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Antiviral Research 177 (2020) 104774

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

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Sebastian Blockus[a,1], Svenja M. Sake[a,1], Martin Wetzke[b,q], Christina Grethe[a], Theresa Graalmann[c,d], Marina Pils[e], Ronan Le Goffic[f], Marie Galloux[f], Hans Prochnow[g,q], Katharina Rox[g,q], Stephan Hüttel[h], Zeljka Rupcic[h], Bettina Wiegmann[i,j,k], Ronald Dijkman[l,m,n], Marie-Anne Rameix-Welti[o], Jean-François Eléouët[f], W Paul Duprex[p], Volker Thiel[l,m], Gesine Hansen[b,r], Mark Brönstrup[g,q], Sibylle Haid[a,*], Thomas Pietschmann[a,q,r,**]

[a] Institute for Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany
[b] Department for Pediatric Pneumology, Allergy and Neonatology, Hannover Medical School, Hannover, Germany
[c] Institute for Experimental Infection Research, TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany
[d] Clinic for Immunology and Rheumatology, Hannover Medical School, Hannover, Germany
[e] Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany
[f] Unité de Virologie et Immunologie Moléculaires, INRA, Université Paris Saclay, Jouy-en-Josas, France
[g] Department of Chemical Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany
[h] Department Microbial Drugs, Helmholtz Centre for Infection Research, Braunschweig, Germany
[i] Lower Saxony Center for Biomedical Engineering, Implant Research and Development, Hannover Medical School, Hannover, Germany
[j] Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany
[k] Member of the German Centre for Lung Research (DZL), Hannover, Germany
[l] Institute of Virology and Immunology (IVI), Bern and Mittelhäusern, Switzerland
[m] Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Switzerland
[n] Institute for Infectious Diseases, University of Bern, Switzerland
[o] UMR1173, Institute National de la Santé et de la Recherche Médicale (INSERM), Université de Versailles St. Quentin, Montigny-le-Bretonneux, France
[p] University of Pittsburgh School of Medicine, Pittsburgh, PA, United States
[q] German Centre of Infection Research (DZIF), Partner Site Hannover-Braunschweig, Germany
[r] Cluster of Excellence RESIST (EXC 2155), Hannover Medical School, Hannover, Germany

ARTICLE INFO

Keywords:
Human respiratory syncytial virus (hRSV)
Labyrinthopeptin
Lanthipeptide
Antiviral activity
Virus entry
Virolytic

ABSTRACT

Acute lower respiratory tract infections (ALRI) caused by respiratory syncytial virus (RSV) are associated with a severe disease burden among infants and elderly patients. Treatment options are limited. While numerous drug candidates with different viral targets are under development, the utility of RSV entry inhibitors is challenged by a low resistance barrier and by single mutations causing cross-resistance against a wide spectrum of fusion inhibitor chemotypes. We developed a cell-based screening assay for discovery of compounds inhibiting infection with primary RSV isolates. Using this system, we identified labyrinthopeptin A1 and A2 (Laby A1/A2), lantibiotics isolated from *Actinomadura namibiensis*, as effective RSV cell entry inhibitors with IC_{50}s of 0.39 μM and 4.97 μM, respectively, and with favourable therapeutic index (>200 and > 20, respectively). Both molecules were active against multiple RSV strains including primary isolates and their antiviral activity against RSV was confirmed in primary human airway cells *ex vivo* and a murine model *in vivo*. Laby A1/A2 were antiviral in prophylactic and therapeutic treatment regimens and displayed synergistic activity when applied in combination with each other. Mechanistic studies showed that Laby A1/A2 exert virolytic activity likely by binding to phosphatidylethanolamine moieties within the viral membrane and by disrupting virus particle membrane integrity. Probably due to its specific mode of action, Laby A1/A2 antiviral activity was not affected by common resistance mutations to known RSV entry inhibitors. Taken together, Laby A1/A2 represent promising candidates for development as RSV inhibitors. Moreover, the cell-based screening system with primary RSV isolates described here should be useful to identify further antiviral agents.

* Corresponding author. Institute for Experimental Virology, Twincore, Hannover, Feodor-Lynen-Strasse 7, 30625, Hannover, Germany.
** Corresponding author. Institute for Experimental Virology, Twincore, Hannover, Feodor-Lynen-Strasse 7, 30625, Hannover, Germany.
E-mail addresses: sibylle.haid@twincore.de (S. Haid), thomas.pietschmann@twincore.de (T. Pietschmann).
[1] equally contributing first authors.

https://doi.org/10.1016/j.antiviral.2020.104774
Received 25 October 2019; Received in revised form 3 February 2020; Accepted 12 March 2020
Available online 18 March 2020

1. Introduction

Acute lower respiratory tract infections (ALRI) are one of the leading causes of childhood morbidity and mortality (Liu et al., 2016; Theodoratou et al., 2014). RSV is the most common cause of ALRI in infants and the most frequent cause of hospitalization among those younger than one year (Leader and Kohlhase, 2002; Olchanski et al., 2018). It is responsible for approximately 33 million episodes of ALRI in children younger than five years, leading to 3.2 million hospitalizations and ca. 60,000 in-hospital-deaths (Shi et al., 2017). Moreover, RSV causes substantial global disease burden in elderly patients (Shi et al., 2019).

To date, only two antiviral treatment options are available for clinical use. Ribavirin, a nucleoside analogue with broad activity against multiple viruses and proven *in vitro* efficacy against RSV, is approved for inhalative treatment of RSV-infected infants. However, due to lack of clinical efficacy in addition to possible teratogenic side effects its routine use is no longer recommended (Mazur et al., 2015). Palivizumab, a RSV fusion protein-targeting monoclonal antibody for prophylactic passive immunization against RSV infection is available. But Palivizumab reduces hospitalization rates only by 55% and cannot be broadly applied due to its high costs (IMpact, 1998). Thus, novel therapeutic strategies to treat and/or prevent severe RSV infection are needed.

RSV is an enveloped virus with a non-segmented, negative strand RNA genome. It is classified in the family of *Pneumoviridae* and the genus *Orthopneumovirus* (Afonso et al., 2016). The viral attachment glycoprotein (G) and the fusion protein (F) mediate infection of respiratory cells. Upon cell entry, the viral RNA genome serves as template for transcription of ten capped and polyadenylated mRNAs and for genome replication. Transcription and genome replication are catalyzed by a ribonucleoprotein complex consisting of the viral nucleocapsid (N) protein that tightly binds to viral genomic and antigenomic RNAs, the viral L protein that serves as catalytic centre of the RNA-dependent RNA polymerase, and the phosphoprotein (P). The M2-1 protein is an essential transcription processivity factor needed for production of full length mRNAs (Collins et al., 1996). A range of RSV inhibitors is in various stages of pre-clinical and clinical development including fusion inhibitors, nucleosidic and non-nucleosidic polymerase inhibitors, as well as G, N and M2-1-targeting antivirals (Bailly et al., 2016; Cockerill et al., 2019; Gaillard et al., 2017; Ouizougun-Oubari et al., 2015). Some of these molecules, including fusion inhibitors like Presatovir (GS-5806) and JNJ-678 have progressed to phase II clinical trials and have proven efficacy in human RSV challenge studies (Cockerill et al., 2019; DeVincenzo et al., 2014). However, the barrier to RSV fusion inhibitor resistance is rather low and several single mutations cause pronounced viral cross-resistance to structurally diverse fusion inhibitors, possibly compromising utility of this type of RSV entry inhibitors (Battles et al., 2016; Yan et al., 2014).

Here, we describe a novel cell-based screening approach for identification of RSV inhibitors and identify Laby A1/A2 as a novel class of RSV-targeting cell entry inhibitors. We show that Laby A1 and A2, class III lanthipeptides, isolated from *Actinomadura namibiensis* DSM6313 (Meindl et al., 2010; Muller et al., 2010; Rupcic et al., 2018), inhibit RSV entry by disrupting the integrity of RSV particles. Because the antiviral activity of Laby A1/A2 is not affected by resistance to Palivizumab or clinical-stage RSV fusion inhibitors, these molecules are interesting compounds for development as RSV inhibitors.

2. Materials and methods

2.1. Media, cells, virus and compounds

All cell culture media were obtained from Life technologies (Thermo Fisher Scientific, Waltham, MA). FCS was obtained from Capricorn Scientific (Ebsdorfergrund, Germany) and heat inactivated for 30 min at 56 °C. HEp-2 cells (CCL-23), A549 cells (CCL-185), 293T cells (HEK293T/17; CRL-11268), RSV-A2 (VR-1540) and RSV-A long strains (VR-26) were originally obtained from ATCC (Manassas, VA). Recombinant viruses rHRSV-A-Luc, rHRSV-A-GFP and rHRSV-B05 eGFP were described recently (Lemon et al., 2015; Rameix-Welti et al., 2014). Laby A1 and A2 were produced and isolated from actinomycete *Actinomadura namibiensis* DSM 6313 using a standardized submerged liquid cultivation in 10 L bioreactors (BBI Biotech, Berlin) and an improved down-stream procedure as described before (Rupcic et al., 2018). Palivizumab was purchased from AbbVie Ltd. (North Chicago, IL) and Ribavirin was purchased from Sigma-Aldrich (Steinheim, Germany). BMS-433771 was a kind gift from Richard Karl Plemper (Georgia State University, Atlanta, GA, USA).

2.2. Cell cultures

All cells were cultured in a humidified incubator at 37 °C and 5% CO_2. HEp-2 cells were grown in Advanced MEM and A549 cells in F12K NutMix, both supplemented with 10% heat-inactivated fetal bovine serum (FCS), non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml Penicillin and 100 U/ml Streptomycin (Thermo Fisher Scientific, Waltham, MA). 293T cells were grown in DMEM media supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml Penicillin, 100 U/ml Streptomycin and non-essential amino acids. Primary human airway epithelia cells (HuAEC) were isolated and cultivated as described elsewhere (Jonsdottir and Dijkman, 2015).

2.3. Primary hRSV isolates

The primary hRSV isolates were collected from children below the age of five years with confirmed RSV infection, hospitalized at Hannover Medical School. Viruses were isolated from nasopharyngeal aspirates and further cultivated on HEp-2 cells. All caregivers gave informed consent for the donation of nasopharyngeal aspirates, and all steps were conducted in compliance with good clinical and ethical practice and approved by the local ethical committee at Hannover Medical School (permission number 63090/2012).

2.4. Screening assay

A HEp-2 cell line expressing a firefly luciferase (HEp-2-F-Luc cells) was generated via lentiviral gene transfer using a self-inactivating vector pWPI-F-Luc transducing firefly luciferase and subsequent selection of transduced cells in the presence of blasticidine (5 μg/ml). 293T cells were transfected with envelope protein expression construct pcz-VSV-G (Kalajzic et al., 2001), HIV Gag-Pol expression construct pCMV-ΔR8.74 (Dull et al., 1998) and firefly transducing vector pWPI-F-Luc-BLR. 48 h post transfection, cell-free supernatant was harvested and used to transduce HEp-2 cells. Transduced cells were passaged in the presence of blasticidine. For compound screening, cells were seeded at a density of 1 × 10e4 cells/well onto a 96-well plate. 24 h post seeding, cells were infected with a clinical RSV strain (RSV-ON1-H1) at an MOI of 3 in the presence of 10 μM compound. 72 h post inoculation, cell viability as direct correlate to virus replication and/or cytotoxicity was analyzed using a firefly luciferase assay and a plate luminometer (Centro XS LB960, Berthold, Germany).

2.5. In vitro infection assay

Cells were seeded at a density of 1 × 10e4 cells/well in a 96-well plate 24 h prior to infection. Virus inoculation was performed at 37 °C for 4 h prior to medium change. If not stated otherwise, number of infection events was analyzed 24 h post inoculation.

2.6. Intracellular FACS analysis

Cells were detached by trypsinization and fixed in PFA-containing fixation buffer (0.5% paraformaldehyde, 1% FCS in PBS) for 30 min prior to permeabilization (0.1% Saponin in PBS) for 20 min at 4 °C. Intracellular RSV phosphoprotein was stained by addition of monoclonal anti-RSV-P antibody (26D6G5C6; final concentration 0.002 mg/ml in permeabilization buffer) (Haid et al., 2015) for 30 min and subsequent washing steps. After addition of a fluorescently labelled secondary anti-mouse antibody (Alexa Fluor488; final concentration 0.01 mg/ml in permeabilization buffer; Thermo Fisher Scientific, Waltham, USA) flow cytometry was performed using an Accuri C6 machine (BD, Heidelberg, Germany) followed by data analysis using FlowJo software (Tree Star, Ashland, USA).

2.7. RSV pseudoparticles

Lentiviral based RSV pseudoparticles were produced as described elsewhere (Haid et al., 2015). In brief, 1.5 × 10e6 293T cells were seeded into a 6-cm diameter plate and transfected with plasmids encoding for lentiviral gag-pol pCMV-ΔR8.74 (Dull et al., 1998), a reporter (pWPI-F-Luc-BLR or pWPI-R-Luc-BLR) as well as the RSV glycoproteins RSV-SH, -G and a c-terminally truncated -F (pcDNA-hRSV-SH, pcDNAhRSV-G or pcDNA-hRSV-F-trc). 30 h post transfection (for hRSV-F K394R mutant already 24 h post transfection), supernatants containing lentiviral particles were harvested, cleared of cell debris and preincubated with the drugs for 30 min prior to infection of A549 cells. 72 h post infection, cells were lysed and luciferase activity was analyzed using a tube luminometer (Lumat LB 9507; Berthold, Freiburg, Germany).

2.8. Liposome assays

Lipids purchased from Avanti Polar Lipids (Alabaster, AL) were dissolved as 10 mM stocks in 67% chloroform/33% methanol and liposomes were generated as described elsewhere (Henriques et al., 2011). HEp-2 cells were seeded on 96-well plates (1.5 × 10e4 cells/well) one day prior to infection. On day of infection, cells were preincubated with medium containing liposomes of different composition together with indicated concentrations of Laby A1/A2 for 30 min at 37 °C prior to addition of RSV-ON1-H1 virus (MOI 0.2) or rHRSV-A-Luc virus (MOI 0.1). Liposomes were used at a final concentration of 100 μM (20% DOPS/40% DOPC/40% DOPE and 20% DOPS/80% DOPC liposomes) or 10 μM (60% DOPC/40% DOPE and 100% DOPC liposomes), respectively. 24 h post infection, the number of RSV-ON1-H1-infected cells was analyzed using an intracellular FACS staining against the RSV phosphoprotein RSV-P. Supernatant of rHRSV-A-Luc virus was changed to fresh medium after 4 h and 24 h post infection cells were lysed for luciferase quantification using a plate luminometer (Berthold, Freiburg, Germany).

2.9. Proteinase K protection assay

The proteinase K protection assay was performed as described elsewhere (Lawless-Delmedico et al., 2000; Mader et al., 2000). In brief, liposomes either existing of phosphatidylcholine alone (DOPC) or a 60:40 ratio of phosphatidylcholine and phosphatidylethanolamine (DOPE) were preincubated with 40 μM Laby A1 for 30 min at room temperature. After 30 min of incubation, either conditioned media or RSV-ON1-H1 virus was added for another 30 min prior to addition of 2 μg/ml proteinase K (Roche, Basel, Switzerland) for 1h on ice. Addition of 5 mM PMSF (AppliChem, Darmstadt, Germany) and heating of the sample for 10 min at 98 °C was used to terminate proteinase K digestion. Virus treated with 2% (v/v) of the detergent Trition X-100 served as positive control. Samples were further analyzed on an 11% SDS-PAGE followed by Western Blot analysis using an antibody against the RSV phosphoprotein P (26D6G5C6) and a HRP-coupled secondary anti-mouse antibody (Sigma Aldrich, Steinheim, Germany). Detection of bound antibodies was performed with the ECL prime detection reagent (GE Healthcare, Freiburg, Germany) on a ChemoStar machine (Intas, Göttingen, Germany).

2.10. Drug-drug interaction

One day before infection, 1x10e4 HEp-2 cells were seeded in 96-well plates and infected with rHRSV-A-Luc virus (Rameix-Welti et al., 2014) at an MOI of 0.1 in the presence of various concentrations of different drug combinations. 24 h post infection, cells were lysed and luciferase activity was analyzed using a plate luminometer (Berthold, Freiburg, Germany). Synergistic, additive or inhibitory effects were determined using equations described by Prichard and Shipman (1990).

2.11. Quantitative RT-PCR to detect viral RNA

Viral RNA was isolated from infected cells or from cell culture supernatant of RSV infected well-differentiated primary human airway epithelial cells using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) or a high pure viral RNA kit (Roche, Mannheim, Germany). 2 μl of purified RNA was subsequently used for quantitative RT-PCR analysis using a one-step RT-PCR LightCycler480 RNA Master Hydrolysis Probe kit and a LightCycler480 machine (Roche, Mannheim, Germany). RSV-A specific probe (6FAM-CACCATCCAACGGAGCACAG GAGAT-BBQ; TIB Molbiol, Berlin, Germany) and primers (s-RSV-A 5'-AGATCAACTTCTGTCATCCAGCAA-3'; as-RSV-A 5'-TTCTGCACATCATA ATTAGGAG-3'; MWG-Biotech, Martinsried, Germany) were used as published elsewhere (Gunson et al., 2005). RSV-B specific viral RNA was detected using a RSV-B specific probe (YAK-ATGCTATGTCCAGG TTAGGAAGGG-BBQ; TIB Molbiol, Berlin, Germany) and primers (s-RSV-B 5'-GTCATAAATTCACAGGATTAATAGGTA-3'; as-RSV-B 5'- GCA TGATATCCAGCATCTTTAAGTA-3'; MWG-Biotech, Martinsried, Germany). Amplification was performed for 40 cycles at 62 °C for RSV-A and 55 °C for RSV-B respectively and RSV copy numbers were calculated using in run standard curves.

2.12. Infection of well-differentiated human air-liquid interface cultures

Human airway epithelia cells of bronchial origin (hBEC) were isolated from explanted human lungs as described elsewhere (Jonsdottir and Dijkman, 2015). In brief, human tissue was enzymatically digested for 48 h at 4 °C prior to isolation of epithelial cells from the lumen of the bronchus. Cells were seeded on collagen-coated flasks for proliferation prior to seeding on collagen-coated transwells with 0.4 μM pore polyester membrane inserts (Corning Costar). Upon confluency, the medium from the apical compartment was removed and cells were washed with HBSS (Thermo Fisher Scientific, Waltham, MA) and exposed to the air. Medium change in the basolateral compartment was performed every other day and the apical cell layer was washed once weekly with HBSS until cells differentiated into a well-differentiated human airway epithelium. At day of infection, cells were apically washed twice with HBSS prior to addition of RSV reporter virus (rHRSV-B05 eGFP or rHRSV-A-GFP) to the apical side for 2 h at 37 °C. Virus inoculum was removed and cells were washed two times with HBSS followed by basolateral medium change. For the prophylactic experiment setup, newly produced and released viruses were harvested on a daily basis by addition of 100 μl HBSS to the apical compartment for 30 min. Viral RNA was isolated using a high pure viral RNA kit (Roche, Mannheim, Germany) and analyzed on a LightCycler480 machine (Roche, Mannheim, Germany). For therapeutic treatment, cells were infected with rHRSV-A-GFP 8 h prior to start of treatment and treatment was performed twice daily. Therefore, Laby A1 and A2 were diluted in HBSS and cells were treated for 1 h from the apical side. 72 h post infection, cells were lysed and total RNA was isolated (Macherey-

Nagel, Düren, Germany) and analyzed on a LightCycler 480 machine (Roche, Mannheim, Germany). All patients gave informed consent for the donation of lung tissue samples, and all steps were conducted in compliance with good clinical and ethical practice and approved by the local ethical committee at Hannover Medical School (permission number 3346/2016).

2.13. Animal studies and ethics statement

BALB/cOlaHsd mice were purchased from ENVIGO and housed with food and water *ad libitum* under specific pathogen free conditions at the animal facilities of Twincore, Hannover. All experiments were conducted in compliance with good animal practice as defined by the Federation of Laboratory Animal Science Associations (FELASA) and German welfare regulations and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, permission number 33.9-42502-04-17/2416).

14 week old female mice were infected with 1.9 × 10e5 PFU of rHRSV-A-Luc or PBS intranasally. Starting 1 h before infection, mice were treated intranasally with 30 µl of 2 mg/ml of Laby A1/A2 or vehicle control (20% DMSO, 80% PBS) once daily. Every second day, *in vivo* imaging of luciferase-based luminescence was measured using an *in vivo* optical imaging system (IVIS SpectrumCT; Perkin Elmer) after intranasal delivery of 30 µl of 10 mM D-Luciferin in PBS. For all treatments, infections and luciferase measurements, mice were anesthetized with Isoflurane. At the end of the experiments, mice were anesthetized with Ketamin (100 g/kg BW) and Xylazin (10 g/kg BW) and perfused with PBS. Lungs were extracted for histological and flow cytometric analysis.

3. Results and discussion

3.1. HEp-2-F-Luc reporter cells for screening of RSV inhibitors with primary isolates

To enable rapid identification of RSV inhibitors by screens with a primary clinical RSV isolate, we transduced HEp-2 cells with a lentiviral vector encoding a firefly luciferase reporter gene. The resulting HEp-2-F-Luc cells constitutively express firefly luciferase (F-Luc) and therefore the amount of F-Luc produced is proportional to the number of viable cells. Since RSV lytically infects HEp-2 cells, inoculation of HEp-2-F-Luc cells results in cell killing and subsequent elimination of luciferase reporter signal unless a non-cytotoxic RSV inhibitor prevents viral infection, replication and spread. The dynamic range of the assay comprises approximately two orders of magnitude, which permits robust and accurate quantification of even minor inhibitory effects. The principle of the screening assay is depicted in Fig. 1A. As expected, Ribavirin dose-dependently inhibited infection of HEp-2-F-Luc cells with a primary RSV-A isolate (RSV-ON1-H1). The antiviral effect was evident from maintenance of cell viability in RSV-ON1-H1 inoculated HEp-2-F-Luc cells as visualized by crystal violet staining (Fig. 1B). Notably, addition of Ribavirin also dose dependently increased detectable luciferase expression (Fig. 1C), and at a level of 100 µM Ribavirin the reporter signal reached ca. 50% of the signal in uninfected control cells (Fig. 1C).

Next, we used this assay to screen a small library of natural products with proven biological activity (Gentzsch et al., 2011) for inhibitors of RSV-ON1-H1 infection. Palivizumab and varying doses of Ribavirin were used as control and effectively restored luciferase activity upon virus infection (Fig. 1D). Among the tested compounds, Laby A1 and A2 showed the greatest antiviral effect maintaining luciferase reporter expression at 76% and 62% of uninfected control cells. Notably, Ferir et al. had previously described antiviral activity of Laby A1 against HIV-1 and HSV-1, suggesting a broad antiviral activity (Ferir et al., 2013). However, an antiviral activity of Laby A2 had not yet been described and an activity against a respiratory virus had not been reported for both Laby A1 and A2. Next, we confirmed the antiviral activity of these two lanthipeptides by inoculation of HEp-2 cells with RSV-ON1-H1 in presence of varying doses of these compounds. Infected cells were quantified by intracellular flow cytometry of virus-expressed P protein (Fig. 1E). Using this direct virus detection assay, we confirmed the antiviral activity of both molecules with an IC_{50} of 0.39 µM for Laby A1 and of 4.97 µM for Laby A2, respectively. The cytotoxic concentration 50% (CC_{50}) for Laby A1 was 79 µM and could not be determined for Laby A2 due to a limited solubility of this compound beyond 100 µM (Fig. 1F). Collectively, these results show that Laby A1 and A2 effectively inhibit RSV infection in cell culture in a low micromolar range and with a therapeutic index of 204 for Laby A1 and >20 for Laby A2, respectively.

3.2. Laby A1/A2 inhibit RSV entry through acting on virus particles

The effect of Laby A1/A2 on the RSV lifecycle was determined using time of addition experiments and cell entry assays with lentiviral pseudotypes (Haid et al., 2015) (Fig. 2). HEp-2 cells were either pretreated with Laby A1/A2, heparin or Palivizumab, or these compounds were present during RSV-ON1-H1 inoculation or compounds were added directly after removal of the inoculum for another 4 h (Fig. 2A and B). Comparable to the cell entry inhibitors Palivizumab and heparin, LabyA1/A2 only inhibited RSV-ON1-H1 infection when present during inoculation (Fig. 2A). Moreover, a more time-resolved assay revealed that RSV-ON1-H1 infection is essentially resistant to addition of Laby A1/A2, when these compounds are added 90 min after virus inoculation (Fig. 2B). When A549 cells were infected with lentiviral pseudotypes carrying either the G protein of the vesicular stomatitis virus (VSV-G), RSV-F protein or RSV-F, -G and -SH proteins, the infection was dose-dependently blocked by Laby A1/A2 (Fig. 2C). Finally, RSV-ON1-H1 particles were incubated with Laby A1 or A2, with Ribavirin or Palivizumab for 30 min. Subsequently, residual virus infectivity was quantified by serial dilution end point titration and reported as tissue culture infectious dose 50% (TCID50) (Fig. 2D). Similar to Palivizumab, which directly binds to the F protein of virus particles, Laby A1/A2 also inhibited RSV infection after pre-incubation of RSV particles. Collectively, these results show that Laby A1/A2 inhibit RSV cell entry, likely by directly acting on RSV particles.

3.3. Laby A1 and A2 disrupt RSV particle integrity

Recently, Prochnow et al. reported an antiviral activity of Laby A1/A2 against dengue virus and they noted that Laby A1/A2 specifically interact with the membrane lipid phosphatidylethanolamine (PE) to exert their antiviral effect (Prochnow et al., 2020). To investigate if Laby A1 and A2 act on RSV particles and inhibit their infectivity also through binding to PE, we prepared unilamellar vesicles composed of phosphatidylserine (PS) together with PE and phosphatidylcholine (PC) or, as control, PC and PS. Subsequently, HEp-2 cells were inoculated with RSV-ON1-H1 in presence of increasing doses of Laby A1 or A2 either in the absence of liposomes or in the presence of PS/PC/PE or PS/PC-containing liposomes (Fig. 3A). Addition of PS/PC-containing liposomes did not change the antiviral activity of Laby A1 or A2. In contrast, in presence of PS/PC/PE-liposomes the antiviral activity of both Laby A1 and A2 was strongly attenuated. Liposomes also interfered with the antiviral effect of Laby A1 when they were composed of PC and PE only, ruling out an important role of PS (Fig. 3B). These results suggest that excess PE binding sites on the liposomes compete for binding of Laby A1 and A2, thereby reducing their antiviral activity. Next, we examined whether addition of Laby A1 destabilizes the membrane of RSV particles and whether addition of PE-containing liposomes rescues virus membrane destabilization. To this end, we used a proteolytic protection assay that quantifies the resistance of the virus

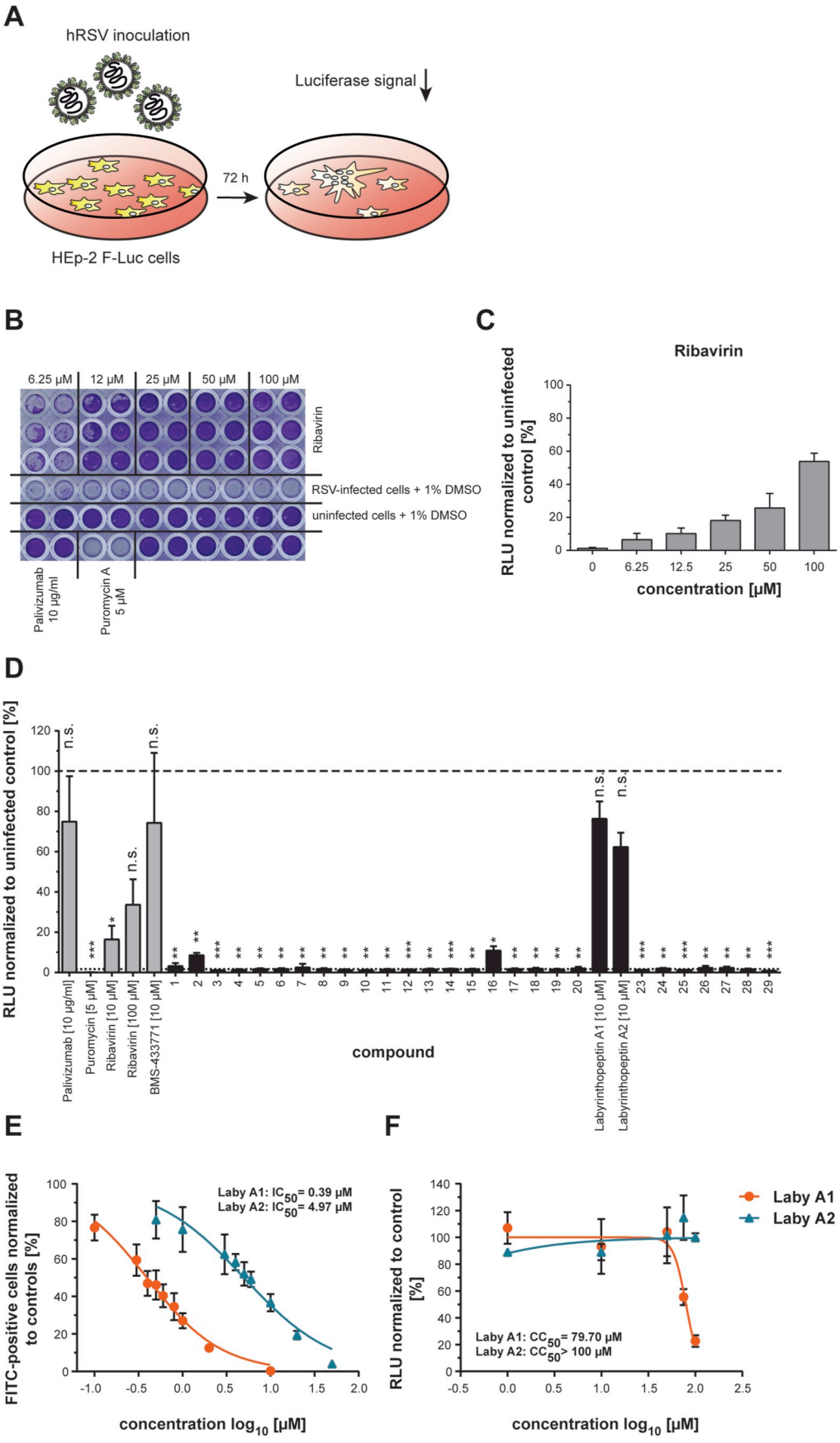

Fig. 1. Identification and characterization of Laby A1/A2 as anti-RSV drugs. A) Screening principle. HEp-2 cells expressing a firefly luciferase reporter gene (HEp-2-F-Luc cells) were infected with RSV. Infection leads to cell lysis thereby decreasing F-Luc expression. (B) Cell survival visualized by crystal violet staining. HEp-2-F-Luc cells were infected with RSV-ON1-H1 in the presence of indicated drugs. 72 h post inoculation cells were fixed and stained with 1% crystal violet solution. Known inhibitors of RSV infection (Ribavirin and Palivizumab) rescued cell survival, visible by the dark violet staining. Puromycin A, a translation inhibitor, killed the cells. (C) Quantification of dose-dependent inhibitory effect of Ribavirin. D) HEp-2-F-Luc cells were infected with RSV-ON1-H1 (MOI 3) in the presence of compounds (Gentzsch et al., 2011) and survival of cells was analyzed 72 h later. (RLU: relative light unit). RLU counts were normalized to uninfected control cells and are given as percentage of cell viability. Mean of 2 independent experiments and standard deviation are given. One sample *t*-test comparing all sample means to hypothetical 100% cell survival without correction for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant. The dotted line indicates signal of infected control cells, the dashed line marks 100% cell survival. E) HEp-2 cells were inoculated with RSV-ON1-H1 in the presence of indicated concentrations of compounds. 24 h post infection, an intracellular FACS analysis detecting RSV-P was performed. The number of RSV-infected cells was normalized to solvent-treated control. Mean values of 3 independent experiments with standard deviation is given. F) Cytotoxicity of Laby A1/A2 was analyzed in HEp-2-F-Luc cells by incubating the cells with the indicated concentrations of compounds for 72 h prior to measurement of firefly luciferase activity. Mean values of 3 independent experiments normalized to solvent control and standard deviation is given.

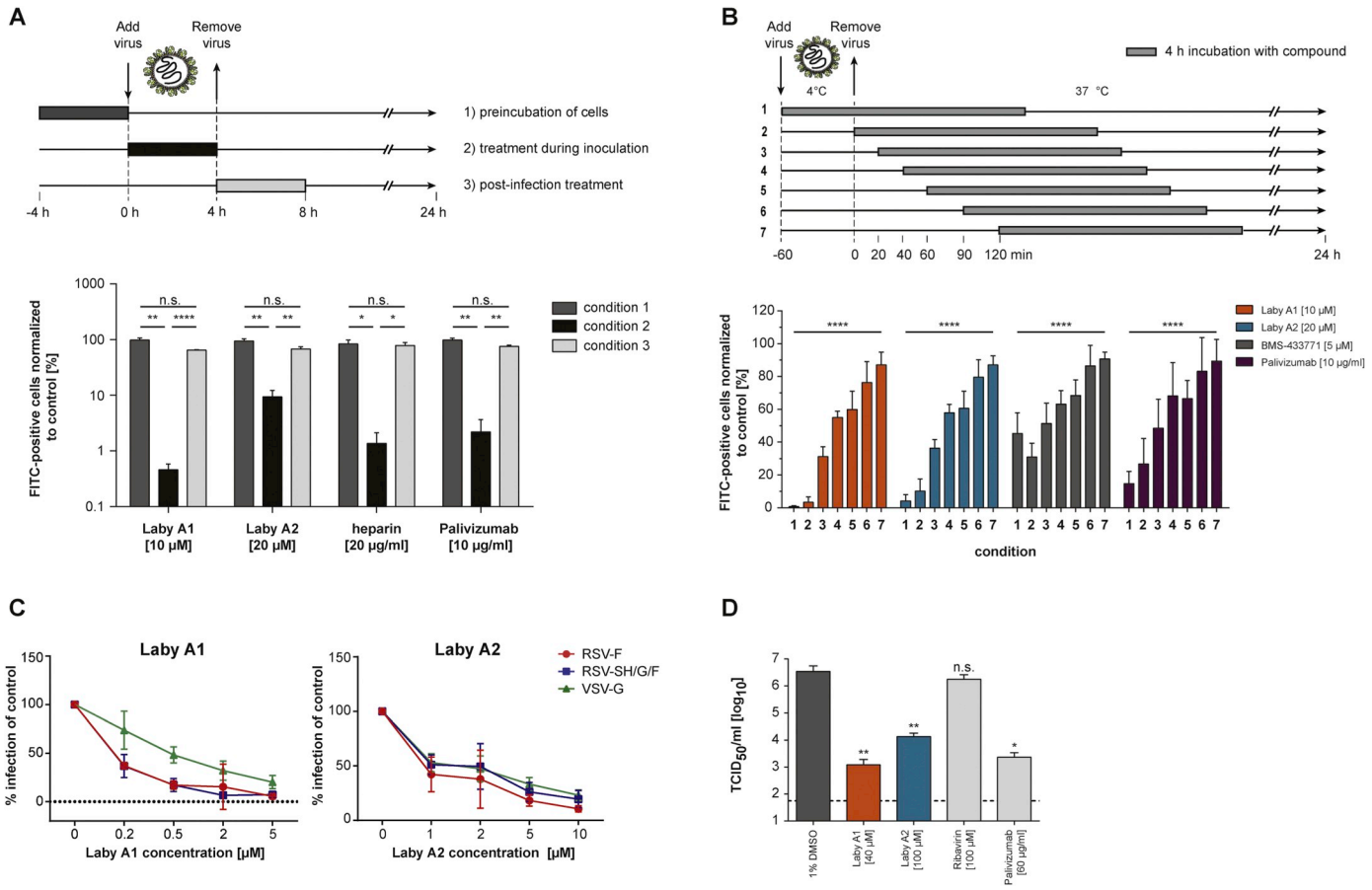

Fig. 2. Laby A1/A2 inhibits RSV entry. A) Experimental setup. HEp-2 cells were either pretreated (dark grey bar), treated during RSV-ON1-H1 virus inoculation (black bar) or treated after virus infection (light grey bar) with indicated concentrations of compounds for 4 h. 24 h post infection, the number of RSV-positive cells was determined by intracellular RSV-P protein FACS staining. Data are given as percentage of solvent control. Repeated measures 2way ANOVA followed by Tukey's multiple comparison correction, with time of addition means compared within each treatment. Mean values and standard deviation of 3 independent experiments are depicted. B) Schematic depiction of experimental setup. HEp-2 cells were incubated with RSV-ON1-H1 virus at 4 °C for 1 h to allow virus attachment and binding. Unbound virus particles were subsequently removed by PBS washes, pre-warmed media was added and the cells were shifted to 37 °C to allow incorporation of bound viral particles. Antiviral treatments were added at different time points after temperature shift for a total of 4 h and an intracellular anti-RSV-P staining was performed and the number of RSV positive cells was detected by FACS analysis 24 h post infection. Repeated measures 1way ANOVA followed by test for linear trend, with means compared from left to right within each treatment group. Mean of 3 independent experiments with standard deviation are given. C) Two different firefly-luciferase encoding lentiviral RSV pseudotypes (red circle and blue square) or VSV-G pseudotypes (green triangle) were pretreated with increasing concentrations of Laby A1/A2 prior to infection of A549 cells. 72 h post infection, A549 cells were lysed and the luciferase activity was measured with a tube luminometer (Lumat LB9507; Berthold, Freiburg, Germany). Mean values and SD of at least 4 independent experiments are shown. D) RSV-ON1-H1 virus was preincubated for 1 h at room temperature with indicated concentrations of compounds prior to serial 5-fold dilutions and infection of HEp-2 cells. 8 wells of a 96-well plate were inoculated with the same virus dilution and 24 h post inoculation RSV-infected cells were histochemically stained with a RSV-P antibody and the virus titer was determined via TCID50 analysis. Dashed line indicates the limit of detection (LOD). Repeated measures 1way ANOVA of logarithmic transformed values followed by Dunnett's multiple comparison correction, with treatment means compared to DMSO control. Mean of 3 independent experiments including standard deviation is given. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; n.s., not significant.

particle associated P protein to exogenously added proteinase K digestion (Fig. 3C). RSV particles contain a lipid membrane, and therefore P protein, which is associated with the polymerase complex, is not accessible to exogenously added proteinase K (Fig. 3C; line 8), unless the viral membrane is disrupted, as for instance by treatment with the detergent Triton X-100 (line 16). RSV-ON1-H1 particles were incubated with proteinase K in either presence or absence of Laby A1 that had been preincubated with liposomes of different composition and concentration. When RSV particles were incubated with proteinase K in the absence of Laby A1 (lane 8), P protein was readily detectable. In contrast, presence of Laby A1 increased the P protein susceptibility to proteinase K (line 9). Co-incubation of particles with proteinase K and Triton X-100 caused degradation of P protein to a level below the detection limit of our assay (line 16). Addition of PE-containing liposomes protected P protein from degradation in a dose dependent manner (line 10, line 12, line 14), whereas PC-containing liposomes were less potent in protecting the viral particles from proteinase K digestion (line 11, line 13, line 15). These results suggest that Laby A1 likely inhibit RSV particle infectivity by binding to PE within the particle membrane and by disrupting the viral lipid envelope. The observation that addition of PE- but not PC-liposomes competes with the antiviral activity of both Laby A1 and Laby A2 supports the notion that an interaction between Laby A1/A2 and virus particle associated PE is critical for the antiviral activity. In turn, the abundance and accessibility of PE in the lipid membrane of virus particles may govern susceptibility of virus particles to Laby A1/A2. Along similar lines, the concentration of PE in cellular membranes may influence Laby A1/A2 cytotoxic effects. It is worth mentioning that both Laby A1 and A2 have favourable therapeutic indices (204 and > 20 for A1 and A2, respectively). The pronounced selectivity for disruption of virus particles over cytotoxicity could be related to differential

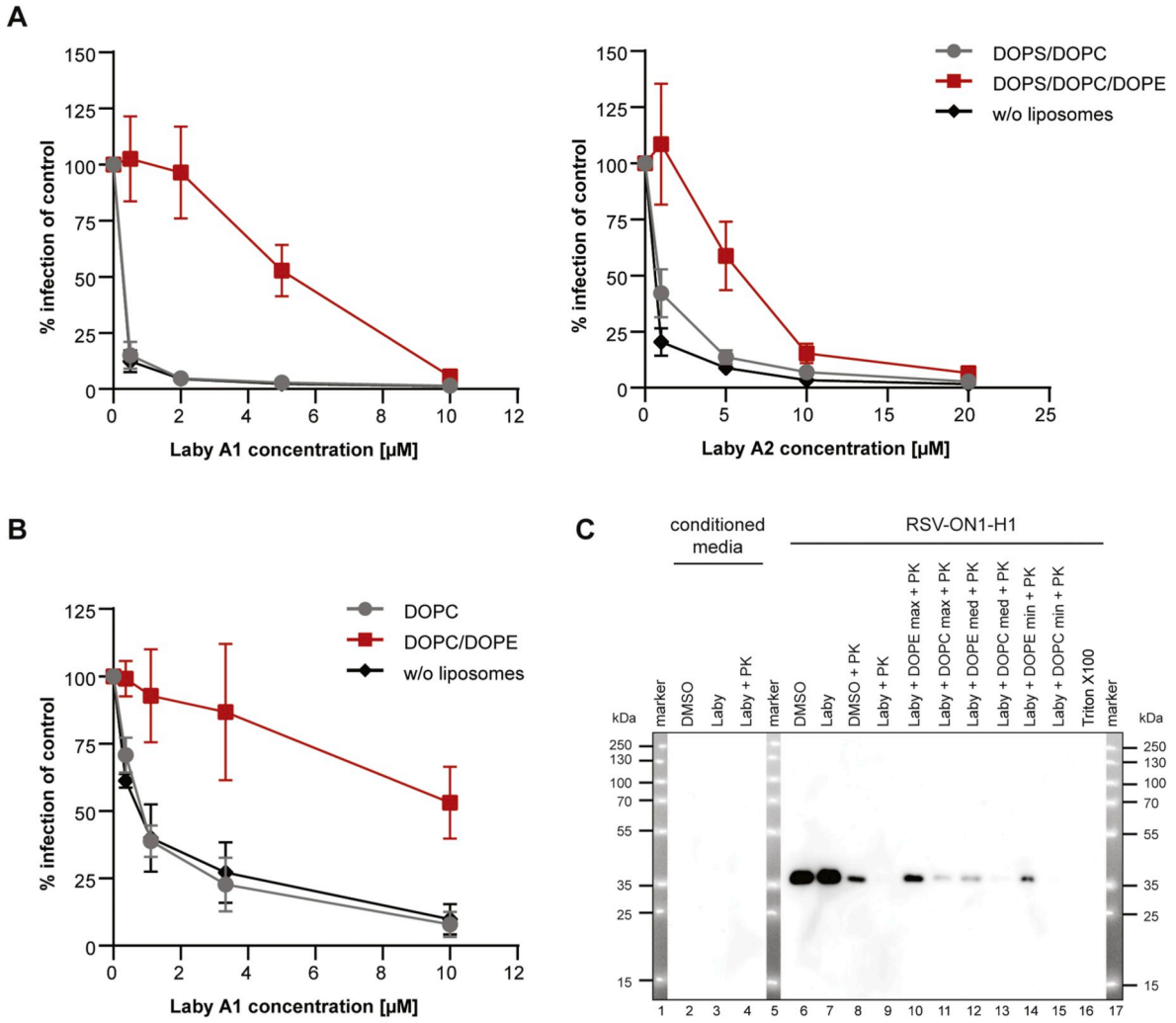

Fig. 3. Laby A1/A2 bind to phosphatidylethanolamine and thereby destabilize the particle integrity. A) HEp-2 cells were pretreated for 30 min at 37 °C with increasing concentrations of Laby A1/A2 and a 10% fixed concentration of liposomes with varying composition (DOPE, DOPC, DOPS red squares; DOPC, DOPS grey circles) or without addition of liposomes (black diamonds) prior to infection with RSV-ON1-H1 at an MOI of 0.2. 24 h post inoculation, number of RSV-infected cells was determined by intracellular RSV-P staining and subsequent FACS analysis. Mean values of 3 independent experiments and standard deviation are given. B) Liposomes composed of DOPC alone or of a mixture of DOPC and DOPE were incubated with given amounts of Laby A1 prior to incubation with RSV luciferase reporter virus particles. Mean values of two independent experiments are shown normalized to control infections performed in the absence of laby. C) 40 µM of Laby A1 or DMSO were preincubated for 30 min at room temperature with 3 different concentrations (max, med, min) of either PE-containing (DOPE) or pure PC-containing liposomes (DOPC). Subsequently, RSV-ON1-H1 virus or cell culture medium derived from uninfected cells (conditioned media) were added for another 30 min at room temperature prior to addition of proteinase K (2 µg/ml final concentration) for 1 h on ice and subsequent inactivation of the proteinase K at 98 °C for 10 min. Samples were separated on a 11% SDS-PAGE followed by Western Blot analysis using an antibody against RSV-P. One out of 2 independent experiments is shown.

abundance of PE in viral and host cell membranes. Alternatively or in addition, cellular membrane repair mechanisms, which are absent in virus particles, may prevent lethal disruption of cell membrane integrity up to very high Laby A1/A2 doses (Brito et al., 2019).

3.4. Laby A1/A2 inhibit primary RSV strains and viruses resistant to entry inhibitors

RSV strains are classified in two antigenic subgroups (RSV-A and RSV-B) (Anderson et al., 1985) and multiple genotypes based on the variability of the glycoprotein G (Griffiths et al., 2017). To examine the breadth of the anti-RSV activity of Laby A1 and A2, we used 2 additional primary clinical isolates representing the RSV subtypes B and GA2 along with the well-known cell culture passaged RSV-A2 and RSV-long strains. As is depicted in Fig. 4A, all primary strains were inhibited by both Laby A1 and A2 in a dose-dependent manner and with comparable IC50 values.

Single point mutations are sufficient for pronounced resistance of RSV to Palivizumab (Zhao et al., 2006; Zhu et al., 2011) or fusion inhibitors as for instance BMS-433771 (Battles et al., 2016; Cianci et al., 2004; Yan et al., 2014). To test cross-resistance of known resistance mutations to Palivizumab or fusion inhibitors like BMS-433771, we performed a lentiviral-based pseudoparticle infection assay (Fig. 4B) (Haid et al., 2015) or infections with a RSV-ON1-H1 virus population that is resistant to Palivizumab (Fig. 4C). Susceptibility to Laby A1/A2 was not affected by the BMS-433771 resistance mutation (K394R) and decreased only minimally by the resistance mutations against Palivizumab (K272E). However, because we did not observe cross resistance of the RSV-ON1-H1 population to LabyA1/A2 we conclude that Laby A1/A2 have a different mode of action that is not affected by resistance mutations against other classes of RSV entry inhibitors.

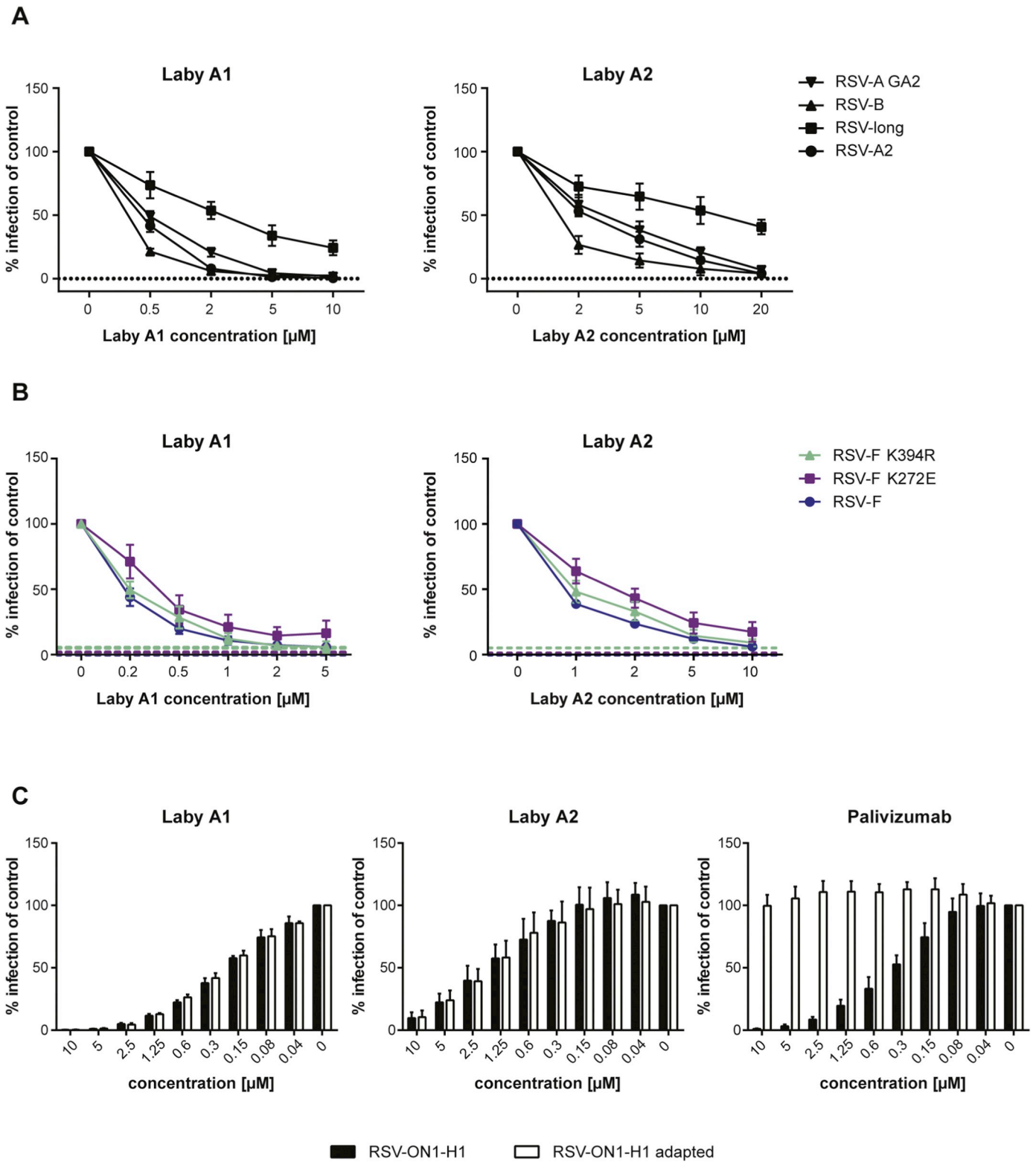

Fig. 4. Drug-resistant mutant viruses are sensitive to Laby A1/A2 treatment. A) Clinical RSV isolates obtained from nasal lavage of RSV-infected children and subsequently cultured on HEp-2 cells were preincubated with Laby A1/A2 at the given concentrations for 20 min prior to infection of HEp-2 cells (MOI 1) for 4 h. Infected cells were quantified 24 h later by FACS analysis using RSV-P-specific antibodies. Means of 3 independent experiments and standard deviations are given. For RSV-B and RSV-A-GA2 only n = 2 is depicted for 20 µM Laby A2. B) A549 cells were inoculated with Renilla-luciferase expressing lentiviral RSV pseudoparticles harboring a 4 aminoacid truncated wild type F-protein (blue circle), a truncated mutant F-protein resistant to Palivizumab treatment (purple square; K272E) or a truncated mutant F-protein resistant to treatment with the fusion inhibitor BMS-433771 (green triangle; K394R) in the presence of indicated concentrations of Laby A1/A2. Results are normalized to solvent treated control. Dashed lines indicate the measurement background for the individual pseudoparticles. Means of 4 independent experiments and standard deviation are given. C) RSV-ON1-H1 virus was cultured for 5 passages in the presence of increasing concentrations of Palivizumab to generate a naturally occurring mutant virus resistant to Palivizumab treatment (Zhu Q et al., JID2011). Amino acid exchange (K272T) in the fusion protein responsible for Palivizumab resistance has been verified by Sanger sequencing (data not shown). Wildtype (black) and resistant mutant (white) of the RSV-ON1-H1 isolate were treated with increasing concentrations of Laby A1, Laby A2 or Palivizumab prior to infection of HEp-2 cells. 24 h post infection, an intracellular FACS analysis against the RSV-P protein was performed. Mean values and standard deviations of 3 independent experiments normalized to solvent control are shown.

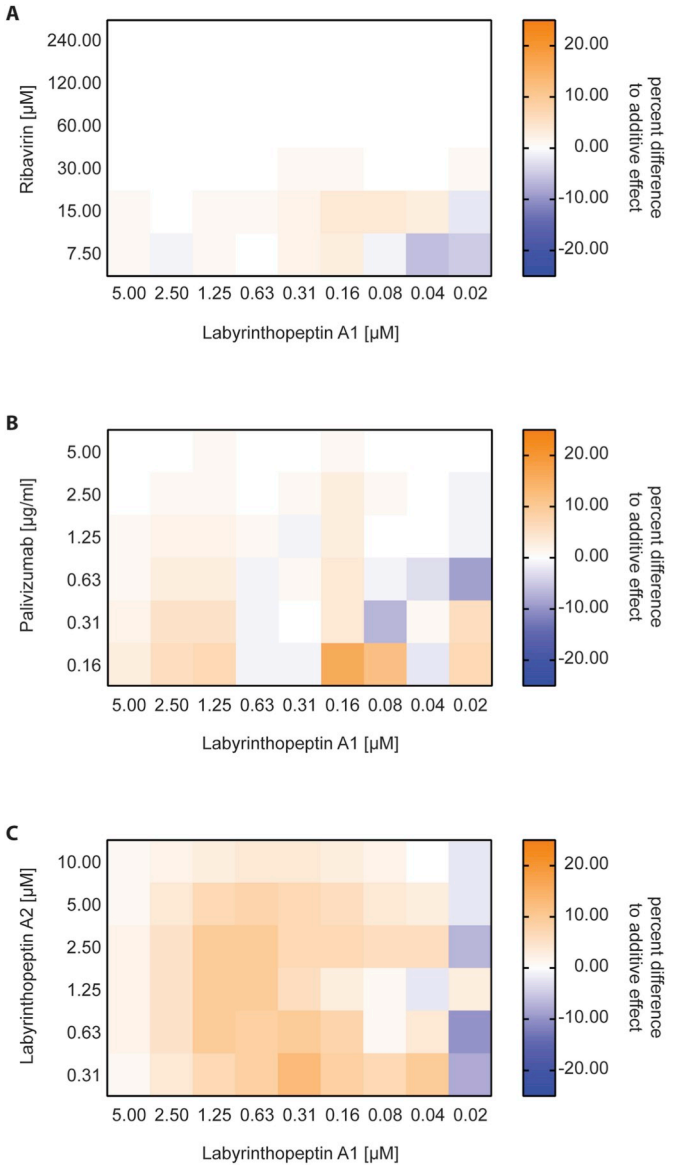

Fig. 5. A) Antiviral effect of Laby A1 and Ribavirin drug combinations in HEp-2 cells. The dose ranges of compounds are given. Cells were lysed 24 h post infection with rHRSV-A-Luc virus in the presence of different drug combinations and luciferase activity was measured. Luminescence values obtained from single drug treatment were normalized to the DMSO control and the theoretical additive effect was calculated using equations described by Prichard and Shipman. Normalized values of combined compounds were compared to the theoretical additive surface and the difference was plotted. Shading indicates different ranges above or below the expected value. Mean of 2 independent experiments is shown. B) Drug-drug interaction of Laby A1 and Palivizumab. Mean of 2 independent experiments is given. C) Drug-drug interaction of Laby A1 and Laby A2. Means of 3 independent experiments are given.

3.5. Synergistic effect of Laby A1/A2 in combination therapy

As resistance to RSV entry inhibitors arises rapidly, we tested whether a combination of different inhibitors had antagonistic, additive or synergistic effects. In Fig. 5 we display deviations from theoretical additive effects, which were calculated as described by Prichard and Shipmann (Prichard and Shipman, 1990) for the combination of Laby A1 with Ribavirin (Fig. 5A), Palivizumab (Fig. 5B) or Laby A2 (Fig. 5C). None of the combinations tested exerted an antagonistic effect. Combined treatment of Laby A1 with Ribavirin was additive across the entire tested dose range (Fig. 5A). For combination of Laby A1 with Palivizumab we observed a modest synergistic activity in selected dose combinations. The combination of Laby A1 with Laby A2 exerted a synergistic effect across a broad range of doses (Fig. 5B and C). The mechanism of synergism between Laby A1 and A2 is presently unknown, however, it is possible that these peptides may cooperatively bind and destroy virus particles leading to the observed synergism.

3.6. Laby A1/A2 inhibit RSV infection in primary human airway epithelial cells and in vivo

In human *ex vivo* cell culture models, RSV infects well-differentiated and polarized lung cells that form a pseudostratified epithelium of the respiratory tract (Villenave et al., 2012). To investigate the antiviral effect of Laby A1/A2 in this most authentic cell culture model, we infected well-differentiated human primary epithelial cells grown in air-liquid interface cultures with RSV. The cells were either treated with Laby A1/A2 during virus inoculation (Fig. 6A), or treatment was initiated 8 h post infection (Fig. 6B). The drugs were applied to the apical side of the epithelium to mimic an inhalative administration route, and the effect on RSV infection was analyzed by qRT-PCR. Prophylactic treatment of RSV infection with Laby A1 and A2 significantly reduced production of viral progeny approximately 100-fold for Laby A1 and 30-fold for Laby A2 48 h post infection (Fig. 6A). The antiviral effect was less pronounced but still significant 72 h post infection, as the drug was only given once. In the therapeutic regimen, treatment was initiated 8 h post virus infection with subsequent twice daily treatments. In this setting, a dose-dependent decrease in viral RNA from cell lysates 72 h post treatment onset was observed for both Laby A1 and Laby A2 (Fig. 6B), but the antiviral effect was less pronounced compared to prophylactic treatment.

Finally, to analyze the antiviral effect of Laby A1/A2 *in vivo*, we pretreated Balb/c mice intranasally with Laby A1/A2 for 1 h prior to intranasal infection with an rHRSV-A-Luc reporter virus (Rameix-Welti et al., 2014). Treatment was repeated daily over eight days, and RSV infection was monitored on an IVIS machine by measuring the luminescence signal intensity. Signal intensity was significantly lower in the noses of animals treated with Laby A1 or A2 between days 2–6 after virus inoculation (Fig. 6C and D). However, no significant decrease in viral load related luminescence was detectable in the lungs of treated animals, most probably due to poor delivery of the compounds to the lower respiratory tract (Fig. 6C and D). One RSV-infected, vehicle-treated animal but none of the Laby A1 or A2 treated mice died during anaesthesia (day 6 post infection). Laby A1 or A2 treated animals showed an initial weight loss, which was significant for Laby A1 treated compared to untreated animals, but plateaued over time (Fig. 6E). Histological analysis did not reveal any cytotoxic effects in the lungs of Laby A1 or A2 treated animals (data not shown) and no further adverse drug effects were observed. It is possible that Laby A1 interactions with membrane lipids of host lung cells cause weight loss for instance by impeding gas exchange. Future studies focus on the development of improved drug delivery formulations for a parenteral and/or inhalative administration and establishment of a safety profile for both drugs.

4. Conclusion

Labyrintopeptin A1 and A2 represent a novel class of RSV entry inhibitors with IC50's in the low micromolar range, with favourable therapeutic index, and with efficacy against a broad range of RSV strains including primary isolates. They exert a synergistic antiviral activity by directly acting on virus particles, likely through binding to virus membrane-resident phosphatidylethanolamine and through exerting a virolytic effect. Due to this novel mode of action, viral resistance to licensed entry inhibitors or clinical-stage virus fusion

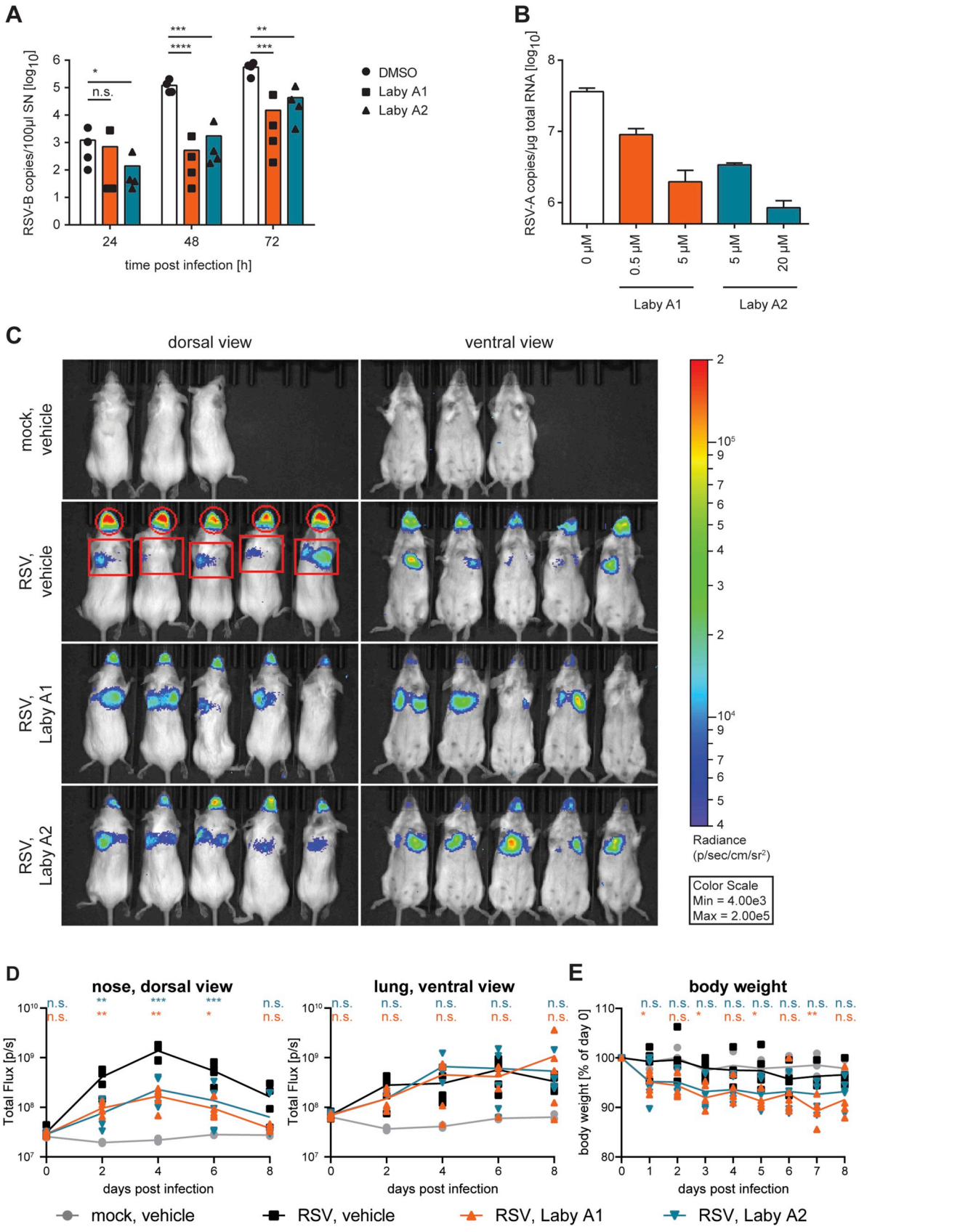

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Fig. 6. Laby A1/A2 reduces viral load in well-differentiated human airway epithelial cells as well as *in vivo*. A) Well-differentiated human airway epithelial cells grown under air-liquid interface conditions were infected with 1 × 10e4 infectious hRSV-B05 eGFP virus particles (Lemon et al., 2015) in the presence of the solvent control DMSO, 5 μM Laby A1 or 10 μM Laby A2 respectively. 24 h, 48 h and 72 h post infection, the amount of newly produced and released RSV particles into the supernatant was determined by RSV-B specific qRT-PCR analysis. Mean of one experiment with 4 individual donors, each measured in duplicates, is given. Repeated measures two-way ANOVA of logarithmic transformed values followed by Dunnett's multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. B) Well-differentiated human airway epithelial cells from a single donor were infected with rHRSV-A-GFP virus 8 h before onset of therapeutic treatment with Laby A1/A2. Treatment from the apical side was repeated twice daily for 1 h and 72 h post treatment onset, cells were lysed, total RNA isolated and measured via qRT-PCR analysis. Mean and SD of duplicate measurements from one donor is given. C) & D) Balb/c mice were treated intranasally with 30 μl of 2 mg/ml Laby A1/A2 or solvent control every 24 h for 8 days. One hour after first treatment, mice were infected intranasally with rHRSV-A-Luc. C) Pictures of mice and visualization of luminescence signal intensity measured on an IVIS Spectrum CT on day 4 post infection. Luminescence signal was measured for 180 s with f/stop of 1 and binning of 8. Pseudocolor photon emission images were generated and show the average radiance (sum of photons/second/cm^2/steradian) as indicated by the scale. Regions of interest (ROI) used for quantification in D) are exemplarily shown in red circles (nose) and squares (lung) for the dorsal view of RSV infected, vehicle treated animals. D) Quantification of luminescence in the nose (dorsal view) or lung (ventral view) as total photon flux (photons/second per ROI) over time. Individual values (dots) and group means (line) of one experiment with 3–5 animals per group are shown. Repeated measures mixed-effects analysis of logarithmic transformed values followed by Dunnett's multiple comparison test, with Laby A1 and A2 means compared to vehicle treated, infected animals. E) Weight of mice during the experiment normalized to the starting weight on day 0 of the respective animal. Individual values (dots) and group means (line) of one experiment with 3–5 animals per group are sown. Repeated measures mixed-effects analysis followed by Dunnett's multiple comparison test, with Laby A1 and A2 means compared to vehicle treated, infected animals. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant.

inhibitors does not cause cross-resistance. Finally, Laby A1 and A2 inhibit RSV infection in prophylactic and therapeutic treatment regimens in primary human lung cells and show promising activity in a murine model *in vivo*.

Declaration of competing interest

The antiviral activity of Laby A1/A2 against a broad spectrum of enveloped viruses has been patented (PCT/EP2016/078143). Otherwise, no competing financial interests exist.

Acknowledgements

This work was supported by the Innovation Fonds of the Helmholtz Association, by the Pre-4D-Fonds of the Helmholtz Centre for Infection Research, and by the Helmholtz-Alberta Initiative for Infectious Disease Research (HAI-IDR). T.P. and G.H. are funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC 2155 “RESIST” – Project ID 39087428. M.W. received a clinical leave scholarship from the German Centre for Infection Research (DZIF) and was funded by a scholarship from the Young Academy of Hannover Medical School. R.D. and V.T. were supported by the Swiss National Science Foundation (grants 310030_179260 (R.D.) and 310030_173085 (V.T.). We are grateful to Richard Karl Plemper for gift of BMS-433771. We thank all members of the Institute for Experimental Virology at TWINCORE-Centre of Experimental and Clinical Infection Research for helpful comments and discussion of this work. TWINCORE is a joint venture of the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research, Braunschweig (HZI).

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