Antiviral activity of influenza A virus defective interfering particles against SARS-CoV-2 replication *in vitro* through stimulation of innate immunity

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Abstract

SARS-CoV-2 causing COVID-19 emerged in late 2019 and resulted in a devastating pandemic. Although the first approved vaccines were already administered by the end of 2020, vaccine availability is still limited. Moreover, immune escape variants of the virus are emerging against which the current vaccines may confer only limited protection. Further, existing antivirals and treatment options against COVID-19 only show limited efficacy. Influenza A virus (IAV) defective interfering particles (DIPs) were previously proposed not only for antiviral treatment of the influenza disease but also for pan-specific treatment of interferon (IFN)-sensitive respiratory virus infections. To investigate the applicability of IAV DIPs as an antiviral for the treatment of COVID-19, we conducted *in vitro* co-infection experiments with produced, cell culture-derived DIPs and the IFN-sensitive SARS-CoV-2. We show that treatment with IAV DIPs leads to complete abrogation of SARS-CoV-2 replication. Moreover, this inhibitory effect was dependent on janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling. These results suggest an unspecific stimulation of the innate immunity by IAV DIPs as a major contributor in suppressing SARS-CoV-2 replication. Thus, we propose IAV DIPs as an effective antiviral agent for treatment of COVID-19, and potentially also for suppressing the replication of new variants of SARS-CoV-2.

Keywords: SARS-CoV-2, COVID-19, antiviral, influenza A virus, defective interfering particles, OP7, DI244

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19), poses a severe burden to public health, economy and society. To date, almost 2.5 million cases of deaths were reported in the context of SARS-CoV-2 infection (WHO, covid19.who.int). Starting early 2020, there has been a unprecedented race for the development of novel vaccines, their production, and safety and immunogenicity studies in clinical trials (Krammer, 2020, Walsh et al., 2020, Voysey et al., 2020, Jackson et al., 2020, Logunov et al., 2020, Palacios et al., 2020). First individuals were already vaccinated at the end of 2020. While vaccination typically provides the best protection against virus infections and disease onset, the production capacity of COVID-19 vaccines, and the infrastructure required for vaccination is still limiting. In addition, vaccination cannot be used for therapeutic treatment of acute infections. Therefore, as an alternative option, the use of antivirals for treatment of COVID-19 is essential. Yet, remdesivir (in clinical use) showed only limited efficacy (Wang et al., 2020, Beigel et al., 2020, Pan et al., 2020), while other repurposed drug candidates (e.g., hydroxychloroquine and lopinavir-ritonavir) showed a lack of efficacy (Boulware et al., 2020, Cao et al., 2020). In addition, corticosteroids (i.e., dexamethasone (Tomazini et al., 2020)) and cocktails of monoclonal antibodies (e.g., bamlanivimab (Chen et al., 2020b)) are used in the clinic and show an antiviral effect. However, the appearance of new SARS-CoV-2 variants poses a constant risk to lose efficacy of highly specific treatments, including vaccination and therapeutic antibodies. Thus, there is a need to develop more broadly acting, cost-effective antivirals that ideally are easily scalable in production.

Influenza A virus (IAV) defective interfering particles (DIPs) were previously proposed for antiviral treatment against Influenza A infections (Zhao et al., 2018, Vignuzzi and Lopez, 2019, Meir et al., 2020, Tapia et al., 2019, Yang et al., 2019, Harding et al., 2019, Tanner et al., 2016, Huo et al., 2020b), but also for pan-specific treatment of other respiratory viral diseases (Dimmock and Easton, 2014, Dimmock and Easton, 2015). IAV DIPs typically carry a large internal deletion in their genome, rendering them defective in virus replication (Alnaji and Brooke, 2020, Ziegler and Botten, 2020, Andreu-Moreno and Sanjuan, 2020). Furthermore, DIPs suppress and interfere specifically with homologous viral replication in a co-infection scenario, a process known as replication interference. As a result, administration of IAV DIPs to mice resulted in full protection against an otherwise lethal IAV infection (Dimmock et al., 2008, Huo et al., 2020b, Hein et al., 2021)(Hein et al., submitted). In the ferret model, treatment of IAV-infected animals resulted in a reduced severity of disease pathogenesis (Dimmock et al., 2012). Intriguingly, mice were also protected against a lethal infection with the unrelated influenza B virus (Scott et al., 2011) and pneumonia virus of mice (PVM) from the family *Paramyxoviridae* (Easton et al., 2011). Here, protection was not attributed to replication interference but to the ability of IAV DIPs to stimulate innate immunity.

SARS-CoV-2 replication seems to modulate and inhibit the interferon (IFN) response in infected target cells (Chen et al., 2020a, Konno et al., 2020, Lei et al., 2020). Still, it was also shown to be susceptible to inhibition by exogenously added IFNs *in vitro* (Busnadiego et al., 2020, Felgenhauer et al., 2020), *in vivo* (Hoagland et al., 2021) and in clinical trials (Monk et al., 2020). Therapies using recombinant IFNs, however, are cost intensive and pose the risk of unwanted side effects including the formation

of auto-antibodies against the cytokine (reviewed in (Sleijfer et al., 2005)). To prevent this, we wondered whether IAV DIPs would be able to suppress SARS-CoV-2 replication through their ability to stimulate a physiological IFN response in target cells. To test this, we produced two promising candidate DIPs: a prototypic, well-characterized conventional IAV DIP "DI244" (Dimmock and Easton, 2014), and a novel type of IAV DIP "OP7", that contains point mutations instead of a large internal deletion in the genome (Kupke et al., 2019) using a cell culture-based production process (Hein et al., 2021)(Hein et al., submitted).

Here, we used Calu-3 cells (human lung cancer cell line) for *in vitro* co-infection experiments with SARS-CoV-2 and DI244 or OP7, respectively. Both DIPs were able to completely inhibit SARS-CoV-2 replication and spreading in a range comparable to IFN-β or remdesivir treatment. Moreover, we show that the inhibitory effect of IAV DIPs was due to their ability to induce innate immune responses that signal via janus kinase/signal transducers and activators of transcription (JAK/STAT). Yet, additional mechanisms may also play a role. Thus, we propose IAV DIPs as effective antiviral agents for the treatment of COVID-19 and, potentially as universal antiviral agents not only against different influenza subtypes but also against other (including newly emerging) IFN-sensitive respiratory viruses.

Results

SARS-CoV-2 replication is abrogated by IAV DIP treatment *in vitro*

In order to test the antiviral efficacy of IAV DIPs on replication of SARS-CoV-2, we conducted *in vitro* co-infection experiments in Calu-3 cells. For this, we infected cells with SARS-CoV-2 (multiplicity of infection (MOI) = 0.03) and highly concentrated IAV DIPs (DI244 or OP7), respectively, from cell culture-based production and chromatographic purification (Marichal-Gallardo et al., 2017, Hein et al., 2021)(Hein et al., submitted). At 3 days post infection (dpi), cells were stained for the SARS-CoV-2 spike (S) protein (Fig. 1A). Indeed, S protein expression was significantly reduced in cells co-treated with DI244 or OP7 compared to cells infected with SARS-CoV-2 only, indicating efficient suppression of SARS-CoV-2 replication by IAV DIP co-infection. In agreement with this observation, images from live-cell microscopy show a significant amelioration of the cytopathic effect upon DIP co-infection (Fig. 1B).

Next, different concentrations of IAV DIPs were tested for the treatment of SARS-CoV-2-infected cells (Fig. 1C). As a read-out for SARS-CoV-2 replication, we determined the plaque titer from supernatants at 3 dpi using adherent Vero-6 cells. Please note that infection with only defective, replication-incompetent IAV DIPs does not result in the release progeny virions, as demonstrated by negative plaque titers (Hein et al., 2021)(Hein et al., submitted). Strikingly, SARS-CoV-2 replication was severely diminished upon IAV DIP co-infection. In particular, at high DI244 and OP7 concentrations, no SARS-CoV-2 plaque titers were detectable anymore, while untreated cells showed a titer of 7.5×10^4 plaque-forming units (PFU)/mL. Suppression of SARS-CoV-2 replication decreased with increasing dilution of DIPs.

Remarkably, though, the treatment with both DIPs at a dilution of 1:1000 still resulted in a pronounced inhibition of SARS-CoV-2 replication, corresponding to a 26-fold and 210-fold reduction in plaque titers for DI244 and OP7 treatment, respectively. For comparison, we also tested the inhibitory capacity of IFN-β or remdesivir treatment on SARS-CoV-2 replication in infected target cells. Both agents were also able to diminish SARS-CoV-2 plaque titers to below the LOD, until a concentration of 633 U/mL for IFN-β and 0.32 µM for remdesivir. Yet, inhibiting effects ceased significantly faster with increasing dilutions, most apparently observed for remdesivir, for which treatment with a concentration of 0.03 µM already did not result an inhibitory effect anymore.

Fig. 1D illustrates SARS-CoV-2 inhibition caused by inactivated DIPs. These DIPs were previously UV irradiated until no interfering efficacy against IAV replication was observed anymore *in vitro* (Hein et al., 2021)(Hein et al., submitted). This suggests the complete inactivation of the causative interfering agent, i.e., the defective interfering (DI) viral RNA (vRNA). Nevertheless, inhibition of SARS-CoV-2 replication by inactivated DIPs was still detectable (Fig. 1D). More specifically, we still observed a residual suppression of plaque titers. This may be explained by an unspecific stimulation of the innate immunity by inactivated virus particles, which still have the capacity to enter target cells and to switch on antiviral processes that ultimately suppress SARS-CoV-2 replication. However, the finding that the inhibition caused by active DIPs was more efficient clearly suggests a specific activity of IAV DIPs, leading to interference with SARS-CoV-2 replication and spreading. Of note, active DIPs still conferred a pronounced antiviral effect even when applied 24 h after preceding SARS-CoV-2 infection (Fig. 1E).

158 In conclusion, treatment with both DI244 and OP7 IAV DIPs completely abolished
159 SARS-CoV-2 replication during *in vitro* co-infections. While the inhibitory potential
160 was comparable to IFN-β and remdesivir treatment, the antiviral effects of IAV DIPs
161 were more sustained with increasing dilution.

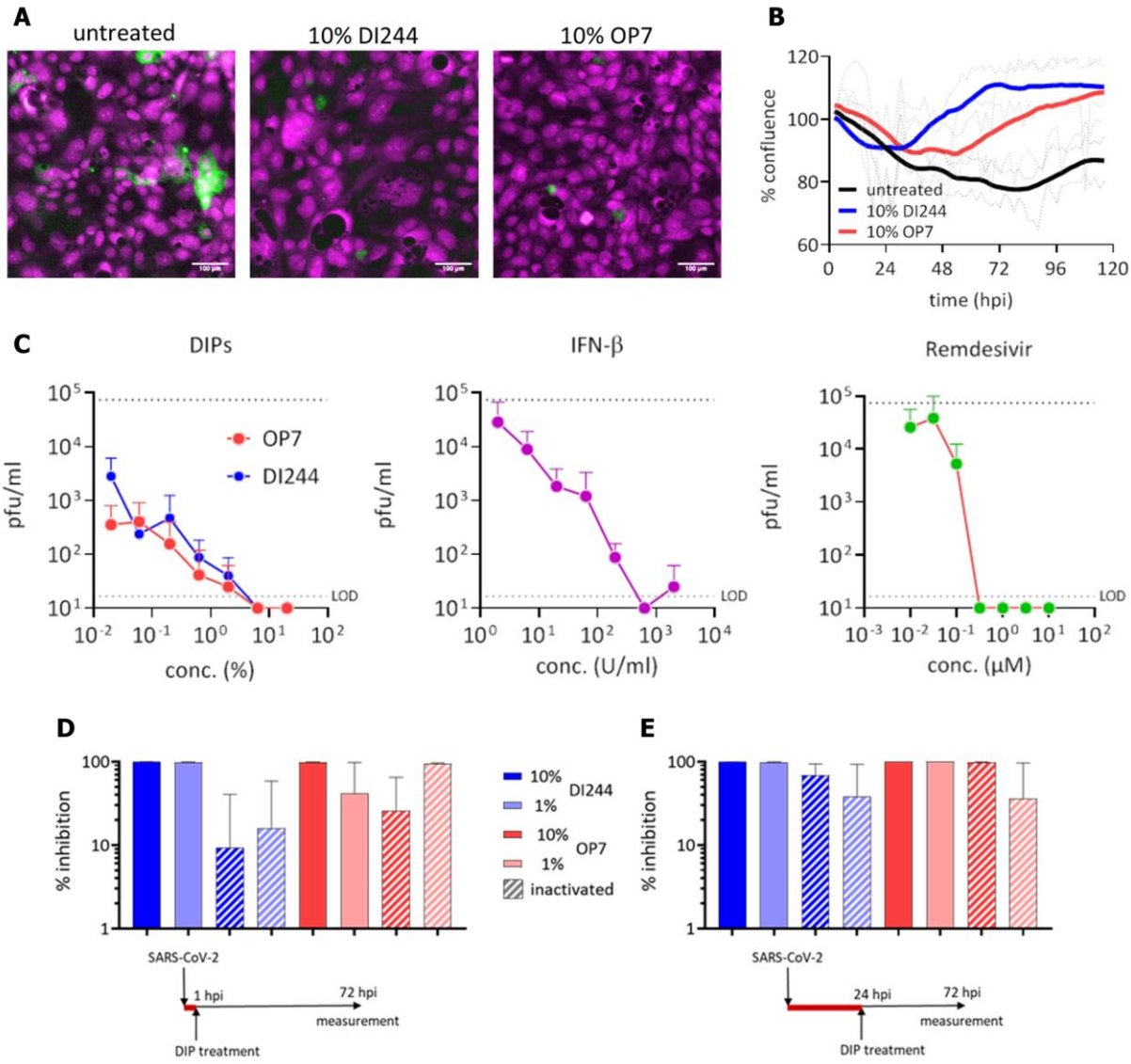

Fig. 1. Inhibition of SARS-CoV-2 replication and spreading by IAV DIPs. SARS-CoV-2-infected Calu-3 cells (MOI=0.03) were treated with IAV DIPs (DI244 or OP7), IFN-β, or remdesivir at 1 hour post infection (hpi). For DI244 and OP7 treatment, highly concentrated produced, cell culture-derived DIP material (Hein et al., 2021)(Hein et al., submitted) was used. % (v/v) indicates the fraction with respect to the cell culture volume of 100 µL. Stock concentration, 5.6 x 10^8 and 1.12 x 10^{11} DI vRNAs/mL for DI244 and OP7, respectively. (**A**) Immunofluorescence analysis of the SARS-CoV-2 S protein expression (green, magenta: DNA) at 3 dpi. Scale bar, 100 µm. (**B**) Cytopathic effect. Confluence (% of initial) was measured by live-cell microscopy at 2 h intervals. Thick lines represent smoothened data (Savitzky-Golay filter), dotted lines show SD of original data (n=2, independent experiments). (**C**) Effective concentration range of DI244 and OP7 compared to IFN-β and remdesivir. Viral titers were determined from the supernatant at 3 dpi by plaque assay. Upper dotted line indicates virus titer in untreated cells, lower dotted line shows the limit of detection (LOD). Independent experiments were conducted; mean +/- SD (n=3) is shown. (**D**) and (**E**) SARS-CoV-2 growth inhibition by inactivated DIPs. SARS-CoV-2 infected cells were treated with active or UV-

177 inactivated DIPs at 1 hpi (**D**) or 24 hpi (**E**). Percentage inhibition of viral growth relative to mock
178 treatment is shown; mean +/- SEM (n=3) is depicted.

Inhibition of SARS-CoV-2 replication by IAV DIPs caused by stimulation of innate immunity

Next, to investigate our hypothesis whether inhibition of SARS-CoV-2 replication by DIPs was due to their ability to stimulate the IFN system, we used ruxolitinib in co-infection experiments. This small molecule drug is an efficient inhibitor of JAK, which are key effectors in the IFN system. Upon IFN sensing, JAKs typically recruit STATs, ultimately leading to the upregulation of IFN-stimulated gene (ISGs). ISGs encode for effector molecules that limit viral replication by inducing an antiviral state in the infected as well as uninfected neighboring cells. Fig. 2 shows the results of SARS-CoV-2 and IAV DIP co-infection upon treatment with ruxolitinib. While DI244 and OP7 co-infection almost completely inhibited SARS-CoV-2 replication, additional treatment with ruxolitinib abrogated the suppressive effect of both IAV DIPs. Specifically, virus titers under JAK signaling inhibition were comparable to SARS-CoV-2 infection in the absence of DIPs. In conclusion, these results suggest a major contribution of unspecific innate immune activation by IAV DIPs in interfering with SARS-CoV-2 replication.

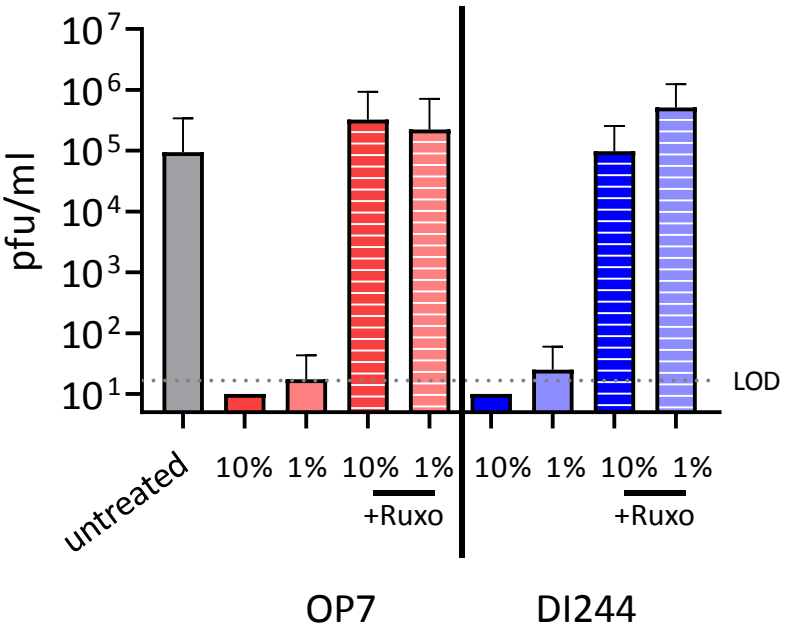

Fig. 2. Suppression of SARS-CoV-2 replication by IAV DIPs under JAK inhibition. SARS-CoV-2-infected Calu-3 cells (MOI=0.03) were treated with IAV DIPs (DI244 or OP7) at 1 hpi in the presence of absence of ruxolitinib (JAK inhibitor). % (v/v) indicates the fraction of DIPs (highly concentrated produced, cell culture-derived material (Hein et al., 2021)(Hein et al., submitted)) with respect to the cell culture volume of 100 µL. Stock concentration, 5.6 x 10^8 and 1.12 x 10^{11} DI vRNAs/mL for DI244 and OP7, respectively. Viral titers were determined from the supernatant at 3 dpi by plaque assay. Dotted line shows the LOD. Independent experiments were conducted; mean +/-SD (n=3) is depicted.

Discussion

Despite the recent availability of vaccines against COVID-19, options for antiviral treatment are urgently needed for therapeutic application. Here, we show that produced, cell culture-derived IAV DIPs are highly potent inhibitors of SARS-CoV-2 replication in human lung cells. In addition, our data obtained in *in vitro* experiments suggest that suppression of SARS-CoV-2 replication by IAV DIPs is predominantly attributed to their ability to stimulate innate immune responses ultimately inducing an antiviral state in target cells.

In the clinic, already approved antivirals for treatment of COVID-19 showed only very limited efficacy. For instance, treatment with the polymerase inhibitor remdesivir did not result in an overall decrease in mortality (Beigel et al., 2020, Pan et al., 2020). For patients receiving supplemental oxygen, however, an improvement in the survival rate from about 4% to 12% was observed (Beigel et al., 2020). In addition, the time required to recover from COVID-19 was decreased by five days (Wang et al., 2020, Beigel et al., 2020). Another option to treat COVID-19 is the use of monoclonal antibodies that target the receptor binding domain of the SARS-CoV-2 S protein, thereby inhibiting engagement with the host cell entry receptor angiotensin-converting enzyme 2 (ACE2) (Hoffmann et al., 2020, Abraham, 2020). Here, it was suggested to use antibody cocktails to prevent the emergence of viral escape variants in treated individuals (Baum et al., 2020). In clinical trials, treatment of outpatients with one such an antibody cocktail (i.e., bamlanivimab) accelerated the decrease in viral load and reduced the fraction of patients requiring hospitalization from 6.3% to 1.6% (Chen et al., 2020b). The administration of the corticosteroid dexamethasone (in clinical use) resulted in an overall lower mortality in critically ill

COVID-19 patients (Horby et al., 2020, Sterne et al., 2020). This has a caveat, though, as a decrease in mortality was observed for patients requiring oxygen (including mechanical ventilation), but an increase in mortality was reported for patients not requiring oxygen (Horby et al., 2020).

Treatment of COVID-19 patients with IFNs has not been approved yet. In general, SARS-CoV-2 infection modulates and inhibits the IFN response (Chen et al., 2020a, Konno et al., 2020, Lei et al., 2020). Moreover, it was recently shown that the host cell entry receptor ACE2 is indeed an ISG, and it was speculated that SARS-CoV-2 may exploit the IFN-driven upregulation of ACE2 to enhance infection (Ziegler et al., 2020). However, SARS-CoV-2 replication was also shown to be susceptible to inhibition by exogenously added IFN. For instance, all IFNs (type I, II and III) exhibited potent antiviral activity with SARS-CoV-2 replication *in vitro* (Busnadiego et al., 2020, Felgenhauer et al., 2020), suggesting that the antiviral activities of IFNs may counterbalance any proviral effects derived from ACE2 induction. In agreement with this, intranasal IFN-I administration (in hamsters) pre- or post-virus challenge was shown to reduce SARS-CoV-2 disease burden (Hoagland et al., 2021). Moreover, in a placebo-controlled phase 2 clinical trial, administration of inhaled, nebulized IFN beta-1a resulted in a higher chance of disease improvement and a more rapid recovery from COVID-19 (Monk et al., 2020).

In our cell culture experiments, IAV DIPs completely abrogated SARS-CoV-2 replication. Notably, the UV-irradiated and thus inactive DIP material also showed a residual inhibitory effect. Yet, the observation of a much stronger antiviral effect upon treatment with active DIPs hints to a specific activity of IAV DIPs in the context of SARS-CoV-2 suppression. DIPs are defective in virus replication, and thus they fail

to complete the entire infection cycle. However, the incoming genomic vRNAs, packaged into a viral ribonucleoprotein (vRNP) complex (Eisfeld et al., 2015), still show polymerase activity and transcribe viral mRNAs (Vreede et al., 2004, Heldt et al., 2012, Vreede and Brownlee, 2007). In particular, the short DI vRNAs (and likely, also the resulting short DI mRNAs) were shown to be preferentially bound by the retinoic acid inducible gene I (RIG-I) protein (Baum and Garcia-Sastre, 2011), which subsequently leads to the activation of an IFN-response (Rehwinkel et al., 2010).

Our results support the notion that IAV DIPs do not only protect host cells from IAV infection but, in addition, may generally confer protection against other heterologous IFN-sensitive respiratory viruses (Easton et al., 2011, Scott et al., 2011, Dimmock and Easton, 2015). Considering the emergence of new SARS-CoV-2 variants that render the efficacy of various vaccine candidates questionable, the unspecific stimulation of innate immunity by IAV DIPs may be advantageous; in particular, regarding a potential universal efficacy against such new (and future) variants. Furthermore, *in vitro* and *in vivo* experiments revealed an antiviral effect of IAV DIPs (derived from strains originally isolated in 1933 and 1934) against a variety of different IAV subtypes that have been isolated between 1933-2014, including pandemic and highly pathogenic avian IAV strains (Dimmock et al., 2008, Dimmock et al., 2012, Zhao et al., 2018, Huo et al., 2020a).

Future work to investigate the feasibility to use IAV DIPs against SARS-CoV-2 infection will comprise animal trials in Syrian hamsters, which are (in contrast to mice) highly permissive to SARS-CoV-2 and develop a similar lung disease compared to human COVID-19 (Kaptein et al., 2020, Boudewijns et al., 2020, Chan et al., 2020). As an alternative approach, SARS-CoV-2/IAV DIP co-infection studies to clarify

the therapeutic effects of IAV DIPs on the outcome of SARS-CoV-2 infection and to decipher in more detail the underlying mode of action may be performed in humanized K18-hACE2 mice. These mice are genetically modified to express the human ACE2 receptor rendering them susceptible for SARS-CoV-2 infection and have recently been shown to develop respiratory disease resembling severe COVID-19 in humans (Yinda et al., 2021). Animal experiments will help to elaborate on the potential applicability of IAV DIPs as a pre- and post-exposure treatment for instance in acute SARS-CoV-2 outbreak scenarios in the clinics or geriatric institutions. In addition to vaccination, this would represent an interesting option for prophylactic treatment to boost antiviral immunity in persons at acute risk for an infection or for therapeutic treatment during an early phase post infection and as such may prevent fatal COVID-19 outcomes.

Materials and methods

Cells and viruses

Vero-6 cells (ATCC CRL-1586) were maintained in DMEM medium (Gibco, 4.5 g/L glucose, w/o pyruvate) supplemented with 10% fetal calf serum (FCS, Biowest, S1810-6500), 100 IU/mL penicillin, 100 µg/mL streptomycin, 1x GlutaMax (Gibco) and 1x sodium pyruvate (Gibco). Calu-3 cells (ATCC HTB-55) were cultured in MEM (Sigma) supplemented with 10% FCS (Biowest, S1810-6500), 100 IU/mL penicillin, 100 µg/mL streptomycin, 1x GlutaMax (Gibco) and 1x sodium pyruvate (Gibco). Caco-2 cells (ATCC HTB-37) were grown in MEM (Gibco) supplemented with 20 % FCS (Biowest, S1810-6500), 100 IU/mL penicillin, 100 µg/mL streptomycin, 1x GlutaMax (Gibco) and 1x non-essential amino acid solution (Gibco). All cells were maintained or infected at 37°C in a 5% CO_2 atmosphere.

The IAV DIPs DI244 and OP7 were produced in a cell culture-based process using a 500 mL laboratory scale stirred tank bioreactor, followed by purification and concentration by membrane-based steric exclusion chromatography (Marichal-Gallardo et al., 2017), as described previously (Hein et al., 2021)(Hein et al., submitted). Production titers of 3.3 and 3.67 log hemagglutination (HA) units/100µL (quantified by the HA assay (Kalbfuss et al., 2008)) and 5.6 x 10^8 and 1.12 x 10^{11} DI vRNAs/mL (quantified by real-time RT-qPCR (Kupke et al., 2019, Hein et al., 2021, Wasik et al., 2018)) were achieved for DI244 and OP7, respectively.

The SARS-CoV-2 isolate hCoV-19/Croatia/ZG-297-20/2020 was used. All experiments with infectious SARS-CoV-2 were performed in the BSL-3 facility at the Helmholtz Centre for Infection Research (Braunschweig, Germany). The SARS-CoV-2 seed virus

was produced in Caco-2 cells, and virus particles were enriched in Vivaspin 20 columns (Sartorius Stedim, Biotech) via centrifugation. Collected virus was stored at -80°C. SARS-CoV-2 titers were quantified by plaque assay.

Plaque assay

Quantification of SARS-CoV-2 was performed by plaque assay. Samples were serially diluted in 10-fold steps, and used to infect a confluent monolayer of Vero-6 cells (on 96-well plates) for 1 h. Then, the inoculum was removed and cells were overlaid with cell culture medium containing 1.5% methyl-cellulose (SIGMA, #C9481-500). At 3 dpi, cells were fixed with 6% formaldehyde and stained with crystal violet. Wells were imaged using a Sartorius IncuCyte S3 (4x objective, whole-well scan) and plaque counts were determined.

SARS-CoV-2 infection and antiviral treatment

Confluent Calu-3 cells in 96-well plates (~6 x 10^4 cells/well) were infected with SARS-CoV-2 (2000 PFU per well). At 1 or 24 hpi, we added active or inactive IAV DIPs (DI244 or OP7) at indicated fractions (% v/v) with respect to the cell culture volume of 100 µL. Whenever indicated, we additionally added 0.8 µM ruxolitinib (Cayman Chemical, Cat. #Cay11609-1) to these wells. Alternatively, remdesivir (MedChem Express, #HY-104077) or human IFN-β-1A (PBL assay science, #11415-1) (instead of IAV DIPs) were added at indicated concentrations at 1 hpi. Supernatants were

collected at 3 dpi. Quantification of SARS-CoV-2 titers was performed using the plaque assay.

Immunofluorescence staining

SARS-CoV-2 infected cells were fixed with 6% paraformaldehyde in PBS for 1 h at room temperature, followed by washing with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, washed with PBS, and blocked with 2% BSA in PBS for 1 h. Antibody labelling was performed with mouse anti-SARS-CoV-2 S protein (Abcalis, clone AB68-A09, #ABK68-A09-M) and secondary antibody anti-mouse Alexa488 (Cell Signaling Technology, #4408), each step followed by three washing steps with PBS containing 0.05% Tween-20. Finally, cells were overlaid with Vectashield mouting medium (Biozol, #VEC-H-1000).

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Declaration of interests

A patent for the use of OP7 as an antiviral agent for treatment of IAV infection is pending. Patent holders are S.Y.K. and U.R. (Udo Reichl).

Another patent for the use of DI244 and OP7 as an antiviral agent for treatment of coronavirus infection is pending. Patent holders are S.Y.K., U.R. (Udo Reichl), M.H., U.R. (Ulfert Rand) and D.B.

P.M.G. and U.R. (Udo Reichl) are inventors in a pending patent application detailing the technology used for the chromatographic purification of the influenza virus particles used in this study.

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