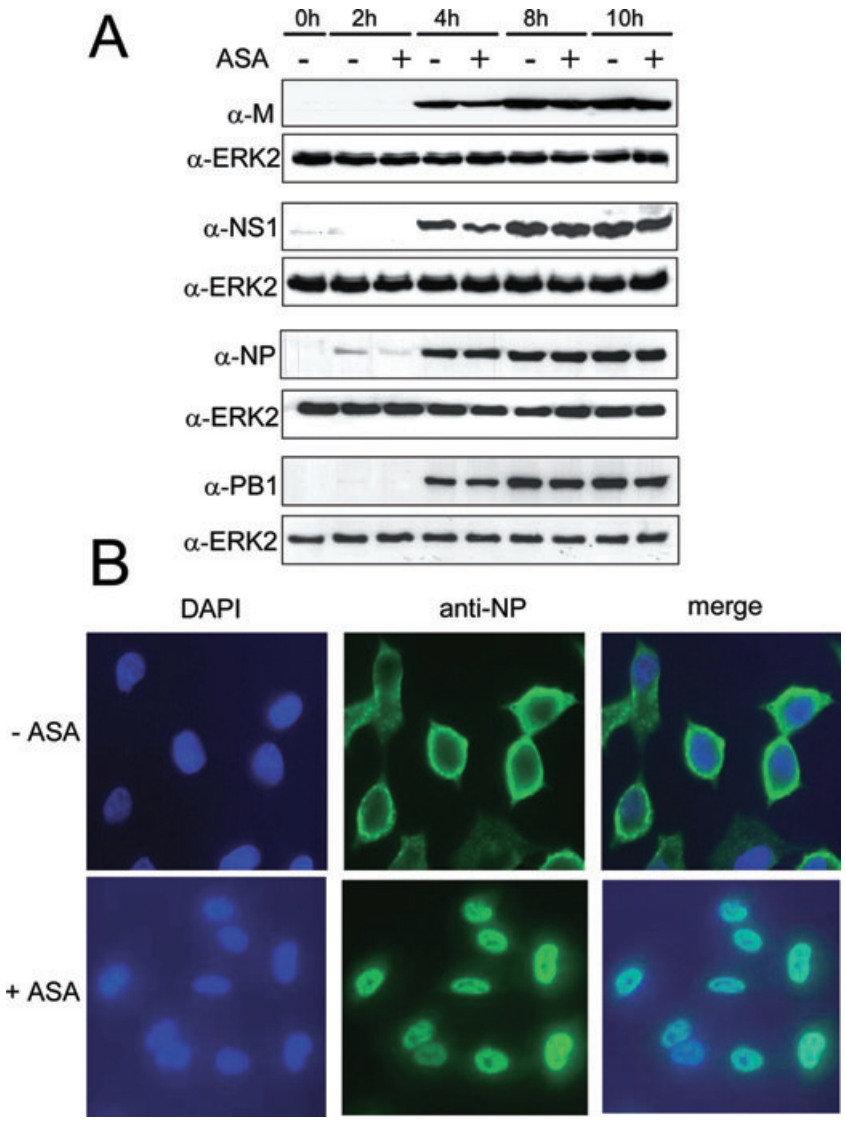

Fig. 4. ASA does not affect accumulation of viral proteins but results in nuclear retention of viral RNP complexes in the nucleus of infected cells.
A. A549 cells were infected with FPV (moi = 5) in the absence or presence of 5 mM ASA and lysed after different time points as indicated. Cell lysates were subjected to Western blot analyses with antisera against the viral M1, NS1, NP and PB1 proteins. Equal protein load was verified with an ERK2 blot.
B. A549 cells were infected with FPV (moi = 10) in the absence or presence of 5 mM ASA. Four hours p.i. cells were subjected to immunofluorescence analyses using an anti-NP antiserum to stain for viral RNP complexes. Cell nuclei were counterstained with DAPI.

Antiviral acting concentrations of ASA are not toxic and show no tendency to induce resistant virus variants

Anti-NF-κB as well as anti-influenza viral activity of ASA is observed in relatively high concentrations of the drug. This raises the concern of toxic side-effects. A first indication that ASA might not be toxic for the cells in the concentrations used came from an experiment in which the morphologies of infected cells treated or not treated with ASA were compared. While at 24 h post infection (p.i.) a significant cytopathic effect of virus-infected and untreated cells is detectable, in the presence of ASA the morphology of infected A549 cells was indistinguishable from uninfected cells (Fig. 5A). The same was observed in other cells, such as Madin–Darby canine kidney (MDCK) cells (data not shown). Thus, not even a double-hit of viral infection and drug treatment resulted in an evident toxicity of ASA. In accordance with this finding the compound had no significant effect on cell viability of MDCK or A549 cells during an observation period of 48 h as determined in a cell staining procedure with propidium iodide (data not shown). Finally, ASA showed no significant effect in MTT cell proliferation assays, indicating that in healthy cells the drug has no negative impact on cell viability, cell proliferation or cell activation in the concentrations used (Fig. 5B).

The fact that only a few anti-influenza drugs are in clinical use so far is in part due to the high variability of the virus. Thus, resistant virus variants can emerge quite rapidly, especially if a viral protein is directly targeted by a drug. This is very evident for the drug amantadine that blocks the viral ion channel protein M2 and induces resistance within a few passages (Hay *et al*., 1985). However, drug-resistant variants have also been observed with the novel neuraminidase inhibitors, such as oseltamivir and

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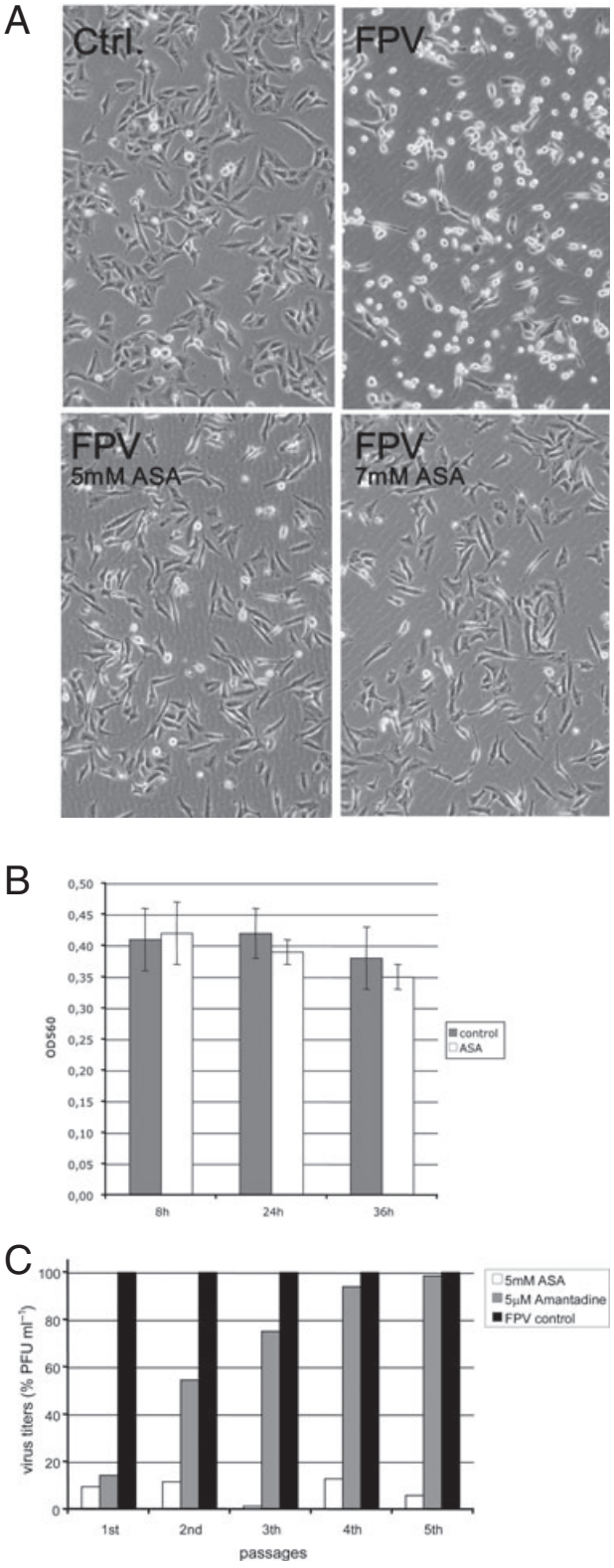

Fig. 5. Antiviral acting concentrations of ASA are not toxic for A549 or MDCK cells and do not show the tendency to induce resistant virus variants.
A. A549 cells were treated with either 5 mM or 7 mM ASA, subsequently infected with FPV (moi = 0.01) as indicated and were examined morphologically under the light microscope 24 h p.i.
B. A549 cells were incubated with 5 mM aspirin for 8 h, 24 h and 36 h. Thereafter cells were washed in PBS and incubated in 5 mg ml^{-1} MTT for 30 min at 37°C. Subsequently DMSO was added and cell proliferation/viability was estimated via a colorimetric assay as described in *Experimental procedures*.
C. MDCK cells were infected with FPV (moi = 0.001) in the absence (black bars) or presence of either ASA (5 mM, white bars) or amantadine (5 μM, grey bars). Twenty-four hours p.i supernatants were used to infect fresh cells under the same conditions. This procedure was repeated three further times. After each passage virus titers were determined in plaque assays. Titers are shown in percentage to the untreated control as described previously (Ludwig *et al*., 2004).

might arise more frequently than previously expected, especially in children (Kiso *et al*., 2004). To address whether ASA, an inhibitor of a cellular process needed for virus propagation, has a tendency to induce resistant variants, we analysed the compound in an established multipassaging experiment (Scholtissek and Muller, 1991; Ludwig *et al*., 2004). While in the presence of amantadine viral titers steadily increased from passage to passage due to selection of resistant variants, ASA does not show any similar effects (Fig. 5C). The drug still efficiently blocked virus propagation after the fifth passage when viral replication capacity in amantadine treated cells was fully back to the wild-type situation. This indicates that the virus cannot easily adapt to the missing cellular function.

Acetylsalicylic acid acts as an anti-influenza virus drug in vivo

According to the data presented so far, ASA action involves impaired NF-κB-dependent transcription of proapoptotic factors, the subsequent inhibition of caspase activity and the resulting block of RNP export. This is relevant in cell culture but may be different *in vivo* where apoptotic cells are rapidly eliminated by the immune system. Thus, we tested the antiviral activity of ASA *in vivo* in mice infected with a lethal dose of a mouse-adapted highly pathogenic avian influenza virus (HPAIV) strain FPV (H7N7). This model strain kills chicken and mice very rapidly. With regard to cleavability of its haemagglutinin as a major determinant of pathogenicity this strain belongs to HPAIV, a group also including the H5N1 'bird-flu' viruses observed in Asia in the last couple of years. To ensure that the drug acts locally in the primary infected tissue we applied ASA directly into the mouse lung as an aerosol by tracheal intubation. In these experiments up to 60% of the treated animals survived a lethal virus dose (10x LD_{50}) (Fig. 6A). This beneficial effect appears to be due to a direct targeting of viral multiplication rather than an

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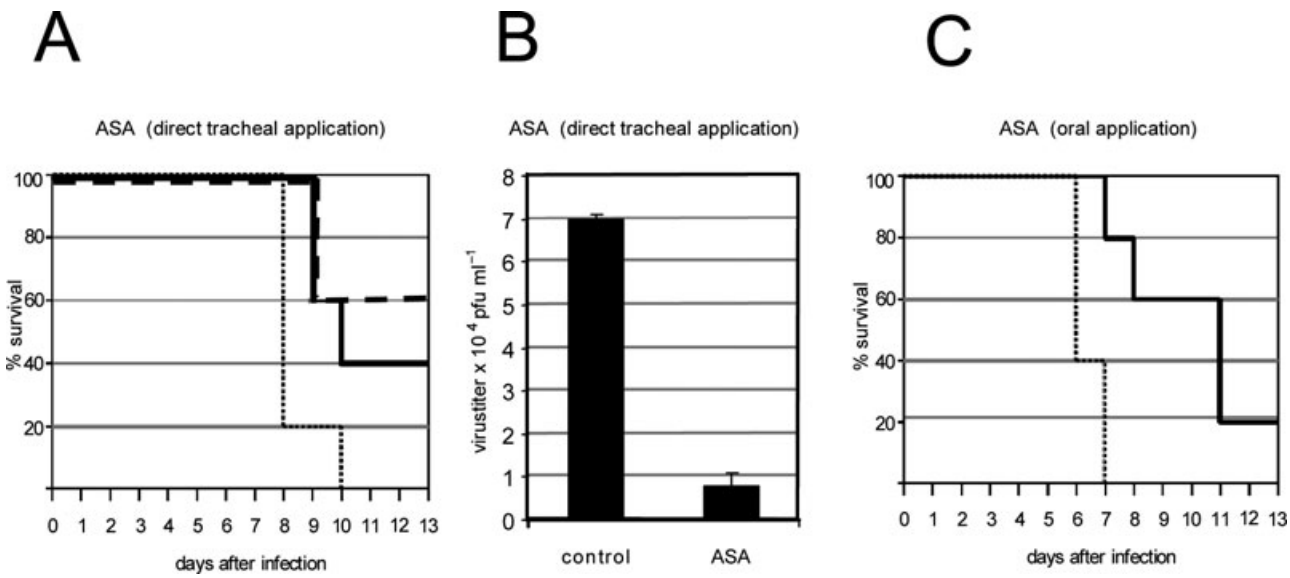

Fig. 6. ASA acts as an anti-influenza drug *in vivo* in lethally infected mice.
A. Survival curve of FPV-infected mice (5×10^3 pfu) that were treated by intubation directly into the trachea with either aerosolic PBS (control, $n = 5$, dotted line) or aerosolic ASA from a 2 mM ($n = 5$, bold line) or 20 mM stock solution ($n = 5$, dashed line) ($P = 0.05$).
B. Mice were infected and treated as described in A with aerosolic ASA in a 10 mM stock solution. Twenty-four hours p.i. mice were sacrificed and virus titers were determined in lung tissue by common plaque assays.
C. Survival curve of FPV-infected mice (10^4 pfu) that were treated with either ASA ($n = 5$, solid line) or PBS (control, $n = 5$, dotted line) orally as described in *Experimental procedures*.

indirect effect on the immune response, as a reduction of virus titers in the lung of infected animals was observed at a time-point (24 h p.i.) when adaptive immune responses have not been established (Fig. 6B). Interestingly, the drug also showed significant effects when applied systemically via the drinking water. A significant delay of death was observed and 20% of the mice survived (Fig. 6C). Finally, survival rates were significantly increased when ASA and other NF-κB-inhibiting agents such as PDTC or MG132 were applied as an aerosol by cage inhalation, while indometacin showed no effect (data not shown). Thus, the antiviral effect of pharmacological inhibitors of NF-κB, including ASA, in cell culture can also be observed *in vivo*, indicating that the proviral action of NF-κB is also evident during a genuine influenza virus infection of an animal. Although the results would favour a direct aerosolic application into the lung, beneficial effects of ASA could also be observed when applying the drug systemically by oral treatment.

Discussion

The recent emergence in South-east Asia of highly pathogenic avian strains that infected and killed humans raises the concern of a new influenza pandemic in near future. In such an event the development and production of an efficient vaccine is expected to require several months and it is unlikely that there will be sufficient supplies of the currently available neuraminidase inhibitors for all patients. Thus, considering that more than 25% of the world's population could be affected by a future pandemic there is an urgent need of amply available and cost-effective antiviral drugs to treat and control such an outbreak in industrialized and developing countries.

On the basis of earlier findings that NF-κB is required for efficient influenza virus propagation we have identified a mechanism by which the IKK2 inhibitor ASA acts on viral propagation. Consistent with earlier data obtained upon expression of dominant-negative mutants (Wurzer *et al.*, 2004) the compound blocked synthesis of proapoptotic factors, such as TRAIL and FasL and subsequently reduced onset of viral caspase activation. ASA did not significantly affect viral protein synthesis, which indicates that it does not interfere with early steps of the replication cycle due to unspecific toxicity. The compound rather appeared to specifically impair export of the viral RNP complexes. Although the mechanism by which TRAIL and FasL-induced caspase activation may promote RNP export is still elusive, there are indications that caspases result in an increase of the diffusion limit of nuclear pores (Faleiro and Lazebnik, 2000). This would allow passive diffusion of larger protein complexes from the nucleus. Involvement of such a passive mechanism is supported by the observation that caspase-mediated RNP export could not be blocked by an inhibitor of the active transport machinery, leptomycin B (Wurzer *et al.*, 2003).

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We cannot fully rule out that still other NF-κB-dependent mechanisms may be involved in the observed effects. Recently it has been shown that in cells lacking the NF-κB factors p50 and p65, levels of IFN responsive genes increase upon stimulation with the cytokine, indicating that NF-κB negatively interferes with the type I IFN response (Wei *et al*., 2006). However, we could not confirm these results in the context of an influenza virus-infected cell. In a global gene expression analysis of virus-infected cells NF-κB inhibition resulted in decreased rather than increased levels of IFN-induced genes (M. Schmolke, D. Viemann, J. Roth and S. Ludwig, unpubl. data). Thus the mechanism proposed by Wei *et al*. (2006) may not play a prominent role in the block of virus replication by ASA.

Acetylsalicylic acid was suspected to inhibit influenza virus production quite a while ago (Huang and Dietsch, 1988) but our study only now identified some of the molecular mediators of this inhibition and confirmed the antiviral action *in vivo*. This is a further example of a blocker of cellular signalling that acts antiviral (Pleschka *et al*., 2001; Ludwig *et al*., 2004; Ludwig, 2007). These data demonstrate that it is in principle possible to fight virus infections by targeting host-cell functions without severe side-effects. The advantages of ASA or salicylates as anti-influenza agents further include that the drugs are widely accepted and show no tendency to induce resistant virus variants, a very crucial aspect for an anti-influenza viral compound.

Unfortunately, the anti-NF-κB and anti-influenza virus action of ASA in cell culture only occurs in a millimolar concentration range. We have shown that these concentrations do not affect the viability of cultured cells, which is not a big surprise because aspirin as a drug usually gets taken up orally in water at concentrations of roughly 10 mM without side-effects. However, it is unlikely that serum concentrations equivalent to the cell culture conditions used here can be achieved by oral administration. Nevertheless, it is striking that beneficial antiviral effects were even observed when ASA was administered to the drinking water of infected mice. This may indicate that *in vivo* lower amounts of the drug might be effective compared to the cell culture conditions that are optimized for virus growth. However, according to the data presented a local administration of the drug as an aerosol into the lung, the primary infected tissue, would be the application of choice. The advantages of this application route would be that higher concentrations of the compound could be delivered directly to infected cells in the respiratory tract and that possible risks of systemic side-effects are minimized. Indeed, results from clinical studies show that patients could be treated with doses of 720 mg of inhaled salicylates twice a day for several weeks without significant side-effects (Bianco *et al*., 1995).

Besides the direct antiviral effect of ASA an infected patient may further benefit from the analgetic and anti-inflammatory properties of the compound that makes it already today a preferred drug to treat flu-like symptoms in general. It may not be ruled out that the anti-inflammatory action also contributes mechanistically to the beneficial effect observed in the mice *in vivo*.

Finally, ASA, as an inhibitor of NF-κB, may also prevent the overshooting induction of NF-κB-regulated cytokines (cytokine storm), a process suspected to contribute to the high pathogenicity of H5N1 avian influenza viruses (Cheung *et al*., 2002).

One point to consider would be that ASA and salicylates may have some rare side-effects. The long-term systemic use of salicylates during influenza infections appears to be statistically linked to the incidence of Reye syndrome in children, an encephalitis-like illness that is not well understood (Davis *et al*., 1985; Larsen, 1997). Thus, in these groups one would have to balance the potential antiviral benefit with the risks of such a treatment.

Nevertheless, given the fact that aerosolic forms of salicylates already successfully passed clinical trials (Bianco *et al*., 1995) it may be worthwhile to test these compounds in the clinics at least as kind of an emergency drug for a severe or pandemic influenza outbreak.

Experimental procedures

Viruses, cell lines and viral infections

Avian influenza virus A/Bratislava/79 (H7N7; FPV) and the human prototype strain A/Puerto-Rico/8/34 (H1N1) were taken from the virus strain collection of the Institute of Virology, Giessen. The human H5N1 strain A/Thailand/1(KAN-1)/2004 (H5N1) was isolated at the Siriraj Hospital, Mahidol University, Bangkok, Thailand. MDCK cells were grown in minimal-essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. A549 human lung carcinoma cells were grown in Ham's F12 medium supplemented with 10% heat-inactivated FBS and antibiotics. For infection cells were washed with PBS and subsequently incubated with virus at the indicated multiplicities of infection (moi) diluted in PBS/BA (PBS supplemented with 0,2% BSA, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin) for 30 min at 37°C. For analysis of events within the first replication cycle, a moi > 1 was used to ensure simultaneous infection of all cells. Low moi (< 1) were applied when effects during multicycle replication should be monitored. After the 30 min incubation period the inoculum was aspirated and cells were incubated with either MEM or Ham's F12 containing 0.2% BSA, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. At the given time points supernatants were collected to assess the number of infectious particles (plaque titers) in the samples. Briefly, MDCK cells grown 90% confluent in 6 well dishes were washed with PBS and infected with serial dilutions of the supernatants in PBS/BA for 30 min at 37°C. The inoculum was aspirated and cells were incubated with 2 ml MEM/BA (medium containing 0.2% BSA and supplements) supplemented with 0.6%

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Agar (Oxoid), 0.3% DEAE-Dextran (Pharmacia Biotech) and 1.5% $NaHCO_3$ at 37°C, 5% CO_2 for 2–3 days. Virus plaques were visualized by staining with neutral-red.

Plasmids, transfections and Western blots

The 3xNF-κB reporter plasmid was described previously (Flory *et al*., 2000). MDCK cells were transfected with Lipofectamine 2000 (LifeTechnologies) according to a protocol by Basler *et al*. (2000). Luciferase-reporter gene assays were carried out as described earlier (Ludwig *et al*., 2001; 2002). For Western blots cells were lysed in Triton lysis buffer (20 mM Tris/HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 50 mM sodium glycerophosphate, 20 mM sodium pyrophosphate, 5 μg ml^{-1} leupeptin, 1 mM sodium vanadate, 5 mM benzamindine) on ice for 30 min. Cell lysates were then centrifuged and protein contents in supernatants were estimated employing a protein dye reagent (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose membranes.

Inhibitors, antibodies and reagents

Acetylsalicylic acid, PDTC, indometacin and MG132 were obtained from Sigma-Aldrich or Roth and used in the concentrations indicated. The BAY 11-7085 inhibitor (Calbiochem) was freshly dissolved in DMSO at a 40 mM stock concentration. Staurosporine was purchased from Sigma-Aldrich. The anti-PARP-1 monoclonal antibody or the caspase 3 antiserum were purchased from BD Transduction Laboratories or R&D Systems respectively. A goat antiserum against the influenza virus protein NP was kindly provided by Robert G. Webster, Memphis, TN. Antisera against the influenza virus proteins NS1 and PB1 were purchased from Santa Cruz. Mouse monoclonal antibodies against the viral NP and M1 proteins were purchased from Serotec. All sera were used at a 1:1000 dilution, except the caspase 3 antiserum used at 1:10 000. To determine activity of the three MAPKs, activation state-specific antibodies against phosphorylated/activated forms of JNK (BD Transduction Laboratories), ERK or p38 (Cell signalling technologies) were used. Antisera against JNK1, ERK2 or p38 for loading controls as well as an antiserum against IκBα were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against TRAIL and Fas/CD95L for flow cytometry analysis are available from Alexis Biochemicals, Grünberg, Germany.

Flow cytometry analysis

TRAIL and FasL were detected by an intracellular staining procedure. A549 cell lines were infected with FPV at a moi of 0.1 for approximately 24 h in the presence of 2 μM monensin to avoid protein secretion. Cells were fixed with 4% paraformaldehyde at 4°C for 20 min and subsequently washed twice in permeabilization buffer (0.1% saponin/1% fetal calf serum/PBS). After incubation with a monoclonal antibody against TRAIL or isotype control antibodies (Becton Dickinson) cells were stained with biotin-Sp-conjugated goat anti-mouse IgG (Dianova) and streptavidine-Cy-chrome (Becton Dickinson). Fluorescence was determined in the FL3-channel using a FACScalibur cytometer (Becton Dickinson). For determination of late stages of apoptotic cell death, apoptotic hypodiploid nuclei were measured in FACS analysis (Nicoletti assay) (Nicoletti *et al*., 1991). Briefly, A549 cells were infected with FPV (moi = 0.01) and treated with 5 or 7 mM ASA or left untreated. As positive control A549 cells were treated with the apoptosis inducer staurosporine (0.5 mM). After 24 h or 36 h cells were harvested by trypsinization, lysed in a hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100) supplemented with 50 mg ml^{-1} propidium iodide, incubated for 4 h and subsequently analysed by FACS. All FACS analyses were repeated at least twice and revealed essentially similar results.

MTT cell proliferation assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of the mitochondrial succinic dehydrogenase enzyme from viable and proliferating cells to cleave the tetrazolium rings of the pale yellow MTT (Mosmann, 1983). This results in the formation of dark blue formazan that is largely impermeable to cell membranes thus resulting in its accumulation within healthy cells. The number of proliferating cells is directly proportional to the level of the formazan product created and can be measured in cell lysates in a colorimetric assay yielding an OD value at 560 nm. A549 cells were incubated with 5 mM ASA for 8 h, 24 h and 36 h. Thereafter cells were washed in PBS and incubated in 5 mg ml^{-1} MTT for 30 min at 37°C. Subsequently DMSO was added and the absorbance was measured at 570 nm.

Indirect immunofluorescence microscopy

A549 cells were grown on 15 mm glass plates. When 50% confluence was reached, cells were infected with FPV at a moi = 10. Thirty minutes p.i., the inoculum was aspirated and medium/BA supplemented with DMSO or inhibitors was added. Four hours p.i., cells were washed twice with PBS, then fixed for 30 min with 3.7% paraformaldehyde (in PBS) at room temperature. After washing, cells were permeabilized with acetone, washed with PBS and blocked with 10% FBS in PBS for 20 min at 37°C. After blocking, cells were incubated with goat antiserum against the viral NP (1:400) in PBS for 30 min. After further washes, cells were incubated with FITC-labelled donkey anti-goat IgG (1:300) in PBS for 30 min. Finally, cells were washed and mounted with Vectashield mounting medium with DAPI. Fluorescence was visualized using a Zeiss Axiovert 135 fluorescence microscope.

In vivo *treatment of mice*

C57Bl/6 mice with the age of 10 weeks were purchased from the breeding facilities of the FLI, Tübingen. For cage inhalation, mice were treated with either 2 ml aerosol of 50 mM aspirin (ASA, Sigma), 100 mM PDTC (Sigma), 2 μM Indometacin (Sigma) or 1 mM MG132 (Sigma) in a cage inhalation unit. Treatment was performed daily starting 1 h prior to infection. Mice were infected with 5×10^3 or 10^4 pfu FPV in 20 μl intranasally. To control the general health status the animals were weighted daily.

Application direct into the lung was performed by a mouse Minivent (Hugo Sachs Elektronik-Harvard Apparatus) connected

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to a Nebulizer (Hugo Sachs Elektronik-Harvard Apparatus). At the day of infection delivery was performed via flexible tubes into the throat of mice. At the three following days an endotracheal tube was inserted under microscopic control to provide aerosol delivery into the lungs of mice (Minivent settings: Strokes/min: 120; Stroke volume: 150 µl). Treatment was performed for about 4 min 30 s to allow a delivery of about 600 µl of 2 mM, 10 mM or 20 mM ASA and control substances (PBS) in aerosolic form. For oral application 500 µl of 50 mM ASA was injected intraperitoneal 30 min prior to FPV infection and ASA in a concentration of 50 mM was applied daily via the water bottle until day 6 p.i. Water was changed daily.

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References

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413:** 732–738.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S., and Randall, R.E. (2004) The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci USA* **101:** 17264–17269.

Basler, C.F., Wang, X., Muhlberger, E., Volchkov, V., Paragas, J., Klenk, H.D., *et al.* (2000) The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proc Natl Acad Sci USA* **97:** 12289–12294.

Bianco, S., Vaghi, A., Robuschi, M., Refini, R.M., Pieroni, M.G., and Sestini, P. (1995) Steroid-sparing effect of inhaled lysine acetylsalicylate and furosemide in high-dose beclomethasone-dependent asthma. *J Allergy Clin Immunol* **95:** 937–943.

Cheung, C.Y., Poon, L.L., Lau, A.S., Luk, W., Lau, Y.L., Shortridge, K.F., *et al.* (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.

Chu, W.M., Ostertag, D., Li, Z.W., Chang, L., Chen, Y., Hu, Y., *et al.* (1999) JNK2 and IKKbeta are required for activating the innate response to viral infection. *Immunity* **11:** 721–731.

Davis, L.E., Green, C.L., and Wallace, J.M. (1985) Influenza B virus model of Reye's syndrome in mice: the effect of aspirin. *Ann Neurol* **18:** 556–559.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303:** 1529–1531.

Faleiro, L., and Lazebnik, Y. (2000) Caspases disrupt the nuclear–cytoplasmic barrier. *J Cell Biol* **151:** 951–959.

Fiedler, M.A., Wernke-Dollries, K., and Stark, J.M. (1998) Inhibition of TNF-alpha-induced NF-kappaB activation and IL-8 release in A549 cells with the proteasome inhibitor MG-132. *Am J Respir Cell Mol Biol* **19:** 259–268.

Flory, E., Kunz, M., Scheller, C., Jassoy, C., Stauber, R., Rapp, U.R., and Ludwig, S. (2000) Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IkappaB kinase. *J Biol Chem* **275:** 8307–8314.

Grilli, M., Pizzi, M., Memorandum, M., and Spano, P. (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. *Science* **274:** 1383–1385.

Hay, A.J., Wolstenholme, A.J., Skehel, J.J., and Smith, M.H. (1985) The molecular basis of the specific anti-influenza action of amantadine. *EMBO J* **4:** 3021–3024.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., *et al.* (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303:** 1526–1529.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., *et al.* (2006) 5′-Triphosphate RNA is the ligand for RIG-I. *Science* **314:** 994–997.

Huang, R.T., and Dietsch, E. (1988) Anti-influenza viral activity of aspirin in cell culture. *N Engl J Med* **319:** 797.

Karin, M. (1999a) The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. *J Biol Chem* **274:** 27339–27342.

Karin, M. (1999b) How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene* **18:** 6867–6874.

Karin, M., Yamamoto, Y., and Wang, Q.M. (2004) The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov* **3:** 17–26.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., *et al.* (2005) Cell type-specific involvement of RIG-I in antiviral response. *Immunity* **23:** 19–28.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., *et al.* (2004) Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet* **364:** 759–765.

Kopp, E., and Ghosh, S. (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265:** 956–959.

Kwak, Y.T., Guo, J., Shen, J., and Gaynor, R.B. (2000) Analysis of domains in the IKKalpha and IKKbeta proteins that regulate their kinase activity. *J Biol Chem* **275:** 14752–14759.

Larsen, S.U. (1997) Reye's syndrome. *Med Sci Law* **37:** 235–241.

Ludwig, S. (2007) Exploited defense: how influenza viruses take advantage of antiviral signaling responses. *Future Virol* **2:** 91–100.

Ludwig, S., Ehrhardt, C., Neumeier, E.R., Kracht, M., Rapp, U.R., and Pleschka, S. (2001) Influenza virus-induced AP-1-dependent gene expression requires activation of the JNK signaling pathway. *J Biol Chem* **276:** 10990–10998.

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Ludwig, S., Wang, X., Ehrhardt, C., Zheng, H., Donelan, N., Planz, O., *et al.* (2002) The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors. *J Virol* **76:** 11166–11171.

Ludwig, S., Planz, O., Pleschka, S., and Wolff, T. (2003) Influenza virus induced signaling pathways – targets for antiviral therapy? *Trends Mol Med* **9:** 46–51.

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

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., *et al.* (2004) Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci USA* **101:** 5598–5603.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:** 55–63.

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., and Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139:** 271–279.

Nimmerjahn, F., Dudziak, D., Dirmeier, U., Hobom, G., Riedel, A., Schlee, M., *et al.* (2004) Active NF-kappaB signalling is a prerequisite for influenza virus infection. *J Gen Virol* **85:** 2347–2356.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., and Reis, E.S.C. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′ phosphates. *Science* **314:** 997–1001.

Pierce, J.W., Schoenleber, R., Jesmok, G., Best, J., Moore, S.A., Collins, T., and Gerritsen, M.E. (1997) Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* **272:** 21096–21103.

Piette, J., Piret, B., Bonizzi, G., Schoonbroodt, S., Merville, M.P., Legrand-Poels, S., and Bours, V. (1997) Multiple redox regulation in NF-kappaB transcription factor activation. *Biol Chem* **378:** 1237–1245.

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

Scholtissek, C., and Muller, K. (1991) Failure to obtain drug-resistant variants of influenza virus after treatment with inhibiting doses of 3-deazaadenosine and H7. *Arch Virol* **119:** 111–118.

Tegeder, I., Pfeilschifter, J., and Geisslinger, G. (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J* **15:** 2057–2072.

Uchide, N., Ohyama, K., Bessho, T., Yuan, B., and Yamakawa, T. (2002) Effect of antioxidants on apoptosis induced by influenza virus infection: inhibition of viral gene replication and transcription with pyrrolidine dithiocarbamate. *Antiviral Res* **56:** 207–217.

Wei, L., Sandbulte, M.R., Thomas, P.G., Webby, R.J., Homayouni, R., and Pfeffer, L.M. (2006) NFkappaB negatively regulates interferon-induced gene expression and anti-influenza activity. *J Biol Chem* **281:** 11678–11684.

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

Wurzer, W.J., Ehrhardt, C., Pleschka, S., Berberich-Siebelt, F., Wolff, T., Walczak, H., *et al.* (2004) NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. *J Biol Chem* **279:** 30931–30937.

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

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