Journal of
Antivirals & Antiretrovirals

Müller et al., J Antivir Antiretrovir 2016, 8:4
DOI:10.4172/jaa.1000151

Research Article Open Access

D, L-lysine acetylsalicylate + glycine Impairs Coronavirus Replication

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Received date: December 05, 2016, **Accepted date:** December 20, 2016, **Published date:** December 30, 2016

Abstract

Coronaviruses (CoV) belong to the large family *Coronaviridae* within the order of *Nidovirales*. Among them, several human pathogenic strains (HCoV) are known to mainly cause respiratory diseases. While most strains contribute to common cold-like illnesses others lead to severe infections. Most prominent representatives are SARS-CoV and MERS-CoV, which can lead to fatal infections with around 10% and 39% mortality, respectively. This resulted in 8098 casualties in the 2002/2003 SARS-CoV outbreak and in 1806 documented human infections (September 2016) during the recent on-going MERS-CoV outbreak in Saudi Arabia. Currently patients receive treatment focusing on the symptoms connected to the disease rather than addressing the virus as the cause. Therefore, additional treatment options are urgently needed which would ideally be widely available and show a broad affectivity against different human CoVs. Here we show that D, L-lysine acetylsalicylate + glycine sold as "Asprin i.v. 500mg®" (LASAG), which is an approved drug *inter alia* in the treatment of acute pain, migraine and fever, impairs propagation of different CoV including the highly-pathogenic MERS-CoV *in vitro*. We demonstrate that the LASAG-dependent impact on virus-induced NF-κB activity coincides with (i) reduced viral titres, (ii) decreased viral protein accumulation and viral RNA synthesis and (iii) impaired formation of viral replication transcription complexes.

Keywords: Coronavirus replication; D, L-lysine acetylsalicylate +glycine; NF-κB inhibition; Replication/Transcription Complexes

Introduction

Coronaviruses (CoV) are enveloped, positive-strand RNA viruses belonging to the family of *Coronaviridae* [1,2] and are known to infect mammals and birds. Prior to 2003 it was believed that circulating human coronaviruses (HCoV), such as HCoV-OC43 [3,4] and HCoV-229E [5], cause low-pathogenic infections of the upper respiratory tract and were not recognized as a significant threat to human health. This changed, after the emerging Severe Acute Respiratory Syndrome-Coronavirus (SARS-CoV) caused an epidemic outbreak in 2002 in Asia, Canada, the U.S. and Europe [6], which resulted in 8098 laboratory-confirmed cases, including 774 deaths (average mortality rate 10%) [6-9]. In 2013, a new human pathogenic CoV with zoonotic origin was discovered in Saudi Arabia: the Middle East Respiratory Syndrome coronavirus (MERS-CoV) causing SARS-like symptoms including lethal pneumonia [10] and renal dysfunction up to complete failure [11-13]. Since September 2012 1806 confirmed cases have been reported of whom 643 died [14].

Against both highly pathogenic emerging viruses no specific anti-viral treatment or vaccination is currently available and treatment of infected individuals presently only aims at relieving symptoms [15,16]. In cell culture, Type 1 Interferon (IFN-1) inhibits MERS-CoV as well as SARS-CoV replication [17-19]. IFN-1 is also used as potential treatment of other +ssRNA viruses [20], but beside high costs it shows massive side effects. Several other drugs were also shown to impair MERS-CoV replication in cell culture including cyclosporine, chloroquine, chlorpromazine, loperamide and lopinavir [21-23]. Whether these drugs will be useful in future treatment of HCoV infections remains to be investigated.

Targeting CoV proteins [24,25] is always prone to lead to the emergence of resistant variants/escape-mutants due to the high mutation rate of RNA viruses [26] including CoV [27]. As all viruses depend on their host cell for their replication, alternative anti-viral strategies target host functions or factors. This approach has a low potential to lead to viral resistance and provides the possibility of broad-spectrum anti-viral applications.

For many years acetylsalicylic acid (ASA) has been therapeutically used as an analgesic, anti-pyretic, anti-rheumatic and also as a non-steroidal anti-inflammatory drug (NSIAD). It can also slow down the progression of Alzheimer's disease and prevent colon cancer [28-30]. However, ASA is only soluble to a limited extent and the rate of bio-absorption is thus limited. By using salts of ASA with basic amino acids, the dissolution rate of the active compound itself can be increased and high blood concentrations of the active compound can be achieved [31]. D, L-lysine Acetylsalicylate (LASA) is the water soluble salt of ASA and the amino acid lysine and due to its improved solubility in water (compared to ASA) it is faster acting and can not only be applied orally, but also intravenously. A further disadvantage of the o-acetylsalicylate was their inadequate stability. The addition of glycine in the production process of D, L-lysine acetylsalicylated + glycine (LASAG) resulted in a stable active compound complex of salts of o-acetylsalicylic acid with basic amino acids and glycine [31].

ASA as well as other salicylates are able to block the activation of NF-κB [32,33] via inhibition of IKK2 in a low millimolar range [34]. The transcription factor NF-κB is essential for cell responses to infection with various pathogens. It controls expression of a variety of anti-viral cytokines and it regulates apoptotic gene expression. Therefore, it is considered to be a main mediator of the anti-viral cell response to viral infection [35]. Besides the wide usage of ASA in pain therapy, it was shown that ASA-mediated inhibition of the NF-κB-

dependent induction of TRAIL and Fas/FasL, reduces influenza virus propagation [36-38]. ASA also displays anti-viral activity against cytomegalovirus [39] and human rhinoviruses [40].

As SARS-CoV was shown to activate NF-κB in the lung tissue of infected mice and NF-κB-inhibition (using CAPE, resveratrol, BAY11-7082 or parthenolide) improved survival rates of SARS-CoV-infected mice [41], we speculated that NF-κB activity might be important for efficient CoV propagation. We therefore investigated whether NF-κB inhibition via the clinically tested and approved LASAG could negatively affect CoV replication. This hypothesis was analysed by LASAG treatment of cells infected with the low pathogenic human strain HCoV-229E as well as the high pathogenic MERS-CoV. Our results could demonstrate that LASAG interferes with virus-induced NF-κB activation, the formation of viral replication transcription complexes, viral RNA synthesis and viral protein accumulation resulting in an overall impaired CoV propagation.

Materials and Methods

Cells and viruses

Huh7 cells (human hepatocellular carcinoma cells) were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, UK) supplemented with 10% foetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin (P/S)).

Confluent cells were infected with human coronavirus 229E (HCoV-229E, strain collection of the Institute of Medical Virology, Giessen, Germany) and MERS-CoV (EMC/2012, kindly provided by Christian Drosten, Bonn, Germany) at the indicated multiplicity of infection (MOI). After 1h the inoculum was aspirated, and the cells were incubated with complete DMEM at 33°C (HCoV-229E) or 37°C (MERS-CoV).

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using the standard Ficoll-Paque gradient centrifugation and maintained in completed DMEM. Briefly, human blood, diluted 1:2 in PBS, was layered carefully on top of Ficoll-Paque PLUS solution (GE Healthcare Life Science; USA) and centrifuged for 30 min at room temperature (RT) at 400 x g without brake. Afterwards the PBMCs containing Plasma-Ficoll interface was collected, the cells were washed twice with PBS (for 10 min at 640 ×g followed by 10 min at 400 ×g) and resuspended in complete RPMI 1640 medium (Gibco Life Technologies, UK) with Glutamax (Gibco Life Technologies, UK) supplemented with P/S and 10% FCS and directly seeded in 24 or 96 well plates.

Inhibitors

D, L-lysine acetylsalicylate + glycine (LASAG, $C_{15}H_{22}N_2O_6$, 326.3 g/mol) was obtained as "Asprin i.v. 500mg®" (Bayer Vital GmbH, Germany) and dissolved in dH_2O to provide a stock concentration of 1 M. BAY11-7082 (Selleckchem, USA) was solved in DMSO at a stock concentration of 50 mM and stored at -20°C until further usage. Lornoxicam (Selleckchem, USA) was solved in DMSO for stock concentration of 20 mM.

Virus titration

Virus titers were determined by focus forming assay [42] as previously described [43]. Briefly, infected Huh7 cells in 96-well plates were fixed and permeabilized (4% paraformaldehyde (PFA, Roth, Germany), and 1 % Triton X-100 (Roth, Germany) in PBS^{++}) and kept at 4°C for 1h. Thereafter, the solution was discarded and cells were washed 3x with PBS^{++}/0.05% Tween-20 (Roth, Germany). Afterwards, cells were incubated with a mouse anti-coronavirus nucleoprotein mAb (Ingenasa, Spain) primary antibody diluted 1:1,000 in PBS containing 3% BSA (PAN Biotech, Germany) for 1h at RT. The cells were washed again and then incubated with a goat anti-mouse HRP-antibody (Santa Cruz, USA) secondary antibody diluted 1:1,000 in PBS containing 3% BSA, for 1h at RT. After additional washing the cells were incubated with 40 µl AEC (3-Amino-9-ethylcarbazole) staining solution (Santa Cruz, USA). Following incubation for 40 min at 37°C, the substrate solution was removed and cells were washed 2x with dH_2O to remove salts. To detect and quantify foci, the plates were scanned with a resolution of 1200 dpi using an Epson Perfection V500 Photo scanner (Epson, Japan) and analysed using Photoshop software (Adobe, USA). Results represent the averages from three biological replicates.

Cell viability (CC_{50})

To determine the median cytotoxic concentration of the compounds at which 50% of the cells are still viable (CC_{50}), Huh7 cells or PBMCs were grown in 96 well micro plates. Growth medium was replaced with DMEM containing different, indicated concentrations of LASAG solved in dH_20 or BAY11-7082 or Lornoxicam both solved in DMSO and were further incubated under conditions of infection for the indicated time periods. Subsequently, an MTT assay was performed as previously described [37].

To determine the CC_{50}, the MTT values were calculated in percentage (% viability = (100/MTT value of untreated sample) x MTT value of inhibitor treated sample) with the control set as 100 %.

Antiviral activity (EC_{50})

To determine the effective concentration at which virus titres are reduced by 50% (EC_{50}), Huh7 were infected with MOI 0.1 in 100 µl for 1h at 33°C (HCoV-229E) or 37°C (MERS-CoV). After removing the inoculum, cells were incubated directly with 500 µl complete DMEM containing different inhibitor concentrations. Samples of the supernatants were collected at the indicated time points post infection (p.i.) and the amount of infectious virus particles was determined by focus forming assay.

In order to define the EC_{50} the viral titre of the untreated virus-infected control was set at 100% and the titres of LASAG-, BAY11-7082- and Lornoxicam-treated samples were calculated in relation to it.

Western blot

Cells were infected in a time course experiment and treated with the indicated concentrations of LASAG or left untreated. After cell lysis, proteins were separated by 12% SDS-PAGE and blotted onto nitrocellulose membrane (Amersham, UK) as previously described [44]. Membranes were incubated with the respective primary antibody (mouse anti-nucleocapsid protein mAb [Ingenasa, Spain], mouse anti-phospho-IκB-α antibody (Cell Signaling, USA] or rabbit anti-actin

antibody [abcam, USA]) in PBS containing 3% BSA). After washing the membranes three times with TBS/T (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.05 % Tween 20), they were further incubated with Infrared IRDye-conjugated anti-mouse and anti-rabbit monoclonal secondary antibodies (Li-Cor, Germany) diluted 1:10,000 in PBS containing 3% BSA and analyzed via Li-Cor Odyssey (Li-Cor, Germany). Western Blots were further graphically analysed by measuring the intensity of the protein bands in regard to the loading control of four different experiments for nucleocapsid protein reduction or two independent experiments for IkB reduction.

Nothern blot

Huh7 cells were infected with HCoV-229E (MOI=3) and treated with LASAG (20, 10, 5 mM) or left untreated. Total RNA was isolated using Trizol (Invitrogen, Germany) 24 h p.i. and 5 µg total RNA was separated in a denaturing gel (1% agarose, 6 % formaldehyde, 1x MOPS) and transferred onto a positively charged nitrocellulose membrane via Vacuum Blot. Northern Blot analysis was done using a ^{32}P-labled probe specific for the HCoV-229E genome (nt 29297 to 27273) as described previously [45].

Immunofluorescence

Huh7 cells were infected with HCoV-229E (MOI=3) and treated with LASAG (20 mM) or left untreated. 24 h p.i. the cells were fixed with ice-cold methanol and stained with mouse anti-dsRNA mAb (J2, English & Scientific Consulting Bt., Hungary) and rabbit anti-HCoV-229E nsp8 mAb (both diluted 1:100 in PBS containing 3% BSA). For detection Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 F (ab') 2 fragment of goat anti-rabbit IgG (Invitrogen, USA) both diluted 1:500 in PBS containing 3% BSA were used. Images were acquired using a confocal laser-scanning microscope (Leica SP05 CLSM, Leica, Germany).

Analysis of NF-κB activation

NF-κB activation was measured as previously described [37] with the commercial available TransAM kit (Active motif, USA) according to the manufacturers instruction (n=4). In our study, HCoV-229E-infected (MOI=3) Huh7 cells that were either treated with LASAG (20 mM) or left untreated were lysed at 4 or 12 h p.i.. Relative NF-κB activation was calculated as fold induction compared to mock-infected control cells.

Temporal LASAG treatment

To determine the effect of LASAG-treatment on the early and the late phase of the CoV replication cycle, Huh7 cells were infected with HCoV-229E (MOI=3) and treated with LASAG (20 mM) either from 3-6 h p.i. or from 9 -12 h p.i.. For this the cells were incubated in regular media, which was replaced with LASAG-containing media for the indicated times. 12 h p.i. the supernatant was collected and pooled and the virus titer was determined via focus forming assay.

Statistical analysis

The results correspond to the mean ± SD of the indicated experiments. The statistical significance of differences between the indicated groups was tested using the unpaired, two-tailed Student's t-test with a threshold of p:*<0.05; **<0.005; and ***<0.0005.

Results

LASAG reduces HCoV-229E and MERS-CoV titres in cell culture as well as in primary human cells at non-toxic concentrations

Based on improved survival rates of SARS-CoV-infected mice, reduced lung pathology following NF-κB inhibition [41] and the fact, that ASA can block NF-κb activation [31,32] we aimed to elucidate whether LASAG (Figure 1) might have a negative effect on CoV propagation *in vitro*.

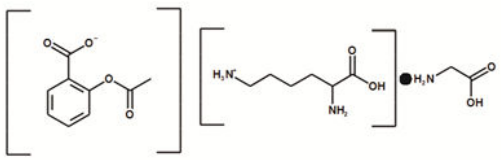

Figure 1: Structural formula of D,L-lysine acetylsalicylate glycine. Treatment was performed with commercial "Aspirin® i.v." containing the active compound acetylsalicylic acid (ASA). D,L-lysine acetylsalicylate glycine (LASAG) is a salt of ASA and the two amino acids glycine and lysine. After dissolving in water, LASAG dissociates readily into ASA and the two amino acids glycine and lysine.

In a first set of experiments the cell viability was investigated via MTT assay after 24 and 48 h incubation for Huh7 cells and after 24 h incubation for PBMCs, applying different amounts of LASAG. For this we determined the cytotoxic concentration 50 (CC_{50}) of LASAG for Huh7 cells, which are regularly used for CoV propagation [46,47] as 20.43 mM and 58.73 mM at 48 and 24 h, respectively (Figures 2A and 2B). Furthermore, we determined the CC_{50} of LASAG for human peripheral blood mononuclear cells (PBMC) at 37.95 mM at 24 h (Figure 2C). PBMCs can be infected with HCoV-229E [48] and served as a primary human cell system.

Next, we analysed whether LASAG affects the replication of the human strain HCoV-229E as well as of MERS-CoV. Therefor we treated HCoV-229E- and MERS-CoV-infected Huh7 cells (MOI=0.1) with LASAG at different concentrations and analysed the viral titres via focus forming assay at 48 h and 24 h post infection (p.i.). The results indicate an effective concentration 50 (EC_{50}) of 1.31 mM for HCoV-229E resulting in a selectivity index (SI: CC_{50}/IC_{50}) of 15.6 as well as an EC_{50} of 3.69 mM for MERS-CoV, leading to an SI of 15.9, similar to that of HCoV-229E (Figures 2D and 2E). The EC_{50} for HCoV-229E-infected PBMCs at 24 h was 6.71 mM resulting in an SI of 5.7 (Figure 2F).

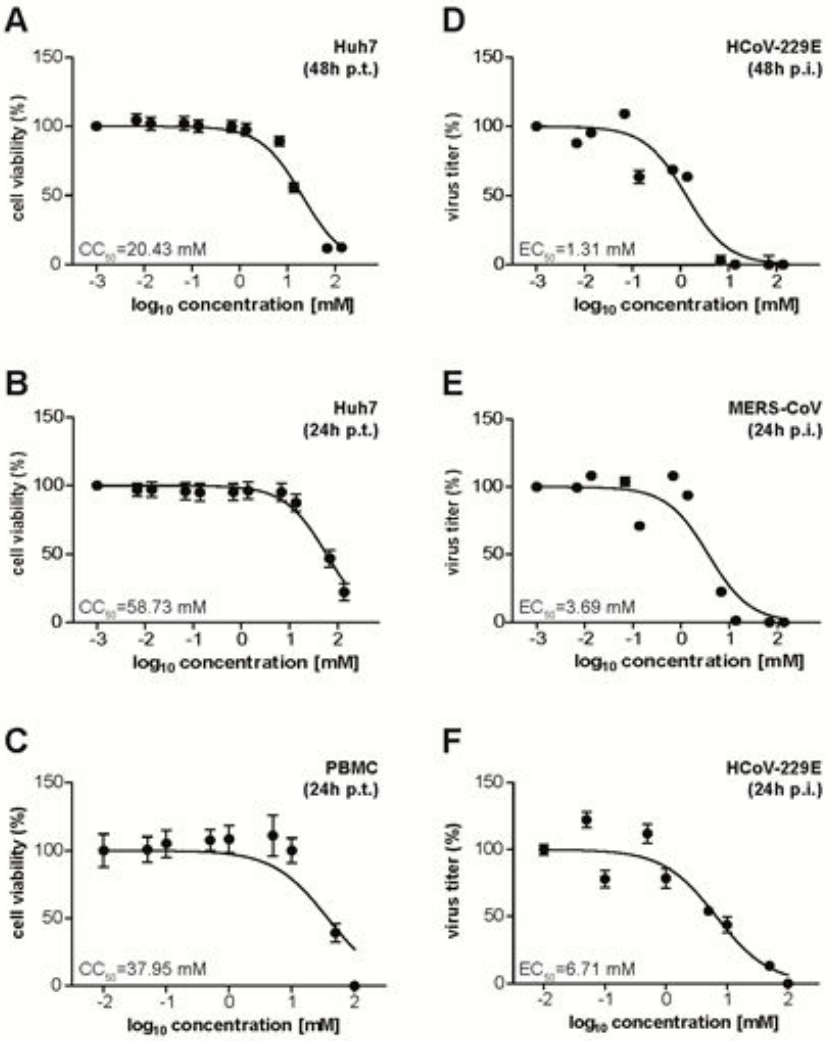

Figure 2: LASAG impairs coronaviral replication. To investigate the effect of LASAG on coronaviral replication we determined the CC_{50} (A, B, C) and the EC_{50} (D, E, F) of LASAG. Huh7 cells were treated with the indicated concentrations of LASAG for 48 h (A, D) and 24 h (B, E), and PBMC were treated for 24 h (C,F). EC_{50} concentrations were determined for HCoV-229E on Huh7 cells (D) and PBMC (F). For MERS-CoV the EC_{50} was determined after 24 h on sHuh7 cells (E).

These results indicate that LASAG can effectively reduce titres of HCoV-229E and MERS-CoV with similar SI values in Huh7 cells and, to a lesser extent, HCoV-229E titers in primary human PBMCs in a dose-dependent manner at non-toxic concentrations. Nevertheless, even though the SI values are distinct, increased SI values for both cell systems, would be advantageous for a therapeutic approach.

LASAG reduces viral protein accumulation and viral RNA synthesis of HCoV-229E

The reduction of viral titres following LASAG treatment led us to speculate that LASAG might impair the intra-cellular replication of CoV. In order to elucidate which step of the viral life cycle is affected by the treatment of LASAG, we first determined viral protein accumulation via Western Blot and also analysed viral RNA synthesis via Northern Blot of HCoV-229E infected Huh7 cells treated for 24 h p.i. at non-toxic LASAG concentrations of 20, 10 and 5 mM.

As shown in Figures 3A and 3B we found that treatment with 20 mM LASAG led to a strong reduction of the viral nucleocapsid (N) protein as well as viral RNA amount (Figure 3C). The effect on viral protein accumulation appears to be specific, as the quantity of cellular actin (loading control) was not affected.

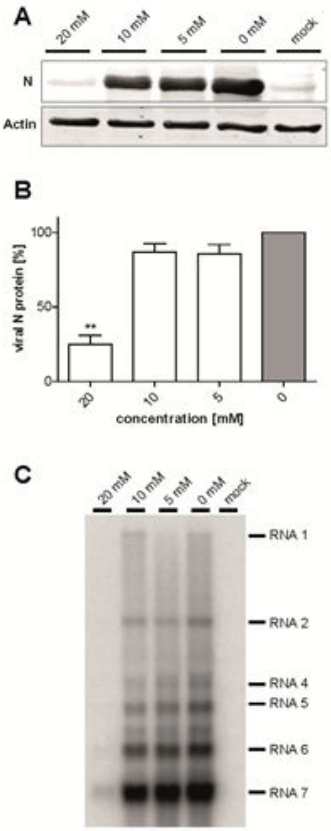

Figure 3: LASAG reduces coronaviral RNA and protein accumulation. Huh7 were infected with HCoV-229E and treated with the indicated LASAG concentrations. 24 h p.i. the amount of viral N protein and actin as loading control in infected and un-infected (mock) cells were detected by Western Blot using mouse anti-nucleocapsid protein mAb and rabbit anti-actin antibody (A) and quantified (B). The amount of viral RNAs was detected by Northern Blot analysis (C).

Compared to the strong reduction of the HCoV-229E titre (EC_{50}=1.31 mM) 48 h p.i. by LASAG the effects of 5 and 10 mM LASAG on viral protein/RNA production are less evident. Nevertheless, it should be considered that these assays (Western Blot/ Northern Blot) were performed 24 h p.i.. This was done, as the CC_{50} for LASAG at 48 h was 20.43 mM, whereas it was 58.73 mM for 24 h (Figure 2), indicating that a LASAG concentration of 20 mM is less toxic at 24 h. Therefore, the effect of the lower LASAG concentrations (5, 10 mM) on viral protein/RNA production might be less evident at this earlier time point. Also, the MOI to determine the EC_{50} was only 0.1, whereas the MOI to analyse viral protein/RNA production was 3. Therefore, the effect of the LASAG concentrations used to analyse the effect on viral protein/RNA production might be weaker. Furthermore, it should be considered that these assays are generally less sensitive than the direct virus titration used to determine the EC_{50}, which could also explain the discrepancy.

Taken together, the results indicate that the reduction in the viral titre observed under LASAG treatment could be related to the negative effect of LASAG on the viral protein and RNA production, which might therefore account for the reduction of infectious progeny virions produced.

LASAG treatment results in reduction of replication transcription complexes

The impaired viral protein accumulation and viral RNA synthesis led us to assume that the mode of action exerted by LASAG on CoV propagation impairs a step of the viral life cycle before viral RNA/ protein synthesis starts. CoVs replicate and transcribe their genome via so called replication/ transcription complexes (RTCs), anchored in virus-induced membrane alterations that consist of double membrane vesicles (DMVs) [49].

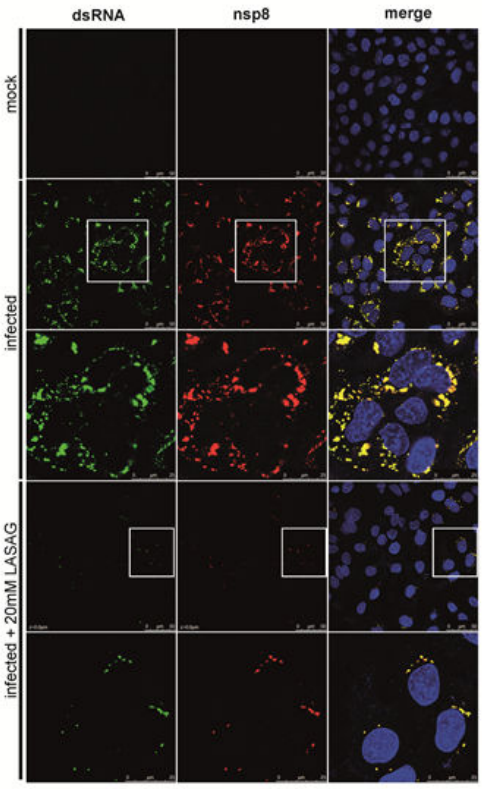

Figure 4: The abundance of coronaviral RTCs is reduced under LASAG treatment. HCoV-229E-infected Huh7 cells +/- LASAG (20 mM) were analysed 24 h p.i. using confocal microscopy. As a marker for viral RTCs, the CoV replication intermediate dsRNA (green) and the viral non-structural protein nsp8 (red) were detected using target specific antibodies. The nuclei were stained with DAPI (blue). Co-localization of dsRNA and nsp8 is shown in the merge (yellow). Open boxes (marked in white) indicate the magnified areas shown underneath the respective panel.

We therefore assessed whether LASAG may impact the formation of these host cell-derived DMVs. To this point, Huh7 cells were infected with HCoV-229E (MOI=3) and analysed 24 h p.i. for the appearance of RTCs. As markers in our immunofluorescence studies (Figure 4) we assessed the presence of dsRNA, a viral replication intermediate, indicating the site of viral genome replication [50,51] and the viral

nsp8 protein as an integral part of the viral RTCs [52]. In infected, untreated cells the characteristic peri-nuclear immunofluorescence pattern for RTCs marked by nsp8 and dsRNA [53], which was absent in the mock-infected cells (Figure 4, mock), was clearly visible (Figure 4, infected). In infected and LASAG-treated cells the amount of cells showing an nsp8/dsRNA signal (RTCs in DMVs) was decreased, compared to the untreated, infected cells (Figure 4, infected+20 mM LASAG). This result demonstrates that 24 h p.i. LASAG-treatment severely affects the formation of viral RTCs and/or DMVs and it is tempting to speculate that this is the cause for the decreased production of viral proteins and RNA. It should be noted that LASAG might also affect a step in the viral life cycle before RTC formation, which needs to be investigated.

HCoV-229E infection triggers NF-κB activation in the early phase of infection, which is decreased by LASAG

Activation of the transcription factor NF-κB is one of the hallmarks of host cell response to invasion by different pathogens. The phosphorylation of the NF-κB inhibitor IκBα leads to its degradation and release of NF-κB [54], which then translocates into the nucleus to transactivate responsive genes [55] related to host defence mechanisms [56].

NF-κB inhibitors have been studied as potential therapeutic drugs in anti-viral therapy [57]. Previously, we and others [36-38,58] demonstrated that NF-κB activity is essential for efficient influenza virus propagation, as inhibition of NF-κB activation results in impaired nuclear RNP export and therefore in reduced virus titers.

For coronavirus infections there are diverting reports of NF-κB activation [41,59-66] as well as NF-κB inhibition during CoV infection [67,68]. To assess whether HCoV-229E infection also activates NF-κB in Huh 7 cells we determined the level of NF-κB activation in HCoV-229E-infected Huh7 cells via TransAM assay and Western Blot analysis. We found that HCoV-229E infection leads to a markedly reduced amount of IκBα at 2 h p.i., which later increases again in the virus-infected cells (Figure 5A). When analysing the activation of NF-κB we found an early (4 h p.i.) activation (Figure 5B, left part), coinciding with decreased amounts of phospho-IκBα early in the infection (Figure 5A). According to the increased amounts of IκBα at later time points of the infection, the activation of NF-kB was found to be lower at 12 h p.i. compared to 4 h p.i. (Figure 5B, right part).

As mentioned before, ASA is known to block NF-κB activation. Therefore we tested whether LASAG treatment also has an effect on HCoV-229E-induced NF-κB activation. The treatment of HCoV-229E-infected cells with 20 mM LASAG resulted in a significant 1.5 fold reduction of NF-κB activation at early (4 h p.i.) and late (12 h p.i.) time points of infection (Figure 5B).

Furthermore, the addition of LASAG in the early stage of infection (3-6 h p.i.), when NF-κB activation was most prominent and viral RTCs are being formed, resulted in a reduction of virus titres. In contrast, the addition of LASAG in later stages of viral life cycle (9-12 h p.i.) resulted in less pronounced titre reduction (Figure 5C). In summary HCoV-229E infection activates NF-κB in the early stages of viral life cycle and the LASAG-dependent reduction of virus-induced NF-κB activation coincides with reduced viral titers.

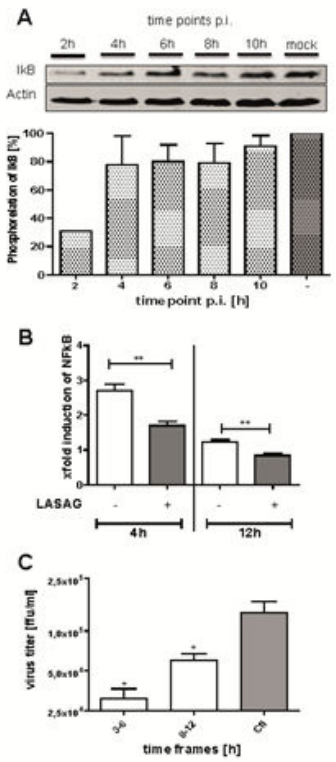

Figure 5: Virus-induced NF-κB activation is affected by LASAG treatment. The total amount of IκBα in HCoV-229E-infected Huh7 cells was analysed at the indicated time points p.i. by Western Blot using mouse anti-phospho-IκB-α antibody (A upper panel) and quantified (A lower panel). HCoV-229E-infected Huh7 cells (+/-) LASAG-treatment (20mM) were analysed for the activation of NF-κB at the indicated time points p.i. via detection of the amount of activated NF-κB (B). HCoV-229E-infected Huh7 cells were treated with LASAG (20mM) for the indicated time frames p.i. or left untreated (control) and virus titres (FFU/ml) were determined 12 h p.i. (C).

NF-κB plays a pivotal role in HCoV-229E infection

ASA is a multi-target compound [69] not only blocking NF-κB activation, but also interfering with several other cellular factors, such as cyclooxygenase 1/2 (COX1/2) a main enzyme in the synthesis of inflammation mediators [70,71] or AMPK/mTOR [72].

To highlight the role of NF-κB activity in CoV infection, we elucidated the effect of the NF-κB-specific inhibitor (BAY11-7082) on HCoV-229E replication. HCoV-229E-infected (MOI=0.5) Huh7 cells were treated with the indicated concentrations of BAY11-7082 and virus titres were analysed 24 h p.i.. Addition of BAY11-7082 at non-toxic concentration of 50 µM (Figure 6A) resulted in a reduction of viral titres by about 4 log10 (Figure 6B). This result supports our assumption that inhibition of NF-κB activity by LASAG impairs virus replication. Beside NF-κB inhibition, one of the major activities of ASA is the inhibition of the cellular COX1/2. We further investigated whether this activity might (also) be responsible for the anti-viral effect

of LASAG against CoV. For this we employed Lornoxicam, a non-steroidal COX1/2 inhibitor, which is used as an anti-inflammatory drug to treat pain, osteoarthritis, and rheumatoid arthritis [73,74]. However, at the indicated non-toxic concentrations (Figure 6C) Lornoxicam had no effect on HCoV-229E propagation in infected Huh7 cells (MOI=0.5) analysed 24 h p.i (Figure 6D). This indicates that the inhibitory effect of ASA on COX1/2 activity does not seem to be important for HCoV-229E propagation.

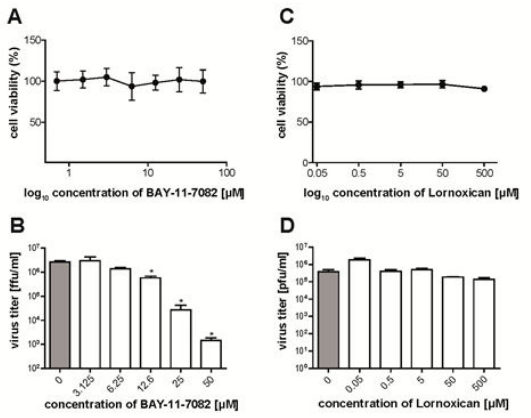

Figure 6: Effect of BAY11-7082 and Lornoxicam on HCoV-229E replication at non-toxic concentrations. Huh7 cells were treated with the indicated concentrations of BAY11-7082 and Lornoxicam for 24h and cell viability was determined via MTT assay (A, C). HCoV-229E-infected Huh7 cells were treated with the indicated concentration of BAY11-7082 and Lornoxicam and virus titres were determined 24 h p.i. (B, D).

Discussion

Among the CoV several human pathogenic strains can cause common cold-like illnesses, while others as SARS-CoV and MERS-CoV lead to severe infections. Currently, no treatment focusing on the virus as the cause is available. Here we show that LASAG, which is an approved drug, impairs propagation of HCoV-229E and of the highly pathogenic MERS-CoV *in vitro*. Our results demonstrate that inhibition of virus-induced NF-κB activity early in the viral replication cycle via LASAG coincides with (i) reduced viral titres, (ii) decreased viral protein accumulation and viral RNA synthesis and (iii) impaired formation of viral replication transcription complexes.

It should be mentioned that upon NF-κB inhibition (BAY11-7082) DeDiego et al. did not observe any virus titre reduction of the beta-coronavirus SARS-CoV [41] which was adapted to murine cell lines. However, it cannot be excluded that this system of a mouse-adapted SARS-CoV in a murine cell line might not reflect the situation of human CoV or wild type SARS-CoV infection of human cells.

Despite the possibility that other cellular factors, which are also targeted by LASAG might affect CoV propagation, the results obtained with the NF-κB inhibitor (BAY11-7082) and the COX1/2 inhibitor (Lornoxicam) further support the notion that NF-κB inhibition is a likely reason for the anti-CoV effect of LASAG. To elucidate the NF-κB-specific effect, further analysis will be needed. It should be noted that similar results were obtained for the treatment of influenza virus (IV) infection with ASA. Here, the COX inhibitor Indometacin also had no effect on the virus titer in cell culture [38].

Even though cell viability is not impaired by the applied LASAG concentrations, the anti-coronaviral action of LASAG in cell culture lies in a millimolar range, likewise to the anti-viral action of ASA against IV [38]. To achieve a 20 mM LASAG concentration in the blood, 6.53 g/L would be needed, which is toxic [75]. The Cmax in the blood after applying 500 mg ASA i.v. is 54.25 mg/L and 4.84 mg/L after oral application [76]. Nevertheless, treatment of patients with a CoV-caused severe acute respiratory syndrome via inhalation might allow achieving locally effective LASAG concentrations. Results from a clinical study investigating the effectiveness of inhaled lysine-acetylsalicylate in the treatment of asthma showed, that patients that received a dosage of 720 mg of inhaled LASAG twice a day over a two-week period did not experience any significant side effects [77]. Also, a dose escalation study of inhaled LASAG in humans for the clinical development of an antiviral treatment of IV infections demonstrated that inhalative doses up to 750 mg LASAG were safe and well tolerated without serious adverse events [78]. Furthermore, administration of aerosolic ASA via intubation directly into the trachea resulted in increased survival rates of mice infected with a lethal dose of IV [38].

In light of the fact that currently no approved anti-viral treatment against severe CoV-infections exist, it should be mentioned that LASAG (i) is widely available and it is tested and approved for humans, (ii) it targets cellular functions, (iii) is so far not known to target CoV functions, which reduces the chance that resistant virus variants emerge and (iv) adjacent to its direct anti-viral property, patients could also benefit from the effects of LASAG on infection-related symptoms based on the analgetic- and anti-inflammatory characteristics of LASAG.

In conclusion, we were able to demonstrate that LASAG inhibits virus-induced NF-κB activity, which might be connected to the anti-viral effect against CoV, including the impaired formation of RTCs and/or DMVs in CoV-infected cells, leading to reduced viral RNA production and consequently decreased production of viral proteins, resulting in an overall diminished virus titre.

Acknowledgments

We want to thank C. Drosten, Bonn, Germany, for providing MERS-CoV EMC/2012. This work was funded in part by the German Centre for Infection Research (DZIF), partner site Giessen, Germany (TTU Emerging Infections to S.P. and J.Z.), and the DFG-funded Collaborative Research Centre 1021 "RNA viruses: RNA metabolism, host response and pathogenesis" (SFB1021; projects A01 and C01 to J.Z. and S.P., respectively). Furthermore the work was supported in part by a fund of the Activaero GmbH (acquired by Vectura). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure Statement

The authors have no conflict of interest.

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