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Influences of cyclosporin A and non-immunosuppressive derivatives on cellular cyclophilins and viral nucleocapsid protein during human coronavirus 229E replication

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ABSTRACT

The well-known immunosuppressive drug cyclosporin A inhibits replication of various viruses including coronaviruses by binding to cellular cyclophilins thus inactivating their cis-trans peptidyl-prolyl isomerase function. Viral nucleocapsid proteins are inevitable for genome encapsidation and replication. Here we demonstrate the interaction between the N protein of HCoV-229E and cyclophilin A, not cyclophilin B. Cyclophilin inhibitors abolish this interaction. Upon infection, cyclophilin A stays evenly distributed throughout the cell, whereas cyclophilin B concentrates at ER-bleb-like structures. We further show the inhibitory potential of non-immunosuppressive CsA derivatives Alisporivir, NIM811, compound **3** on HCoV-229E-GFP and -Luciferase replication in human Huh-7.5 hepatoma cells at 18 and 48 h time points post infection with EC_{50} s at low micromolar ranges. Thus, non-immunosuppressive CsA derivatives effectively inhibit HCoV-229E replication suggesting them as possible candidates for the treatment of HCoV infection. The interruption of interaction between CypA and N protein by CsA and its derivatives suggest a mechanism how CypA inhibitors suppress viral replication.

1. Introduction

Coronaviruses (CoVs) infect a number of mammalian species mostly causing respiratory and gastrointestinal tract pathologies (Perlman and Netland, 2009). The six human CoVs, namely HCoV-229E, -OC43, -NL63, -HKU1, SARS (severe acute respiratory syndrome)-CoV, MERS (Middle East respiratory syndrome)-CoV are of zoonotic origin (Corman et al., 2018). The first four viruses mainly target the respiratory tract and are associated with common colds, whereas SARS- and MERS-CoV are highly pathogenic with high mortality rates (Cui et al., 2019). Although great efforts have been made to discover anti-MERS agents by screening defined drug libraries (Cao et al., 2015; de Wilde et al., 2014; Dyall et al., 2014; LaFemina, 2014) no effective drug treatment is available against CoVs.

In order to identify host-targeting agents (HTAs) we have recently performed virus-host protein-protein interaction screenings by testing individual SARS-CoV ORFs against human cDNA libraries utilizing high-throughput yeast-2-hybrid techniques (Pfefferle et al., 2011). In that study we had identified cyclophilins and FK506-binding (FKBPs) proteins as cellular interaction partners of the viral Nsp1 protein and the cyclophilin-binding immunosuppressive drug cyclosporin A (CsA) as a replication inhibitor of the various human and animal CoVs including SARS-CoV, NL63, 229E and Feline CoV, Transmissible Gastroenteritis Virus, Infectious Bronchitis Virus, respectively. In a follow-up study we found that non-immunosuppressive CsA derivatives Alisporivir (ALV), NIM811 and further compounds inhibit replication of NL63 and that CypA is an essential cellular molecule required for virus replication (Carbajo-Lozoya et al., 2014). Similar inhibitory properties of CsA and derivatives on CoV and Arterivirus replication, both belonging to the order of *Nidovirales* were described (de Wilde et al., 2011, 2013a, 2013b).

Cyclophilins are ubiquitous enzymes catalyzing the *cis/trans* isomerization of prolyl peptide bonds (PPIase activity) thus facilitating protein folding (Lang et al., 1987). The most prominent human

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cyclophilin is CypA with important roles in many biological processes such as protein folding and trafficking (Nigro et al., 2013). In addition, the coincidental binding of the CsA/CypA complex causes immunosuppression, i.e. it prevents activation of the transcriptional regulator Nuclear Factor of Activated T-cells (NFAT). Inhibition of the PPIase activity not only prevents correct folding of cellular, but also of a number of viral proteins indispensable for viral replication. This was shown first for Human Immunodeficiency Virus 1 (HIV-1) and Hepatitis C virus (HCV) (Hopkins and Gallay, 2015; Lin and Gallay, 2013). Thus, cyclophilins are discussed as therapeutic targets of viral liver diseases (Naoumov, 2014).

For treatment of virus infection with relatively low pathogenicity, the inhibition of the PPIase but not the immunosuppressive activity of CsA is desirable. A number of CsA derivatives have been developed which do fulfill these criteria: ALV (Gallay and Lin, 2013), NIM811 (Membreno et al., 2013), SCY-635 (Hopkins et al., 2010), Sangliferins (Sanglier et al., 1999) and a series of new compounds were described recently (Carbajo-Lozoya et al., 2014; Malešević et al., 2013; Prell et al., 2013). ALV has experienced substantial clinical testing and safety database development with more than 2000 patients treated for up to 48 weeks. NIM811 or SCY-635 have been administered in a very small number (< 50 patients) only in short proof-of-concept trials. Compound **3** or sangliferins have not been given to patients yet.

Here we demonstrate the inhibitory effects of non-immunosuppressive CsA derivatives on 229E replication in various Huh-7-derived hepatoma cell lines and the requirement of CypA for interaction with the viral nucleocapsid protein and for virus propagation in Huh-7.5 cells.

2. Materials and methods

2.1. Western blot antibodies and drugs

Mouse antibody 1H11 (1:20,000) recognizing HCoV-229E N-protein was obtained from INGENASA, Spain (Sastre et al., 2011). Anti-Lamin A (A303-433A, [1:20,000]), anti-PPIA (ab3563, [1:500]) and anti-PPIB (PA1-027A, [1:800]) were purchased from Biomol, Abcam and ThermoFisher, respectively. Secondary antibodies were received from Biomol (goat anti-rabbit-Ig-horse radish peroxidase HRP, [1:3000] and rabbit-anti-goat-Ig-HRP [1:3000]) and Sigma Aldrich (anti-mouse-Ig-HRP [1:40,000]).

Alisporivir (formerly DEB025) and NIM811 were provided by Novartis (Basel, Switzerland). CsA and Rapamycin (RAPA) were obtained from Sigma-Aldrich (Germany). Cyclosporin H (CsH) was synthesized according to published procedures (Whitaker and Caspe, 2011). Synthesis of compound **3** was described recently (Carbajo-Lozoya et al., 2014; Malešević et al., 2013).

2.2. Cell culture and cell lines

Human hepatocellular carcinoma cells Huh-7, Huh-7.5 cells (Blight et al., 2002) and sub clones were maintained in Dulbecco's modified Eagle medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. Huh-7D (Feigelstock et al., 2010) and Huh-7 Lunet (Koutsoudakis et al., 2007) cells were described. Cell viabilities were determined by CellTiter-Glo® Luminescent Cell Viability Assay (Promega #G7570).

2.3. Viruses

HCoV-229E viruses expressing Renilla luciferase (LUC) or Green Fluorescent Protein (GFP) (Carbajo-Lozoya et al., 2012; Cervantes-Barragan et al., 2010) reporter genes were used to examine the inhibitory effect of compounds. Generally, Huh-7.5 cells were infected with MOI = 0.1 and incubated for two days in the presence of increasing concentrations of inhibitor in the culture medium. Viral replication was determined by measuring Renilla luciferase activity or GFP fluorescence.

2.4. Fluorescence microscopy

For evaluation of HCoV-229E-GFP replication in Huh7-derived cell lines cells were split onto sterile coverslips, grown to ~80% confluence and infected with respective MOI. After indicated time points non-infected and infected cells were fixed with 2.5% formaldehyde for 15 min, washed twice with PBS and subjected to DAPI (Cell Signalling) staining. After two further washes coverslips were air-dried, mounted with fluorescence mounting medium (Dako, S3023) and inspected using a Leica DMI 4000IB fluorescence microscope at 40× magnification.

For immunofluorescence analysis, Huh7 cells were seeded onto sterile cover slips in a 24-well plate (Costar) at a cell density of 10^5 cells per well. After 24 h, cells were infected with HCoV-229E wt at an MOI of 1 for 1.5 h at 37 °C and 5% CO_2. After infection cells were washed with PBS and incubated with CsA, ALV (20 µM) inhibitors and with ethanol as solvent control in the culture medium. For immunostaining, cells were fixed overnight with 4% paraformaldehyde at 4 °C. Subsequently, they were blocked with 5% BSA in PBS (Gibco–Life Technologies) overnight. Fixed cells were incubated for 72 h at 4 °C with the following primary antibodies diluted in PBS (5% BSA, 0.2% Tween-20): mouse anti-dsRNA (clone J2, 1:1,500; Scicons), rabbit anti-CypA (ab3563, 1:500; Abcam), rabbit anti-CypB (1:800; Thermofisher, PA1-027A, mouse anti-PDI (1:100; ThermoFisher, MA3-019), anti-GM130 (1:100, BD Biosciences, 610822), mouse anti-SQSTM1 (1:200; Thermofisher, MA-5-27800), mouse anti-DCP1 (1:500, SantaCruz, sc100706), mouse anti-PABP (1:1500; Sigma Aldrich, P6246). After incubation cells were washed three times with PBS and incubated for 1 h in the dark with secondary antibodies goat anti-rabbit-FITC (1:1000; Sigma Aldrich, F0382) and goat anti-mouse-Alexa-555, 1:500; ThermoFisher, A21424) in PBS containing 5% goat serum and 0.2% Tween-20) at room temperature. Cells were washed with PBS (3x). Cell nuclei were stained with 1 µg/m1 DAPI (Sigma Aldrich, D9542) for 10 min in the dark. After further three washes with PBS coverslips were air-dried, mounted with fluorescence mounting medium (Dako, S3023), and inspected using a Leica DMI 4000IB fluorescence microscope at 40× magnification.

2.5. Western blotting

N-protein expression in the presence of inhibitors or in Huh-7.5 CypA variants was analysed as described recently (Carbajo-Lozoya et al., 2014). Briefly, Huh-7.5 cells were infected at HCoV-229E-LUC/-GFP virus MOI = 0.1 for 1 h in six-well plates. Virus was washed off with PBS and inhibitors were added to the medium at the respective concentrations. After 48 h cells were harvested and lysed with 250 µl lysis buffer (1% NP-40 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT and Protease Inhibitor Cocktail [Hoffmann La Roche]). Proteins were separated by 8 or 12.5% SDS-PAGE and electro-blotted onto nitrocellulose membranes. Latter were blocked with 5% milk powder in TBST buffer. Primary antibodies were usually incubated at 4 °C overnight. Secondary antibody incubation was performed at room temperature for 2 h. After each incubation step membranes were washed three times with TBST for 10 min. HRP was developed with Immobilon Western blot HRP chemiluminiscent substrate from Millipore. Membranes were exposed to X-ray film (Agfa).

2.6. Split YFP protein-protein interaction assay

Construction of split YFP vectors was described recently (Ma-Lauer et al., 2016). Briefly, pDEST-c-myc-YFP^N (c-myc-YFP^N [amino acids 1–155] fused to N-terminus of a test gene) and pDEST-HA-YFP^c (HA-YFP^C [amino acids 156–239] fused to N-terminus of a test gene) or

pDEST-ct-c-myc-YFP^N (c-myc-YFP^N fused to C-terminus of a test gene) and pDEST-ct-HA-YFP^c (HA-YFP^C fused to C-terminus of a test gene) served as split-YFP vectors. Genes encoding 229E N protein and cyclophilin A (PPIA) were first BP Gateway™-cloned into the pDONR207 vector and consequently LR-cloned into the pDEST-c-myc-YFP^N, pDEST-HA-YFP^c, pDEST-ct-c-myc-YFP^N, pDEST-ct-HA-YFP^c, vectors, yielding constructs for split-YFP assays. 229E-N was amplified with primers 229E_N-for-5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATGG CTACAGTCAAATGGGC and 229E_N-rev-5′GGGGACCACTTTGTAC AAG-AAAGCTGGGTCTCMGTTTACTTCATCAATTATGTCAG. hPPIA was amplified with primers PPIA-att-for: 5′-GGGGACAAGTTTGTACAA AAAAGCAGGCTCCGCCATGGTCAACCCCACCGTGTTCTTCGAC-3′ and PPIA-att-rev: 5′GGGGACCACTTTGTAC-AAGAAAGCTGGGTCTCMTTCG AGTTGTCCACAGTCAGCAATGG -3'. GATEWAY™-cloning (BP-, LR reactions) was performed according to the manufacturer's instructions.

HEK293 cells were seeded directly onto autoclaved coverslips and cultured in 24-well plates. Plasmids pDEST-c-myc-YFP^N-PPIA (YFP^N-PPIA) and pDEST-HA-YFP^C-229E-'N' (YFP^C-229E-'N′), "empty" split YFP vectors or combinations thereof were co-transfected into cells at 80% confluence by using Lipofectamine 3000 (Thermofisher). 24 h after transfection, cells on coverslips were examined directly under a microscope (Leica DM4000 B). Pictures were taken with a 40× objective.

3. Results

3.1. HCoV229E grows in different Huh-7-derived cell lines

Human hepatocellular carcinoma cells (Huh-7) support the replication of a number of viruses including HCV (Bartenschlager and Pietschmann, 2005), HCoV-229E, SARS-CoV and MERS-CoV (Raj et al., 2013). A number of Huh-7 mutant cell lines [Huh-7.5 (Blight et al., 2002), Huh-7D (Feigelstock et al., 2010), Huh-7-Lunet (Friebe et al., 2005)] were generated in order to increase Hepatitis C Virus (HCV) permissiveness for viral and replicon propagation. For instance, Huh-7.5 cells carry a mutation in the cytosolic retinoic acid-inducible gene I (RIG-I) which is a pattern recognition receptor for triggering type I interferon pathways by sensing HCV dsRNA. Huh-7D carry mutations outside of the RIG-I coding region. Huh7-Lunet cells support high level HCV RNA replication.

In order to test the permissiveness of different hepatoma cell lines for HCoV-229E replication we infected cells with HCoV-229E-GFP. As shown in Suppl. Fig. S1 all cell lines were permissive to infection at similar extends. DAPI staining of cell nuclei indicated comparable confluence of HuH7 cells. HCoV-229E-GFP replication efficiency in these cell lines was not analysed in further detail. Although infection of Huh-7.5 was slightly less effective as compared to Huh7D we chose to primarily work with this cell line as there was a CypA knockdown mutant available (von Hahn et al., 2012).

3.2. Non-immunosuppressive CsA derivatives inhibit HCoV-229E replication

We have recently reported on the biochemical and immunological characteristics and on the inhibitory effect of a number of CsA-derived compounds on the replication of HCoV-NL63 (Carbajo-Lozoya et al., 2014). The molecules included CsA, ALV, NIM811, as well as newly developed CsA position 1-modified compound **3** (structure is shown in Fig. S2). Here we tested the inhibitory effect on the replication of HCoV-229E using recombinant viruses expressing Renilla luciferase (229E-LUC) or GFP (229E-GFP). Fig. 1 shows replication in Huh-7.5 cells reflected by Renilla luciferase activity of 229E-LUC at 18 and 48 h p.I. The corresponding EC_{50} (effective inhibitory concentration) values are listed in Table 1. It is clearly demonstrated that similarly to CsA the non-immunosuppressive molecules ALV, NIM811 (Fig. 1A) and compound **3** (Fig. 1B) inhibit 229E-LUC replication in the low micromolar range. Cell viability was only slightly affected at highest

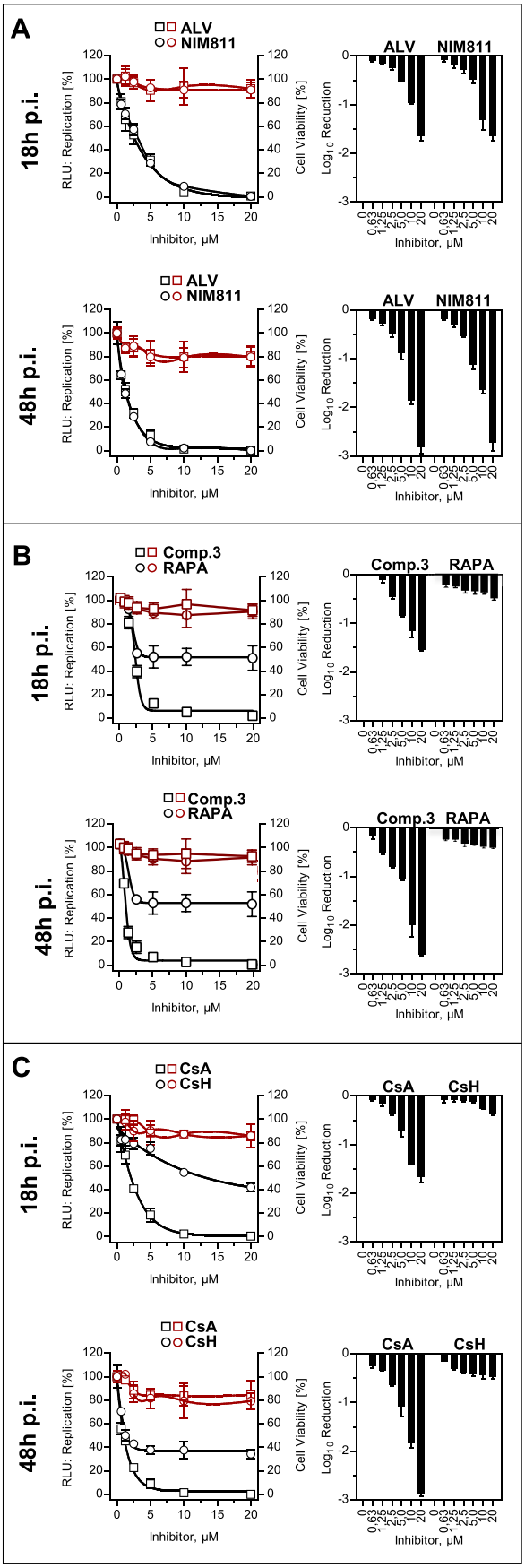

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Fig. 1. Effect of CsA and various non-immunosuppressive derivatives on HCoV-229E-LUC (MOI = 0.1) replication in Huh-7.5 cells. Left Y-axes represent the percentage of reduction of virus replication (RLU = relative light units of Renilla luciferase activity, black lines) in linear (left panels) or in log scale (right panels) at the indicated inhibitor concentrations given on the X-axis. Cell viability (red lines) with mock-treated cells set to 100% is shown on the right Y-axes of the left panels. Measurements were taken 18 and 48 h p.I. CsH and RAPA served as controls. The graphs were plotted using Prism 5 (GraphPad Software, Inc.) and by a non-linear regression with a variable slope algorithm, the curve was fitted for each respective inhibitor and the EC_{50} was calculated.

Table 1

EC_{50} values for the individual inhibitors determined at 18 and 48 h p.I.

The cytotoxicity of the inhibitor compounds was determined by comparing the resulting absorbances with the mean absorbance of the control wells (only solvent) using CellTiter-Glo® Luminescent Cell Viability Assay and was expressed as percentage of cell viability. The 50% cytotoxic concentration (CC_{50}) was defined as the quantity of inhibitor generating 50% of cell viability, compared to the control. The values of the percentages of cell viability were plotted against inhibitor concentrations in GraphPad Prism version 7, and CC_{50} was determined using non-linear regression statistics ("inhibitor vs normalized response"). Dividing CC_{50} by EC_{50} 48 h time point values resulted in the therapeutic index (SI). Experiments were carried out in triplicates in 96 well plate format in parallel to viral replication inhibition assays.

	EC_{50}	EC_{50}	CC_{50}	SI
time p.i. (hrs)	µM	µM	µM	
	18	48	48	
ALV	2.77	1.37	280.1	204.5
NIM811	3.11	1.19	207.9	174.7
compound 3	2.05	0.92	72.71	79
Rapamycin	–	–	49.48	–
CsA	2.09	0.97	185.6	191.3
CsH	–	–	333.9	–

concentrations used. As control we included CsH and Rapamycin.

CsH is a stereoisomer of CsA in which D-methylvaline at position 11 substitutes the natural L-methylvaline. Following the minor affinity for CypA this substitution abrogates the immunosuppressive and anti-inflammatory properties of CsA (de Paulis et al., 1996; LeGrue et al., 1986). Fig. 1C shows a slight residual inhibitory activity of the CsH preparation, which might be a result of the low CypA affinity or of traces of CsA-like impurities present in the CsH preparation. Due to the chemical similarity (stereoisomers) of these molecules, the impurities are very difficult to remove from the product batches. We also observed a very low inhibition of CypA in the PPIase assay with this compound. The immunosuppressive drug rapamycin was used as a further, cyclophilin-independent control molecule to test the effect of immunosuppressants on CoV replication. It binds to FKBP1A (=FKBP12), but as opposed to FK506 it interferes with the mTOR (mechanistic Target of Rapamycin) pathway by inhibiting a serine/threonine protein kinase (Huang et al., 2003). Fig. 1B shows background inhibitory activity of rapamycin. Thus, lack of effective inhibition of viral replication by CsH, which does not affect the PPIAse activities of cyclophilins strongly, argues for the requirement of this function for coronavirus replication.

3.3. Effects of inhibitory drugs on HCoV-229E N protein expression

The N protein is a multifunctional protein (McBride et al., 2014) and it is required for virus replication and for the propagation of replicons (Chang et al., 2014). Its primary function is to encapsidate and protect genomic RNA. Lack of N protein is thus a measure of lacking viral replication. To study the effect of CsA, ALV, NIM-811 and compound **3** on viral N protein expression Huh-7.5 cells were incubated with

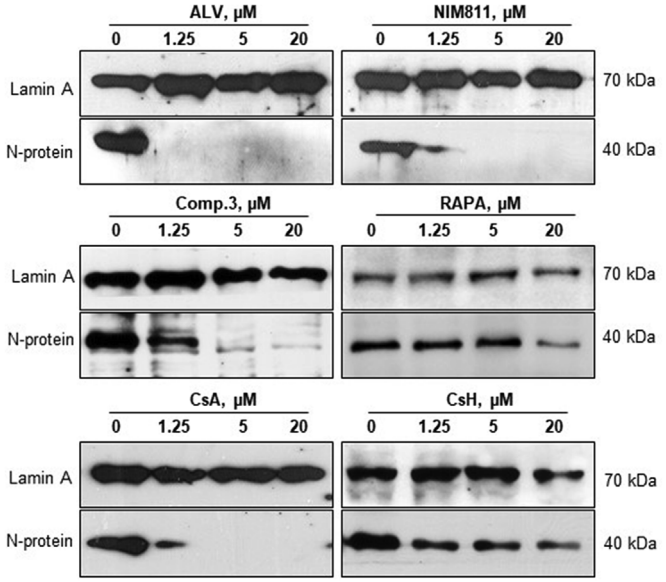

Fig. 2. Western blot analysis of N protein expression in HCoV-229E-LUC-infected Huh-7.5 cells. Cells were treated for 48 h with increasing concentrations of ALV, NIM811, compound **3**, RAPA, CsA and CsH and then processed for WB analysis. As a measure of viral replication 229 N-Protein was detected with a mouse mab against N. A rabbit anti-Lamin A antibody was used to detect Lamin A as a loading control.

concentrations of 0–20 µM of the respective inhibitors for 48 h. Western blot analysis of 229E-infected Huh7.5 cells was performed utilizing an anti-N antibody. Fig. 2 demonstrates the decrease of the N protein between 1.25 µM and 5 µM in the presence of ALV, NIM811 compound **3**. In case of ALV N protein is not detectable anymore at 1.25 µM. Similarly, presence of the positive control CsA also decreases N protein expression between 1.25 and 5 µM whereas CsH and rapamycin allow significant N protein synthesis even at 20 µM. It is not clear whether the inhibitors act on cyclophilin binding to the N protein itself, on other viral proteins or both. However, viral N protein, essential for replication, is not synthesized in the presence of the inhibitors.

3.4. CsA and non-immunosuppressive derivatives inhibit binding of HCoV-229E-N protein to CypA

As SARS-CoV N protein was reported to bind CypA in *vitro* (Luo et al., 2004), we tested whether HCoV-229E N protein binds directly to CypA by split YFP interaction experiments in living HEK293 cells. The experimental protein-protein interaction system has been described recently (Ma-Lauer et al., 2016). It is based on the splitting of YFP into N (YFP^N)- and C (YFP^C)- terminal halves which are fused at the DNA level to two proteins to be tested for interaction. Upon interaction of the two proteins, the two YFP fragments assemble to functional YFP, which is demonstrated by fluorescence. Fig. 3 (left panel) demonstrates the direct interaction between CypA and 229E-'N′ proteins using fusion constructs YFP^N-PPIA and YFP^C-229E-'N'. Control combinations using YFP^N and YFP^C with only one fusion partner were negative. Upon addition of CsA, ALV and NIM811 (45 µM each) the fluorescence signal disappeared almost completely indicating a direct interruption of interaction between CypA and N protein.

Quite interestingly, from our earlier Y2H interaction studies we had hints on the interaction between CypA and the SARS-CoV X-domain also called macrodomain I (Mac1) (Lei et al., 2018; Pfefferle et al., 2011). Here (right panel of Fig. 3) we also found interaction of CypA (PPIA-YFP^N) with the X-domain of HCoV-229E (229E-X-dom-YFP^C). However, CsA, ALV or NIM811 did not block this interaction. As CypB might also be an important player during virus replication, we applied

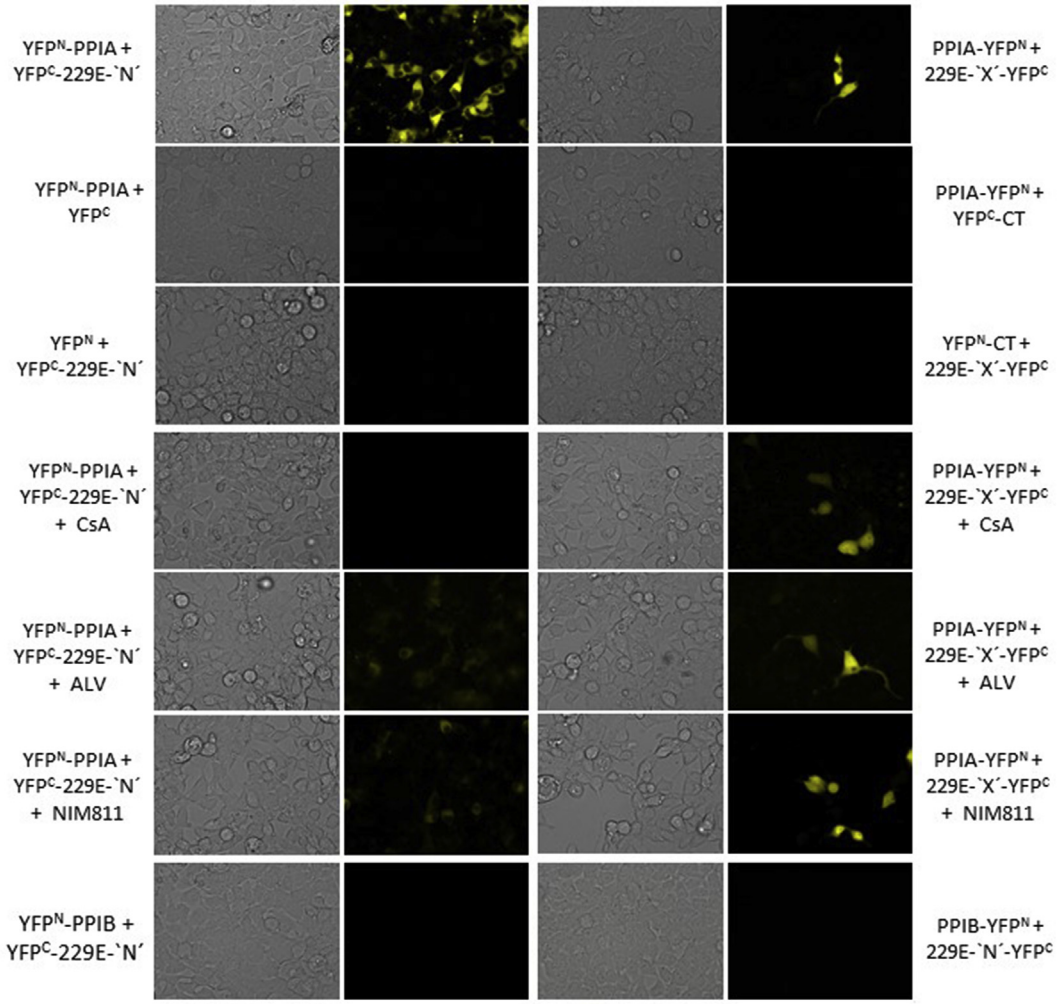

Fig. 3. Interaction analysis of 229E-'N' and -'X' (Mac1) domains with CypA and CypB by split YFP assay in the absence or presence of Cyp inhibitors. YFP^N- and YFP^C- fragments were fused to the N- or C- termini of PPIA (CypA) and PPIB (CypB) genes in respective expression plasmids. 229E-'N' and -'X' ORFs were fused to the YFP^C C- and N- termini, respectively. HEK 293 cells were co-transfected with respective plasmid combinations including "empty" split YFP vectors as negative controls. Cells were cultivated in the absence or presence of 45 μM CsA, ALV or NIM811. After 24 h, strong YFP signals (yellow) were detected in the living cells in case of interaction. The left panel shows strong interaction of 229E-'N' (YFP^N-PPIA + YFP^C-229E-'N') with Cyp A disappearing in the presence of Cyp inhibitors, but not with CypB (lowest panel, YFP^N-PPIB + YFP^C-229E-'N'). The right panel shows strong interactions of 229E-'X'with CypA (PPIA-YFP^N +229E-'X'-YFP^C) also in the presence of Cyp inhibitors.

analogous setups used for CypA for testing possible interaction of CypB with HCoV-229E-N proteins. As shown in Fig. 3 (lower panel) we could not demonstrate interaction irrespective of the orientation of the split YFP fusion proteins.

3.5. Localization and expression of CypB but not of CypA is strongly altered by HCoV-229E and cyclophilin inhibitors

CsA and ALV block the activity of both cyclophilins A/B. So far, the inhibitory effect on HCoV-229E replication cannot exclusively be attributed to only one or to both cyclophilins. We reasoned that a possible co-localization of the cyclophilins with a marker of viral replication at the site of replication might give hints on the relevance of the two proteins. Therefore, we visualized the effect of both inhibitors on the intracellular localization of CypA/B and dsRNA by immunofluorescence assays in presence and absence of virus. Expression levels of CypA were similar, and it was distributed throughout cytoplasm and nucleus of Huh7 cells irrespective of the conditions (Fig. 4, "anti-CypA column"). In contrast, CypB located in untreated cells (no inhibitors and no virus) almost exclusively to the cytoplasm (Fig. 5, "ETOH/mock" and "ETOH/HCoV-229E" panels). From earlier work, it is known that CsA alters CypB trafficking through the secretory pathway (Price et al., 1994). Here we found that CypB Inhibitor treatment causes its re-localization and sequestration to foci in the nucleus and a strong reduction of expression (Fig. 5, right panel: small green dots in CsA/ALV mock- and 229E-infected cells). Most interestingly, virus infection in cells not treated with inhibitors caused the shift of CypB localization from an even cytoplasmic distribution (Fig. 5, "ETOH/mock" row) to large bleb-like structures around the nucleus (Fig. 5 "ETOH/HCoV-229E″ row and Fig. 6 left "anti-CypB" column). The intense yellow co-staining of these structures with anti-PDI and anti-CypB antibodies indicates co-localization of the two proteins. Due to the intense concentration, we refer to virus-induced CypB/ER blebs. Staining with antibodies against other cell organelles (cis-Golgi, autophagosomes, P-bodies, stress granules) did not reveal co-localization with CypB (Fig. 6). Viral dsRNA did co-localize neither with CypA (Fig. 4, "ETOH/HCoV-229E″ row) nor with CypB/ER blebs (Fig. 5, "ETOH/HCoV-229E″ row). These blebs were not observed in infected and CsA/ALV-treated cells as the inhibitors prevent virus replication. Western blot analysis clearly demonstrated down-regulation of CypB but not of CypA in the presence of inhibitors independent of virus infection in Huh7 cells (Fig. 7).

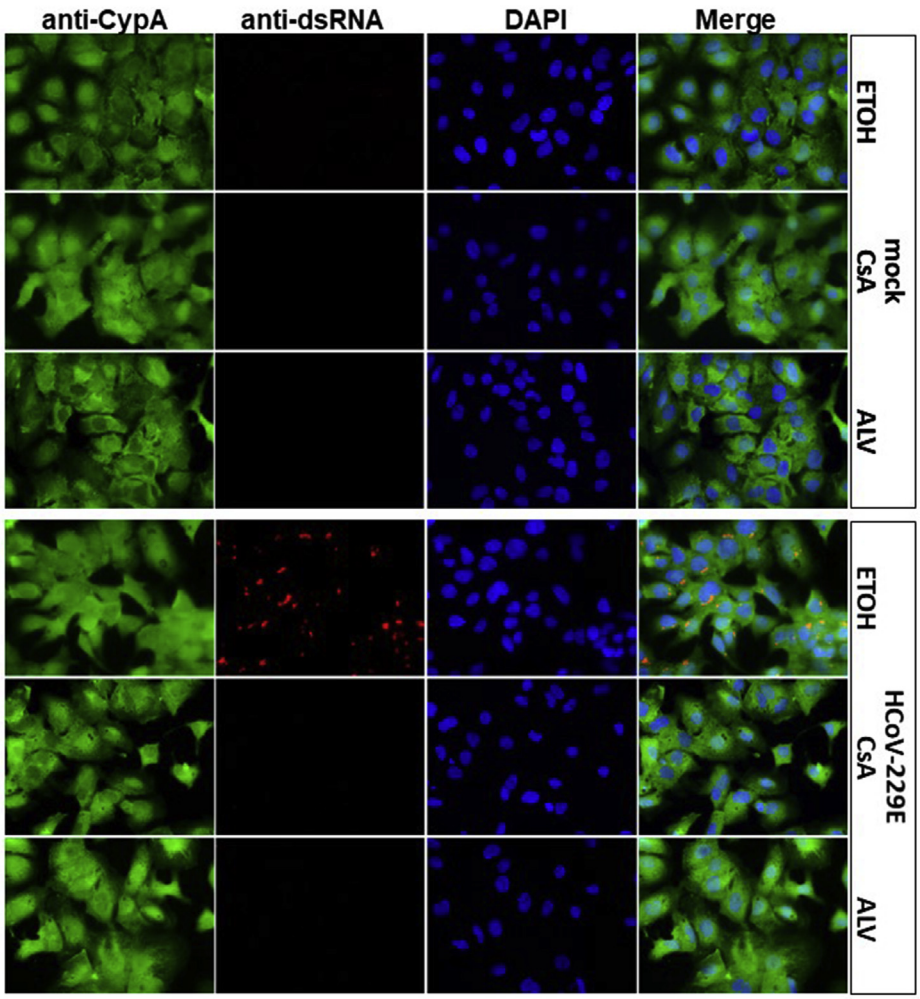

Fig. 4. Immunostaining of CypA in mock- and HCoV-229Ewt-infected Huh7 cells in the presence of ETOH solvent or cyclophilin inhibitors CsA and Alisporivir. After infection (MOI = 1) medium was removed and new medium was added to cells containing 20 μM of inhibitor for 48 h and samples were processed for IF. CypA and dsRNA were stained with anti-CypA (green, ab3563, Abcam, 1:500) and anti-dsRNA J2 (red, Scicons, 1:1500), respectively. Nuclei were visualized with DAPI. Nucleo-cytoplasmic distribution of CypA was not affected by the presence of virus or cyclophilin inhibitors. dsRNA as replication marker was not detected in the presence of CsA or ALV. Exposure times for the respective antibodies were the same in the different samples.

4. Discussion

In a recent study we had identified CsA as a broad-spectrum inhibitor of CoV replication in humans, mouse, cat, pig and bird with cyclophilins as presumable cellular targets (Pfefferle et al., 2011). De Wilde et al. extended this list to MERS-CoV (de Wilde et al., 2013b) and arteriviruses equine arteritis virus (de Wilde et al., 2013a) indicating the broad activity on the two families *Coronaviridae* and *Arteriviridae* of the order of *Nidovirales*.

We have shown the successful inhibition of HCoV-NL63 replication by ALV, NIM811 and position 1-modified CsA derivatives (Carbajo-Lozoya et al., 2014) and the requirement of CypA, not CypB for NL63 replication. Here we demonstrate the inhibition of HCoV-229E by the same compounds as well as their influence of the CypA-229E-N protein interaction. Virus inhibition experiments (Fig. 1, Table 1) clearly demonstrate the highly effective inhibition of the Renilla luciferase-expressing 229E-LUC with decreasing EC_{50} values between 18 and 48 h p.I. Inhibition patterns of ALV, NIM811 (both Fig. 1A), compounds **3** (Fig. 1B) and CsA (Fig. 1C) are very similar with EC_{50} values of 1.37, 1.19, 0.97 and 0.92 μM, respectively. Log titer reductions range between 2.5 and 3 at the 48 h time point. Cell viabilities decrease only slightly at the highest drug concentrations (20 μM) used. Therapeutic index values show rather high levels between 79 and 204.5 (Table 1).

In general, CoV inhibition requires low micromolar ranges of CsA and its non-immunosuppressive derivatives, which was recently found by us (Carbajo-Lozoya et al., 2014; Pfefferle et al., 2011) and by others (de Wilde et al., 2011). Interestingly, inhibition of HCV with ALV and CsA/NIM811 is commonly observed at low nanomolar and low micromolar concentrations, respectively, unraveling ALV as the most effective compound. Currently, we cannot explain why the Cyp inhibitors (especially ALV) are much more potent in preventing HCV as opposed to CoV replication, or why their inhibitory activity on CoVs is rather similar. One explanation might be that several CoV proteins depend on the activity of CypA and/or CypA might have higher affinities to coronaviral as compared to HCV proteins thus requiring higher inhibitor doses to abolish interactions. We used CsH and rapamycin as control compounds. Both allow the transfer of NFAT to the nucleus (not shown). CsH displays only a very weak binding affinity to cyclophilins. Thus, no CsH/CypA complexes exist that can bind to and inactivate Calcineurin (CaN). Rapamycin targets FKBPs similarly to the immunosuppressant FK506. In an earlier report we had described 229E inhibition by the FKBP- binding drug FK506 (Carbajo-Lozoya et al., 2012). However, the rapamycin/FKBP complexes inhibit the unrelated m-TOR pathway in contrast to FK506/FKBP complexes which bind similar to the CsA/Cyp complexes to CaN thus hampering the CaN phosphatase activity and as a consequence the NFAT pathway. Both drugs do not inhibit 229E replication. The minor reduction observed in the case of CsH might be attributed to a very weak binding affinity to cyclophilins or to traces of CsA, from which CsH was synthesized and which can only be separated quantitatively by enormous experimental expenditure. Expression analysis of the 229E N protein further supports the inhibitory effect of the different substances. N is required for virus replication and thus essential for the viral life cycle. Western blot analysis (Fig. 2) demonstrates the lack of N protein expression during 229E-LUC infection of Huh-7.5 cells at ALV, NIM811, Compound **3** and CsA inhibitor concentrations above 1.25 μM and 5 μM, respectively, but

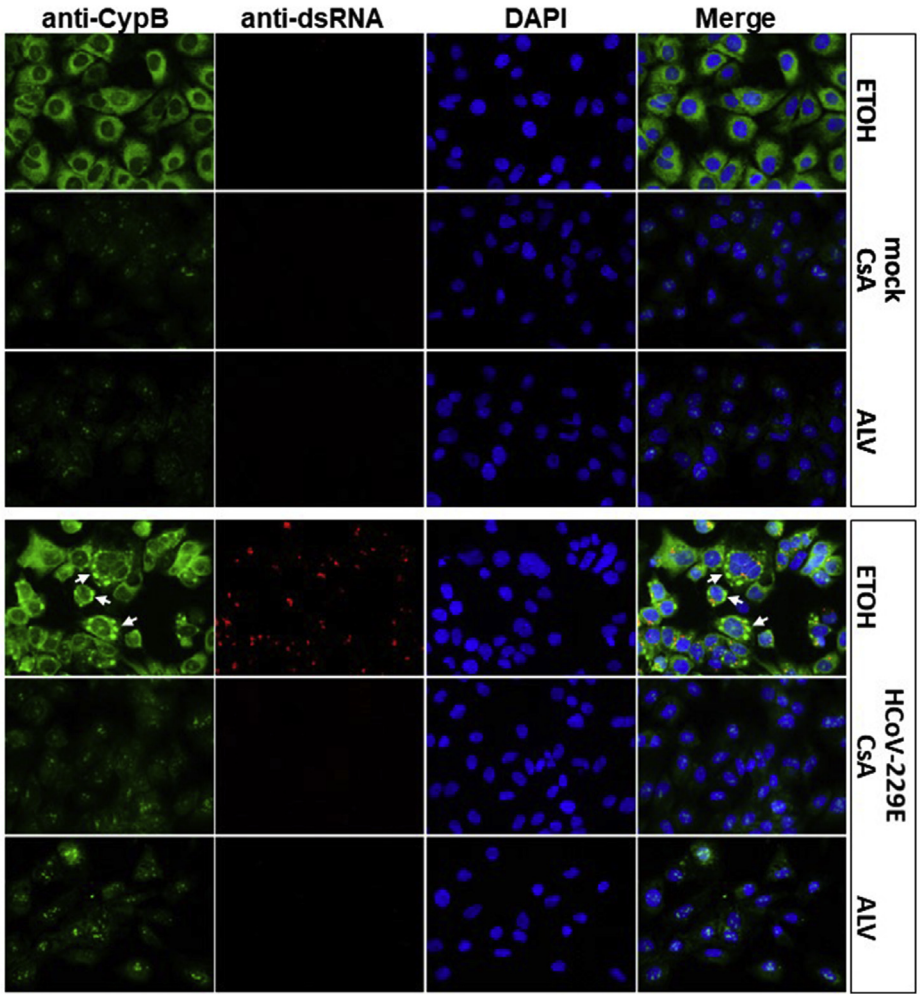

Fig. 5. Immunostaining of CypB in mock- and HCoV-229E-infected Huh7 cells in the presence of ETOH solvent or cyclophilin inhibitors CsA and Alisporivir. After infection (MOI = 1) medium was removed and new medium was added to cells containing 20 μM of inhibitor for 48 h and samples were processed for IF. CypB and dsRNA were stained with anti-CypB (green) and anti-dsRNA J2 (red, Scicons, 1:1500), respectively. Nuclei were visualized with DAPI. CypB shifts from an even cytoplasmic distribution to intense bleb-like structures in the presence of virus (white arrows, ETOH solvent panels). CsA and ALV led to the re-localization of CypB to granular structures in the nucleus and to a massive reduction of expression independent of virus infection. dsRNA as replication marker was not detected in the presence of CsA or ALV. Exposure times for the respective antibodies were the same in the different samples.

not for CsH and rapamycin.

We attribute the reason for the downregulation of N expression in the presence of Cyp inhibitors to the prevention of the interaction of N and CypA. The split YFP protein-protein interaction experiments clearly show the interaction of the two proteins in HEK293 cells and its abolishment in the presence of the inhibitors (Fig. 3). Another viral protein, the X-domain of Nsp3, also interacts with CypA. Cyp inhibitors do not prevent this interaction, suggesting different mechanisms. We can only speculate that different binding sites, chemical bonds or different time points of interaction of CypA with viral proteins might be involved during viral replication.

CsA and its non-immunosuppressive derivatives inhibit replication of a number of viruses including HCV, HBV and HIV-1. In most cases the responsible cyclophilin is CypA (Zhou et al., 2012). CypA and CypB were found to be required for FCoV replication (Tanaka et al., 2017). For HCoV-NL63, we have recently shown that CypA expression is essential in CaCo-2 cells (Carbajo-Lozoya et al., 2014). Utilizing a Huh-7.5 $CypA^{KD}$ (knockdown) cell line, originally constructed for the study of the requirement of stable CypA for HCV replication (von Hahn et al., 2012) we found significantly reduced replication of a HCoV-229E-Renilla luciferase expressing virus (von Brunn et al., 2015). This indicates the involvement of CypA also in replication of HCoV-229E.

Minor replicative activity might be explained by an incomplete suppression of CypA in the shRNA-based knockdown, as the protein is one of the most abundant proteins (~0.1–0.4% of total cellular protein) in the cell cytoplasm (Fischer and Aumuller, 2003; Saphire et al., 2000). Residual molecules of CypA might be sufficient to exert PPIase functions. In addition, other Cyps or PPIases might overtake CypA functions. In any case, from these $CypA^{KD}$ experiments and from interaction data of CypA and viral proteins it is highly likely that CypA plays an important role during replication of human coronaviruses.

On the basis of siRNA-PPIA and -PPIB knockdown experiments a recent report suggested that neither CypA nor CypB are required for replication of SARS-CoV and Mouse Hepatitis Virus (de Wilde et al., 2011). However, in both siRNA knockdowns residual expression of CypA or CypB proteins might have left enough PPIase activity levels in the infected cells to support viral replication. The role of CypB during CoV replication is not clear as we show that its protein level and subcellular localization completely changes in the presence of CsA and ALV. Divergent results are reported in a recent study which found no difference in HCoV-229E replication in Huh7wt as compared to Huh7-$CypA^{KO}$ (knockout) pool cells and to two Huh7-$CypA^{KO}$ clones (de Wilde et al., 2018). MERS-CoV titers were moderately diminished (3-fold), whereas equine arteritis virus was reduced by ~3 logs. These cell lines had been generated by CRISPR/Cas9-technology and it was obviously very difficult to generate clear-cut $CypA^{KO}$s in the Huh7 hepatoma cells as chromosomal translocations, carrying PPIA sequences were observed. Only two heterogeneous clones #1 and #2 carrying eight and six PPIA-specific insertion/deletions (indels), respectively, could be isolated. The clones were identified to be CypA-negative by Western blot with an antibody directed against a short peptide sequence at the C-terminus of CypA. However, this region is located within exon 5 and it cannot be excluded that frameshifts occurred upstream of the antibody-recognized peptide sequence within exon 5, which was not sequenced. Interestingly, it was not possible to generate Huh7-$CypB^{KO}$, Huh7-$CypC^{KO}$, and Huh7-$CypD^{KO}$ cell clones.

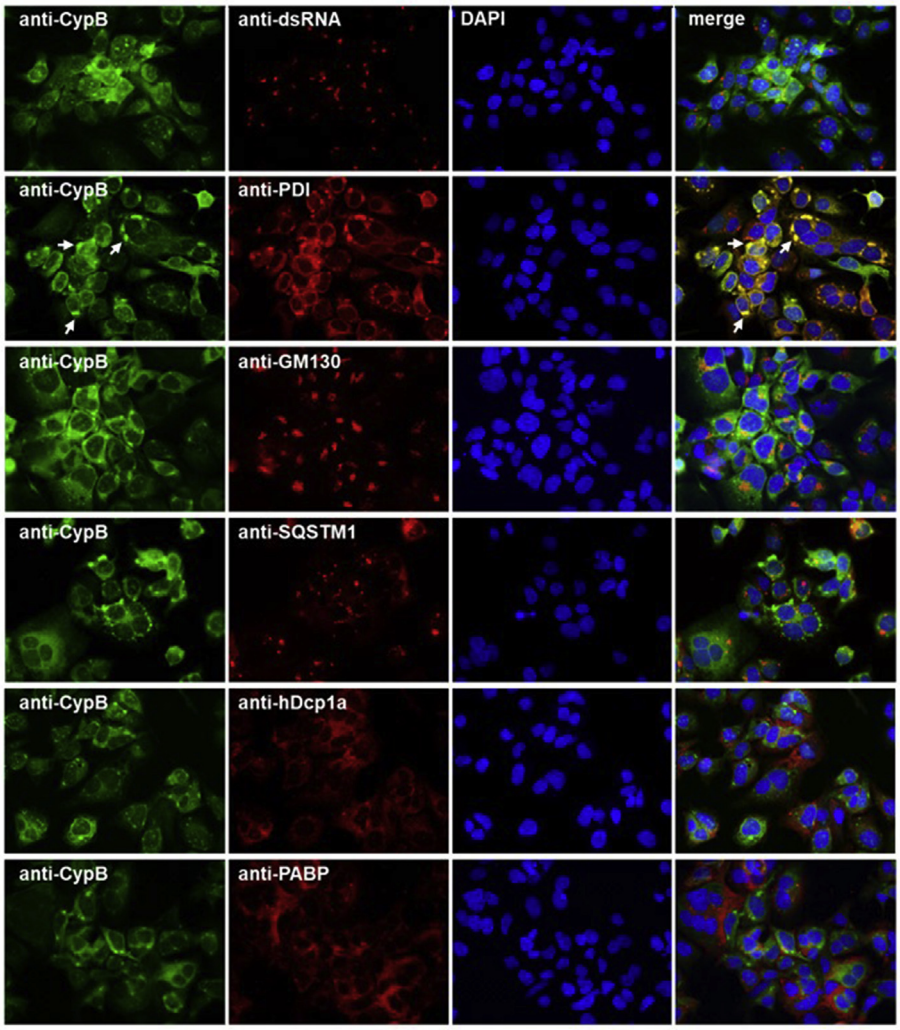

Fig. 6. Co-immunostaining of CypB and cell organelles in HCoV-229E-infected Huh7 cells. For the identification of the intense cyclophilin B bleb-like structures infected cells (MOI = 1; 48 h p.i.) were co-stained with anti-CypB and antibodies directed against markers of the ER (anti-PDI), cis-GOLGI (anti-GM130), autophagosomes (anti-SQSTM1), anti-P-bodies (anti-hDcp1a) and stress granules (anti-PABP). Cyclophilin B normally distributes within the ER. In the presence of HCoV-229E, it intriguingly concentrates at bleb-like structures of the ER.

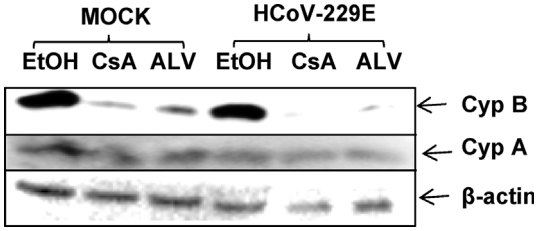

Fig. 7. Downregulation of Cyp B, but not CypA in the presence of Cyp inhibitors CsA and ALV. Huh7 cells were either mock or HCoV-229E (MOI = 1) infected and cultivated in the presence of EtOH solvent, or 20 μM CsA or ALV for 48 h. Cell extracts were subjected to Western blot analysis and staining with anti-Cyp A, anti-Cyp B and anti-beta actin as loading control.

The role of CypB during HCoV-229E replication seems rather peculiar at the moment. The protein contains an ER retention signal and promotes secretion into the medium. Therefore, Price et al. suggested a competition of CsA with endogeneous plasma membrane proteins for the association with CypB favouring its role as chaperone for those proteins (Price et al., 1994). By WB and IF we find strongly decreased CypB, but not CypA expression levels and a re-localization and sequestration of CypB to foci in the nucleus in the presence of Cyp inhibitors. Most intriguingly, we also find a distinct co-localization and concentration of CypB and the ER marker Protein Disulfide-Isomerase (PDI) in virus-induced CypB/ER blebs upon infection with HCoV-229E. These do not occur in mock-infected cells indicating an influence of viral components. We do not know whether this reflects just an accumulation, a secretory block, or another mechanism. Both proteins contain chaperone activities and it will be highly desirable to identify possible effects of both enzymes on viral proteins or vice versa.

We have shown earlier that SARS-CoV Nsp1 protein binds to cyclophilins. The binding of CypA to the SARS-CoV N protein is known from a very early educated guess finding using surface plasmon resonance biosensor technology (Luo et al., 2004). This was supported by a spectrometric profiling study showing the incorporation of CypA into SARS-CoV virions (Neuman et al., 2008). Here we show for the first time the direct interaction between CypA and HCoV-229E 'N' protein and 'X' (ADRP) proteins by split YFP assay (Fig. 3). Most interestingly, only the CypA-'N' but not the CypA-'X' interaction is blocked by CsA and non-immunosuppressive derivatives ALV and NIM811 indicating the importance of CypA for correct folding of 'N'during replication. Mechanistically, we suggest that CoV inhibition is a direct consequence of interruption of the interaction between N capsid protein and CypA by cyclophilin inhibitors. It further remains to be determined what the function of Nsp1 and 'N' binding to CypA is and whether other

coronaviral proteins require the proline-directed binding and PPIase activity of CypA.

From our own and other laboratories results it is clear that non-immunosuppressive CsA derivatives block CoV replication. Furthermore, ALV and NIM811, which have already been tested in human phase II (ALV, NIM811), as well as the new CsA position 1-modified compounds (not tested in humans) are promising, broadly-acting candidate HTAs for anti-coronaviral therapy. According to our results, CypA represents an important player during CoV replication. For final resolution of the requirement of different Cyps for CoV replication, further efforts have to be put on the construction of stable Cyp knockouts in different CoV-infectable cell lines and on the involvement of different viral proteins.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104620.

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