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Chapter 4

Cyclosporin A inhibits the replication of diverse coronaviruses

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ABSTRACT

Low micromolar, non-cytotoxic concentrations of cyclosporin A (CsA) strongly affected the replication of SARS-coronavirus (SARS-CoV), human coronavirus 229E, and mouse hepatitis virus in cell culture, as was evident from the strong inhibition of green fluorescent protein reporter gene expression and up to 4 log reduced progeny titres. Upon high-multiplicity infection, CsA treatment rendered SARS-CoV RNA and protein synthesis almost undetectable, suggesting an early block in replication. siRNA-mediated knockdown of the expression of the prominent CsA targets cyclophilin A and B did not affect SARS-CoV replication, suggesting that either these specific cyclophilin family members are dispensable or that the reduced expression levels suffice to support replication.

MAIN TEXT

The 2003 outbreak of Severe Acute Respiratory Syndrome (SARS) sparked a renewed interest in coronaviruses, a group of positive-strand RNA viruses that can cause respiratory or gastrointestinal disease in humans and livestock (reviewed in [317]). Several inhibitors of coronavirus enzymes (reviewed in [318]) and compounds that inhibit replication in cell culture have been described [143, 159, 319], but effective treatment of coronavirus infections is currently unavailable [187]. An inherent risk of the use of inhibitors directed against viral functions is the development of antiviral resistance due to the rapid adaptive evolution of RNA viruses. Coronavirus replication relies on a variety of host factors [320-322], which also constitute potentially interesting targets for antiviral therapy, as resistance is less likely to develop when host factors are targeted instead of viral proteins.

While aiming to identify host factors involved in SARS-coronavirus (SARS-CoV) replication, we established that the drug cyclosporin A (CsA) inhibited coronavirus replication. CsA affects the function of many members of the cyclophilin family, which consists of peptidyl-prolyl isomerases that act as chaperones and facilitate protein folding (reviewed in [279]). CsA was previously reported to inhibit the replication of human immunodeficiency virus [272], vesicular stomatitis virus [271], hepatitis C virus (HCV) [274, 299, 323] and other flaviviruses [273, 275].

Initially, using GFP-expressing recombinant coronaviruses, we investigated the effect of CsA on the replication of representatives of different coronavirus genera, i.e. human coronavirus 229E (HCoV-229E), mouse hepatitis virus (MHV) and SARS-CoV. In order to rigorously evaluate the inhibitory potential of the drug, each of these viruses was tested in single-cycle, high-multiplicity of infection (MOI) experiments, in which the drug was added upon removal of the inoculum at 1 h post infection (p.i.). Experiments were performed in 96-well plate format and GFP expression was quantified using a Berthold Mithras plate reader. When using SARS-CoV-GFP [324] and Vero E6 cells (MOI 10), a CsA dose range of 0 to 64 μM was used and cells were fixed at 18 h p.i. CsA inhibited SARS-CoV-GFP replication in a dose-dependent manner, with GFP expression becoming undetectable upon treatment with 16 μM CsA (Fig. 1a, upper panel). Cell viability was not affected at any of the CsA concentrations tested (Fig. 1a, lower panel). To confirm that CsA also inhibits SARS-CoV replication in human cells, the experiment was repeated using 293/ACE2 cells, which stably express the SARS-CoV receptor ACE2 [74]. Indeed, in these cells, CsA inhibited SARS-CoV-GFP replication to the same extent as in Vero E6 cells (Fig. 1b).

To investigate whether CsA also inhibits the replication of other coronaviruses, Huh7 cells infected with HCoV-229E-GFP [325] and 17CL1 cells infected with MHV-GFP [326] were given CsA at 1 h p.i. and GFP expression was quantified at 24 and 18 h p.i., respectively (Fig. 1c and d, upper panels). As in the case of SARS-CoV-GFP, MHV-GFP replication
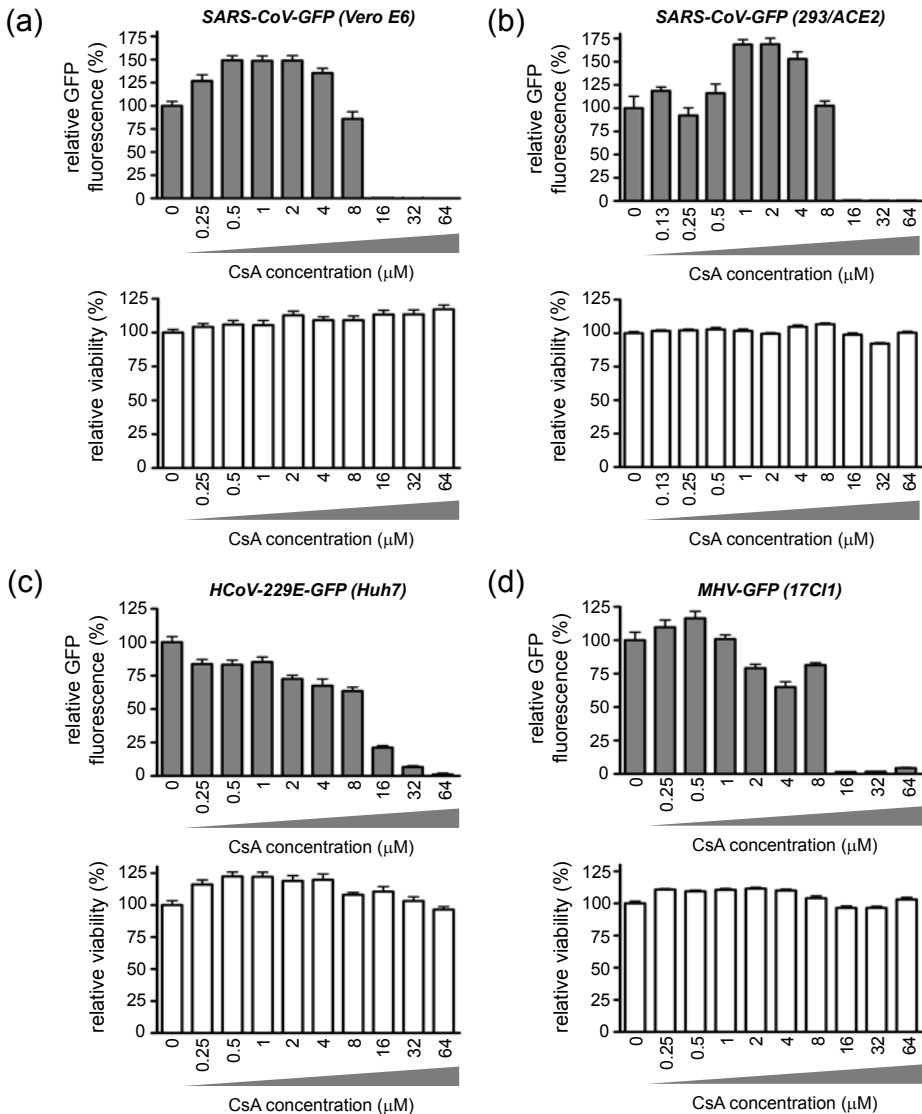
Fig. 1. CsA inhibits the replication of GFP-expressing recombinant coronaviruses. Vero E6 cells (a) or 293/ACE2 cells (b) were infected with SARS-CoV-GFP at a MOI of 10 and at 1 h p.i. the inoculum was replaced by medium containing different CsA concentrations. Cells were fixed at 18 h p.i. and GFP reporter expression was measured and normalized to the signal in control cells (100%) that were treated with DMSO, the solvent used for CsA (upper panels, grey bars). Huh7 cells infected with HCoV-229E-GFP were treated with CsA from 1 h p.i. on and were fixed for GFP measurements at 24 h p.i. (c, upper panel). 17CL1 cells were infected with MHV-GFP, treated with CsA from 1 to 18 h p.i., and GFP fluorescence was quantified (d, upper panel). The effect of CsA treatment on the viability of the various cell lines used, compared to untreated control cells (a-d, lower panels) was determined using the Cell Titer 96 AQ MTS assay (Promega). Graphs show the results (average and SD) of a representative quadruplicate experiment. All experiments were repeated at least twice.

was strongly inhibited by 16 µM CsA. HCoV-229E-GFP appeared to be somewhat less sensitive, as complete inhibition of GFP expression required 32 µM CsA (Fig. 1c). The viability of 17CL1 and Huh7 cells was not affected by the CsA concentrations used (Fig. 1c and d, lower panels). It should be noted that SARS-CoV replication appeared to be somewhat enhanced by low CsA doses (up to 4 µM).

Western blot analysis of SARS-CoV-GFP-infected Vero E6 cells that were treated with 0 to 32 µM CsA from 1 to 10 h p.i. showed that the expression of SARS-CoV non-structural protein (nsp) 8, nucleocapsid (N) protein and GFP was strongly reduced in cells treated with 16 µM CsA (Fig. 2a). This suggested that CsA treatment strongly inhibited an early step in the SARS-CoV replicative cycle. To verify the inhibitory effect of CsA with wild-type (wt) SARS-CoV, we repeated the experiments using the Frankfurt-1 isolate (Fig. 2b) and found that the expression of nsp8 and N protein was barely detectable upon treatment with 16 µM CsA. At lower CsA concentrations, little effect on viral protein synthesis was observed, indicating that the replication of recombinant and wt SARS-CoV is equally sensitive to CsA treatment. The steep dose-response curve, showing a strong reduction in SARS-CoV replication between 8 and 16 µM CsA, is in line with the observations made for several other +RNA viruses, like HCV [274, 299, 327].

The conclusions from Western blot studies were further substantiated by immuno-fluorescence labelling of nsp4 and dsRNA in SARS-CoV-infected cells, as markers for viral protein and RNA synthesis, respectively (Fig. 2c). Hardly any nsp4 or dsRNA was detectable upon treatment with 16 µM CsA and the immunolabelling for these markers was visibly reduced when 8 or 4 µM CsA was given. Remarkably, about 1-5% of the infected cells remained SARS-CoV positive in immunofluorescence analysis, even at CsA concentrations up to 64 µM, suggesting they were somehow insensitive to CsA treatment and remained capable of supporting a certain level of SARS-CoV replication. Compared to untreated cells the signals for nsp4 and dsRNA were clearly reduced in these cells, although - probably due to the relatively high avidity of the antibodies used - the N protein remained readily detectable (data not shown), suggesting that SARS-CoV replication was indeed impaired although not fully blocked.

To assess whether CsA treatment also affected the production of infectious progeny, virus titres were determined for supernatants harvested at 16 h p.i. from CsA-treated Vero E6 cultures infected with wt SARS-CoV or SARS-CoV-GFP (Fig. 2d). CsA indeed dramatically reduced progeny titres, with a 16 µM CsA dose resulting in approximately 4- and 3-log reductions for SARS-CoV-GFP and wt SARS-CoV, respectively. These data correlate well with the barely detectable expression of GFP, nsp4, nsp8 and N protein after treatment with 16 µM CsA (Fig. 1a and 2a-c). The 3-4 log progeny titre reduction also suggested that the low percentage of cells that remained SARS-CoV positive in im-munofluorescence assays upon treatment with 16 µM CsA produced reduced levels of infectious progeny. CsA also affected HCoV-229E-GFP titres (Fig. 2e), although a 32-µM
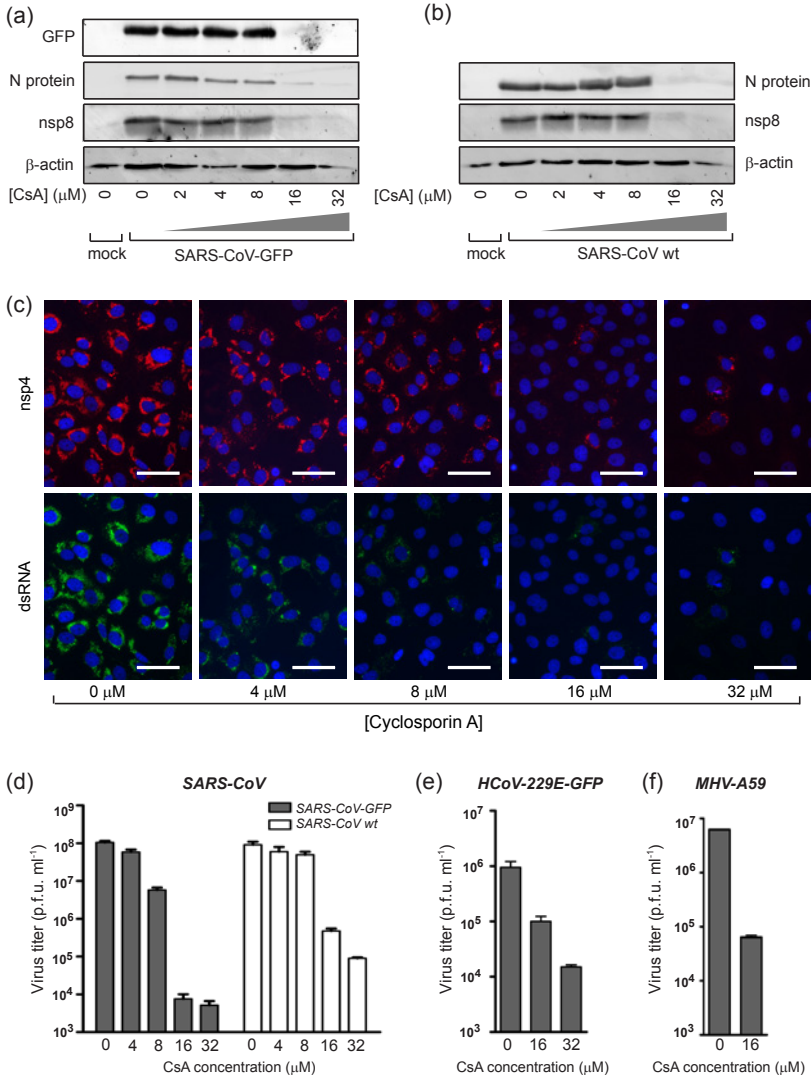
Fig. 2. CsA treatment inhibits coronavirus protein and RNA synthesis, and the production of infectious progeny. Vero E6 cells were infected with SARS-CoV-GFP (a) or wt SARS-CoV (b) and treated with CsA from 1 to 10 h p.i. Viral protein expression was analysed by Western blotting using polyclonal rabbit antisera against nsp8 [53], the N protein [295], and GFP as indicated next to the panels. β-actin, detected with a rabbit antiserum (Sigma), was used as loading control. (c) Immunofluorescence analysis of Vero E6 cells infected with SARS-CoV (MOI 10) and treated from 1 to 10 h p.i. with the CsA concentration indicated below each panel. Cells were stained with an anti-SARS-CoV nsp4 rabbit antiserum (upper panel; [53]) or an anti-dsRNA monoclonal antibody (lower panel; [30]). Scale bar: 50 μm. (d) Vero E6 cells infected with SARS-CoV-GFP (grey bars) or wt SARS-CoV (white bars) were treated with various concentrations of CsA from 1 h p.i. on, and virus titres in the culture supernatant were determined at 16 h p.i. by plaque assay on Vero E6 cells. Huh7 cells infected with HCoV-229E-GFP (e) or 17CL1 cells infected with MHV-A59 (f) were treated with CsA from 1 h p.i. on, and infectious progeny titres were determined at 30 h p.i. and 8 h p.i., respectively. The graphs show the mean of two independent duplicate experiments.

CsA concentration was required to achieve a 2-log reduction. Progeny titres of MHV, the fastest replicating of the three coronaviruses tested, were also 2-log reduced upon treatment with 16 µM CsA (Fig. 2f). Also, as observed for SARS-CoV-infected cells, a sub-population of the HCoV-229E-infected Huh7 and MHV-infected 17CL1 cells appeared to be resistant to CsA treatment.

CsA inhibits the peptidyl-prolyl isomerase activity of several members of the cy-clophilin family [279]. Specifically cyclophilin A (CypA) [271, 307, 308, 328] and B (CypB) [273, 309] have been reported to enhance the replication of several viruses. Furthermore, CypA was identified as interaction partner of the SARS-CoV N protein [312]. CsA might exert its inhibitory effect on coronavirus replication by inhibiting cyclophilin function or - alternatively - by direct inhibition of a virus-specific function. A direct inhibitory effect on the activity of the SARS-CoV nsp12 RNA-dependent RNA polymerase, was excluded using an *in vitro* assay and recombinant nsp12 (data not shown; [329]). We next analysed the effect of siRNA-mediated knock-down of cellular CypA and CypB expression (for 48 h) on the replication of SARS-CoV-GFP in 293/ACE2 cells. Western blot analysis of cells transfected with siRNAs targeting CypA and CypB confirmed that protein levels
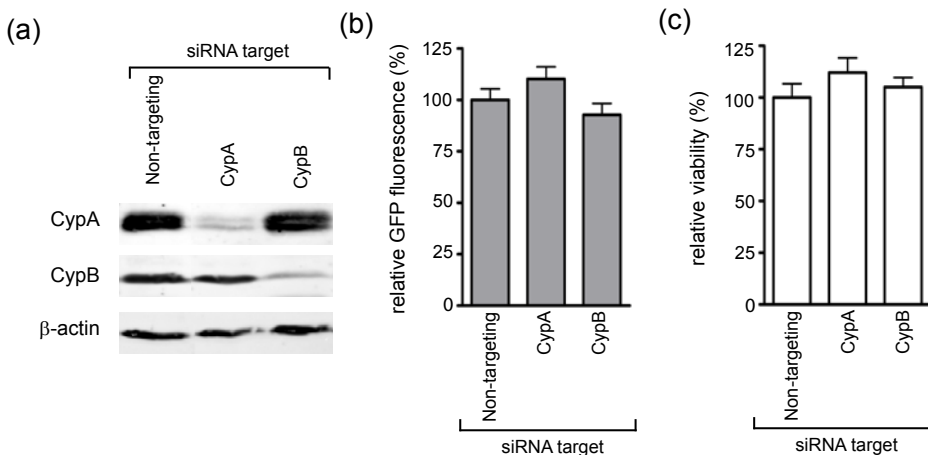

Fig. 3. SARS-CoV-GFP replication in Cyclophilin A- or B-depleted cells. Using DharmaFECT1 (Dharmacon), 293/ACE2 cells were transfected with siRNAs (Dharmacon ON-Target PLUS pools) targeting CypA and CypB mRNAs. Non-targeting siRNA, and siRNA targeting GAPDH expression were used as nega-tive and positive control for transfection and depletion efficiency, respectively. Expression levels of CypA (a, upper panel) and CypB (a, middle panel) in cells transfected with the siRNA pools indicated below the lanes, were analysed by Western blotting using specific antisera (Abcam). β-actin, detected with a rabbit an-tiserum (Sigma), was used as loading control. The viability of cells transfected with the various siRNAs was monitored using the Cell Titer 96 AQ MTS assay (b). Data were normalized to the average MTS assay value of cells transfected with non-targeting control siRNAs (100%). Forty eight hours after siRNA transfection, cells were infected with SARS-CoV-GFP and 24 h later cells were fixed and GFP fluorescence was quantified (c). The level of GFP expression was normalized to that in infected cells transfected with non-targeting siRNA.

were significantly reduced, to approximately 25 % of the original level (Fig. 3a). Depletion of CypA or CypB did not affect cell viability (Fig. 3b), but did also not significantly inhibit the replication of SARS-CoV-GFP in 293/ACE2 cells, compared to infected cells transfected with a non-targeting control siRNA (Fig. 3c). These data suggest that these specific cyclophilins, which have been implicated in the replication of other viruses, are not required for SARS-CoV replication. Alternatively, the remaining cyclophilin levels in siRNA-treated cells may suffice to support normal virus replication.

In conclusion, CsA inhibits the replication of diverse coronaviruses at non-cytotoxic, low-micromolar concentrations. Treatment of infected cells with 16 μM CsA strongly reduced viral and reporter gene expression of SARS-CoV-GFP, the amount of dsRNA in infected cells and the virus titre in culture supernatants (by more than 3 logs). In cells infected with HCoV-229E-GFP and MHV-GFP reporter gene expression and the production of infectious progeny were also significantly decreased upon CsA treatment. Compared to other RNA viruses [272-274, 299, 330], somewhat higher CsA concentrations were required to block coronavirus replication (16 versus 0.5-3 μM), suggesting coronaviruses to be less sensitive to CsA treatment. However, we cannot exclude that this may in part be due to differences in experimental set-up, including the cells and high MOI used and whether or not cells were pretreated with CsA [275, 307, 327].

The inhibitory effect of CsA and its analogues and the role of cyclophilins in virus replication have been studied in considerable detail for HCV and several other RNA viruses. In the case of HCV, cyclophilin inhibitors lacking the undesirable immunosuppressive properties of CsA – NIM811, Debio-025 and SCY-635 - are currently being tested in clinical trials [283-285]. Several mechanism of action studies on the inhibitory effect of CsA identified mainly CypA and CypB to be involved in virus replication. CypA was found to interact with HCV NS2 [331], NS5A [302, 304, 332] and NS5B [306] and was shown to be required for HCV replication. Furthermore, CypA was found to functionally interact with West Nile Virus NS5 [275] and vesicular stomatitis virus N protein [271]. In addition, an interaction between CypB and Japanese encephalitis virus NS4A [273] was documented and CypB also appears to be a functional regulator of the HCV polymerase [309]. Also Cyp40 was found to play a role in HCV replication [303, 333].

Although the exact mechanism by which CsA inhibits coronavirus replication remains to be established, it is likely that the drug also interferes with functional interactions between viral proteins and one or multiple members of the large cyclophilin family. If this indeed proves to be true, it will be interesting to explore the potential of these host proteins for the development of a coronavirus-wide antiviral strategy.

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CHAPTER 4