The quest for broad-spectrum coronavirus inhibitors

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CHAPTER VII

The cyclophilin-dependent calcineurin inhibitor voclosporin inhibits SARS-CoV-2 replication in cell culture

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ABSTRACT

Kidney transplant recipients (KTRs) are at increased risk for a more severe course of COVID-19, due to their comorbidity and maintenance immunosuppression. Consensus protocols recommend lowering immunosuppression in KTRs with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, but the optimal combination remains unclear. Calcineurin inhibitors (CNIs) are cornerstone immunosuppressants in KTRs and some have been reported to possess antiviral activity against RNA viruses, including coronaviruses. We evaluated the effect of the CNIs tacrolimus, cyclosporine A , and voclosporin (VCS), as well as other immunosuppressants, on SARS-CoV-2 replication in cell-based assays. Loss of compound due to plastic binding and interference of excipients in pharmaceutical formulations (false-positive results) complicated the determination of EC50 values in our antiviral assays. We could circumvent some of these issues by using exclusively glass labware. VCS reduced viral progeny yields in human Calu-3 cells at low micromolar concentrations and did so more effectively than cyclosporin A and tacrolimus. Although, we cannot recommend a particular immunosuppressive regimen in KTRs with COVID-19, our data suggest a potential benefit of cyclophilin-dependent CNIs, in particular VCS, which warrants further clinical evaluation in SARS-CoV-2-infected KTRs.

TRANSLATION STATEMENT

As the efficacy of the vaccines is uncertain in immunocompromised hosts like KTRs and effective (antiviral) treatment options icient therapies are limited, finding alternative solutions is crucial to protect these patients. In this study, CNIs demonstrated more potent inhibitory effect of SARS-CoV-2 replication (in cell culture) than other classes of immunosuppressive agents. Strikingly, VCS displayed antiviral activity at 8-fold lower concentrations lower than TAC. Of note, the concentrations of VCS that reduced SARS-CoV-2 viral load can be correlated with tolerable doses in humans that are attainable in KTRs. Therefore, VCS becomes an attractive CNI and it is currently under investigation for COVID-19 infected KTRs.

INTRODUCTION

Between December 2019 and January 2021, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease-2019 (COVID-19), has resulted in over 90 million infections globally. A more severe course of COVID-19 has been correlated to comorbidities commonly present in solid organ transplant recipients[1-3]. Moreover, initial reports showed that the latter are among those at increased risk of COVID-19 related death[4].

Finding the balance between preventing rejection and controlling infections is the conundrum when prescribing immunosuppressive regimens for transplant recipients[5]. The current standard for immunosuppressive therapy in most transplant centers consists of a calcineurin inhibitor (CNI), either tacrolimus (TAC) or cyclosporin A (CsA), an antimetabolite agent such as mycophenolate (MPA/MPS) and most often, maintenance steroids. An mTOR inhibitor such as everolimus (EVL) may be prescribed alternatively to MPA[6]. The precise impact of immunosuppression on the course of COVID-19 is poorly understood. Early in the disease, (over)immunosuppression might prevent a proper antiviral response, whereas later some immunosuppression might protect against pathological immune overactivation, resulting in less severe disease. Consequently, consensus protocols recommend to reduce but not completely cede immunosuppression in SARS-CoV-2 infected KTR's, depending on the risk of rejection and disease severity[7, 8].

Previous reports suggest that CNIs and mTOR inhibitors like EVL in addition to MPA have antiviral activity against human coronaviruses such as SARS-CoV and Middle East respiratory syndrome (MERS-) CoV [9]. TAC (which targets FKBP12) was reported to inhibit CoV replication in cell culture at non-cytotoxic concentrations[10], and was recently proposed as a potential inhibitor of SARS-CoV-2 replication by computational analysis[11].

Next to its immunosuppressive effects[12-17], CsA was reported to inhibit replication of different RNA viruses in cell culture, including influenza virus[18], human immunodeficiency virus-1[19], hepatitis C virus[20], flaviviruses[14], and human and zoonotic CoVs[17, 21-24]. Several non-immunosuppressive CsA derivatives, like alisporivir (Debio-025), also inhibit the replication of CoVs in cell culture[12, 22, 25], including SARS-CoV-2[26, 27]. Collectively, these studies established the broad-spectrum antiviral activity of CsA and derivatives in cell-based infection models. Since cyclophilins (Cyps) were also implicated in CoV replication, CsA has been recommended for KTRs during the COVID-19 pandemic as an alternative to other regimens to prevent rejection[28]. Although Cyp inhibitors can affect CoV replication, the exact role of these host proteins in CoV replication remains elusive[29].

Voclosporin (VCS) is a novel CNI which has been studied in psoriasis, renal organ transplantation, and was recently FDA-approved for treatment of active lupus nephritis in combination with background immunosuppressive therapy[30-32]. Structurally similar to CsA, VCS incorporates a methyl group at the amino acid residue position 1, which enhances its binding to calcineurin, and confers better metabolic stability[33, 34]. (Pre)clinical observations suggest that VCS is more potent and less toxic at therapeutic levels than other immunosuppressants in its class[31, 33-36]. Moreover, VCS was shown to inhibit norovirus replication in a CypA-dependent manner and more effectively than CsA [16]. Therefore, VCS is an interesting candidate to evaluate for inhibitory activity on SARS-CoV-2 replication.
In this study we compared the effect of three calcineurin inhibitors (TAC, CSA, VCS) and other immunosuppressants commonly used in KTR's on SARS-CoV-2 replication using cell-based assays.

RESULTS

Inhibition of SARS-CoV-2 replication in Calu-3 cells by VCS, CsA and TAC

To evaluate the effect of VCS, CsA and TAC on SARS-CoV-2 replication, viral load reduction assays were performed using human lung epithelial cells (Calu-3). VCS is highly lipophilic and binds to plastic, which could compromise its bioavailability in standard cell-based assays using plastic labware. Therefore, we compared the effect of the compound in standard assays using plastics and custom assays using glass tubes, containers and pipettes. RDV was included as a positive control for inhibition of SARS-CoV-2 replication.
Cells were treated with different concentrations of compound before, during and after SARS-CoV-2 (Fig. 1A and 1B) or mock infection (Fig. 1C and 1D). Calu-3 cells in glass remained viable and supported SARS-CoV-2 replication, as titers of 1.7 x 10^6 PFU/ml were measured in the medium at 24 h p.i. (Fig. 1A). Treatment of infected cells with 10 µM of RDV inhibited viral replication resulting in infectious progeny titers just above the limit of detection of the plaque assay (data not shown). Treatment of cells with 3.2 µM VCS caused a more than 1.5 log reduction in SARS-CoV-2 infectious progeny titers, while an ~0.5 log reduction was observed when the same concentration of CsA or TAC was used (Fig. 1A). However, treatment with 3.2 µM VCS or CsA also caused cytotoxic effects, as cell viability dropped to ~75% (Fig. 1C). Therefore, it cannot be excluded that part of the observed antiviral effect is due to pleiotropic effects (toxicity).
In experiments using plastic materials, a dose-dependent reduction in infectious progeny titers was observed when cells were treated with VCS, leading to a more than 1 log reduction at 6.4 µM (Fig. 1B). CsA treatment led to a similar reduction at 25 µM, but at 6.4 µM inhibited

less than VCS. However, at concentrations of 12.5 µM or above CsA displayed significant cytotoxicity while VCS did not (Fig. 1D). TAC did not display much cytotoxicity, but a concentration of 25 µM was required to reduce the infectious virus progeny titer by more than 1 log. VCS had a stronger effect in experiments performed with glass instead of plastic labware, likely due to loss of the compound by binding to plastic. We therefore measured the concentration of free VCS after incubating various solutions in glass containers either with or without cells. No significant loss of compound from solution was observed after a 24h incubation at 37°C in glass without cells (Table 1). When VCS solutions with concentrations from 0.2 to 3.2 µM were incubated in glass bottles with Calu-3 cells, a ~75% reduction of the VCS concentration was measured, suggesting the compound was bound or taken up by cells. We also measured the VCS concentration in the medium of (infected) cells after 24h treatment with 25 µM VCS in experiments performed with standard plastic labware, which turned out to be as low as 0.68 µM. Even considering a 75% reduction due to cellular binding or uptake, it implies that 90% of VCS was lost due to plastic binding. The similar reduction in virus titers by 3.2 and 25 µM of VCS in glass and plastic, respectively, corroborated that when using plastic, the bioavailable amount of VCS is likely ~10% of what was added initially.

Table 1- VCS concentration in samples from experiments using only glass labware, measured by LC-MS/MS

	Concentration of VCS in supplied solution											
	3.2 µM		**3.2 µM**		**1.6 µM**		**0.8 µM**		**0.4 µM**		**0.2 µM**	
	Without cells		**With cells**									
Incubation time	**Conc. in µM**	**% remaining**	**Conc. in µM**	**% remaining**	**Conc. in µM**	**% remaining**	**Conc. in µM**	**% remaining**	**Conc. in µM**	**% remaining**	**Conc. in µM**	**% remaining**
0 h	2.91		2.91		1.77		0.99		0.45		0.33	
24 h	2.79	**96**	0.82	**28**	0.35	**20**	0.15	**15**	0.10	**22**	<0.07*	**ND**

*below detection limit of LC/MS-MS Note: The percentages indicate the ratio of the measured (true) concentration at 24 h and the concentration of the prepared solution administrated to the cells (at 0 h incubation time).

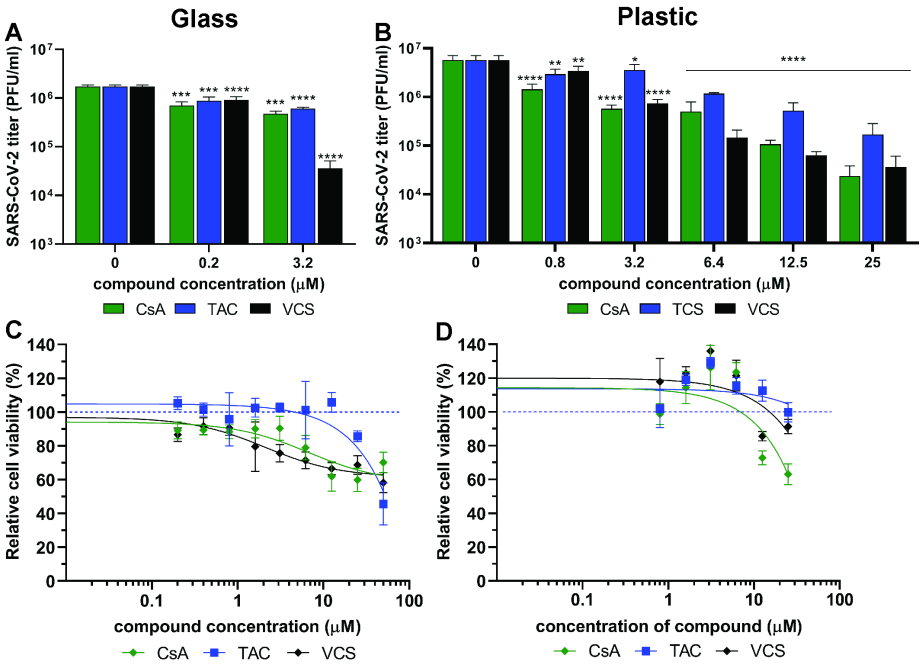

Fig. 1. Impact of CsA, TAC and VCS treatment on the production of infectious SARS-CoV-2 progeny by human Calu-3 cells. Experiments were performed using either glass (A and C) or plastic labware (B and D). Cells were infected with SARS-CoV-2 in the presence of different concentrations of VCS, CsA and TAC using stock solutions prepared from pure powders dissolved in DMSO. The viral load in the medium of infected cells was determined by plaque assay on Vero E6 cells using supernatant harvested at 24 h p.i. Viability of uninfected Calu-3 cells treated with the same range of compound concentrations was measured in parallel by a colorimetric viability assay (C; n=12; D; n=3). Mean values ± SD are shown and statistical significance of the difference between each concentration and solvent control was assessed by one-way ANOVA. *, *p<0.1*; **, *p<0.01*; ***, *p<0.001*; ****, *p<0.0001*.

Inhibition of SARS-CoV-2 replication in cell culture by various immunosuppressive drugs

In order to avoid problems with solubility and plastic binding (as described above) and to compare the antiviral effect of different immunosuppressive drugs commonly used in KTRs, we performed SARS-CoV-2 CPE reduction assays with the pharmaceutical formulations (including excipients, co-solvents and other components) of VCS, CsA, TAC, EVL, and MMF. In parallel, drug cytotoxicity was assessed in non-infected cells. For VCS we confirmed by LC-MS/MS that the intended concentrations were achieved when dissolving stock solutions at 6.4 µM (data not shown).

The CNIs VCS, CsA, and TAC inhibited virus-induced cell death with EC_{50} values in the sub- to low micromolar range (Fig. 2A, 2B, 2C). EVL (Fig. 2D) did not show an inhibitory effect at tested

concentrations. The prodrug MMF (Fig. 2E) was included in our comparison, but was not expected to inhibit virus replication, as it is likely not metabolized into its active form MPA[37] in our assay [38, 39]. Thus, we attributed the apparent antiviral effect of MMF mainly to excipients present in the drug formulation (see below).

The EC_{50} values of VCS, CsA and TAC were 0.22 ± 0.01 µM, 4.3 ± 0.6 µM and 10 ± 1 µM, respectively. Apart from VCS, none of the compounds showed cytotoxicity, and therefore their CC_{50} values were higher than 100 µM. Although VCS displayed higher cytotoxicity, with a CC_{50} of ~4 µM, its EC_{50} was also 18-45 times lower compared to the other compounds tested.

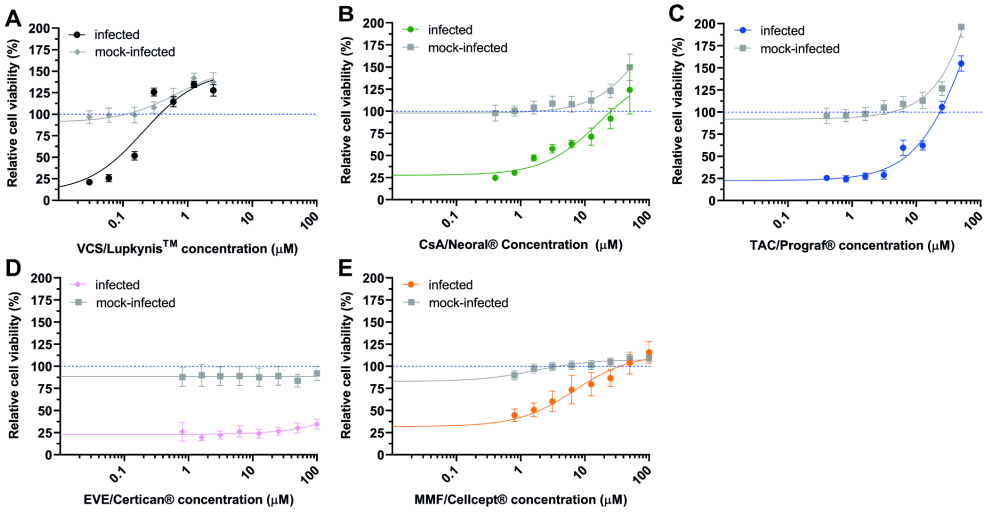

Fig. 2. Effect of immunosuppressive drugs on SARS-CoV-2 replication. Inhibition of SARS-CoV-2 replication (colored symbols and curves) in Vero E6 cells by various drugs were determined by CPE-reduction assay. For each drug, two-fold serial dilutions of the pharmaceutical formulations were tested. (A) VCS, (B) cyclosporine A/ Neoral, (C) TAC/ Prograf, (D) EVL/ Afinitor and (E) MMF/ Cellcept. After preincubation with compound, cells were infected with SARS-CoV-2 and kept in medium containing the drug for 3 days, after which cell viability was measured with a colorimetric assay. Cytotoxicity of the drugs was evaluated in parallel using mock-infected, compound-treated cells (solid grey line). Data points represent the mean ± SD of two independent experiments. The CC50 and EC50 were determined by non-linear regression analysis and the regression curves are plotted in the graphs (solid lines).

An excipient in the pharmaceutical formulation of VCS inhibits SARS-CoV-2 replication in CPE reduction assays

To investigate whether one or more excipients contributed to the low EC_{50} (Fig. 2A) of the pharmaceutical formulation of VCS, CPE reduction assays were performed to assess the

antiviral effect of the content of VCS capsules and placebo capsules. Surprisingly, both the VCS formulation (Fig. 3A) and the placebo (Fig. 3B) inhibited SARS-CoV-2 replication in a similar dose-dependent manner. The absence of VCS in placebo capsules was confirmed by LC-MS/MS analysis (not shown). Therefore, one or more excipients in the drug formulation appear to have an antiviral effect in this experimental setup (further evaluation in supporting information).

To avoid interference by excipients in our antiviral assays we performed CPE reduction assays with DMSO solutions prepared from high purity powders of the various immunosuppressive drugs. In the case of Neoral (CsA microemulsion), CsA powder, the most commonly used CsA derivative in KTR treatment, was evaluated. VCS solutions prepared from pure powder did not confer the same level of protection to SARS-CoV-2 infected-cells (Fig. 4A) as solutions made from the pharmaceutical formulation (Fig. 3A). However, the VCS solution from pure powder also caused less cytotoxicity. The same issue was observed with CsA and MPA (Fig. 4B and 4D), suggesting that also in cell-based assays these drugs need excipients to ensure solubility/bioavailability for optimal activity. Interestingly, TAC solutions prepared from pure powder inhibited SARS-CoV-2 with similar efficacy as the drug formulations, i.e., with an EC_{50} of ~15 µM (compare Fig. 2C and 4C), suggesting that the pharmaceutical formulation of TAC does not contain excipients with antiviral effects.

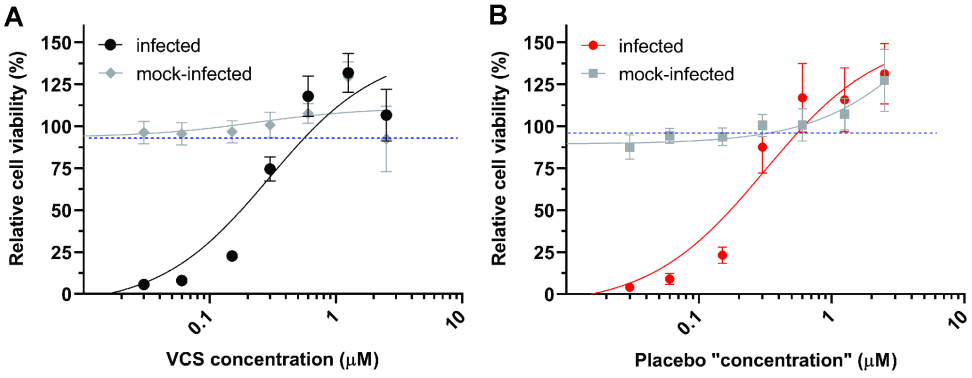

Fig. 3. Comparison of the antiviral effect of VCS and placebo pharmaceutical formulations. The inhibition of SARS-CoV-2 replication in Vero E6 cells treated with the content of VCS pharmaceutical formulation (A) or placebo (B) was determined by CPE reduction assays as described in the legend of Fig. 2.

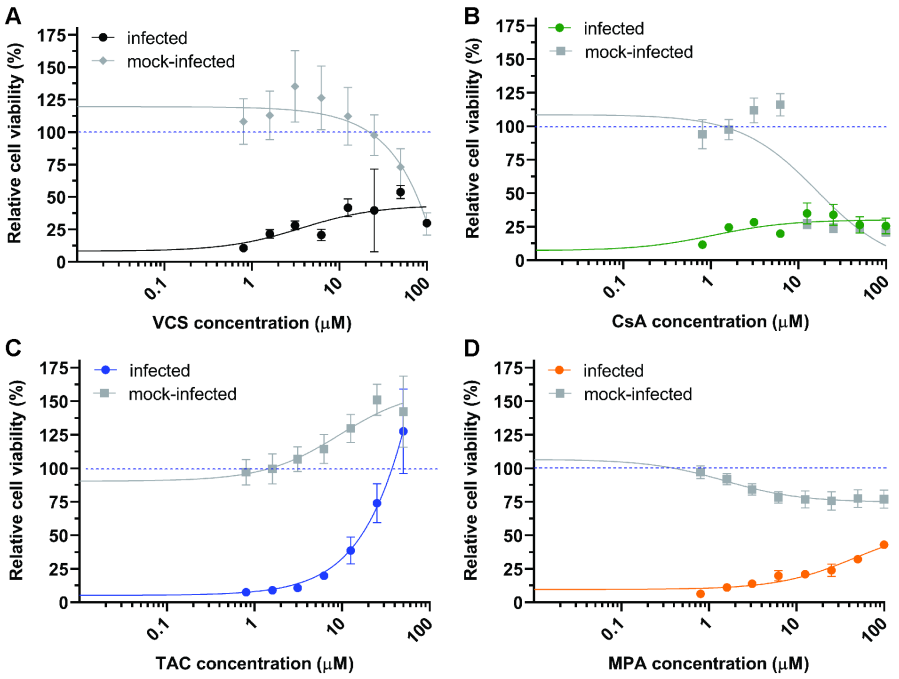

Fig. 4. Inhibition of SARS-CoV-2 replication by immunosuppressive compounds in CPE-reduction assays with stocks prepared from pure compound powders. (A) VCS, (B) CsA, (C) TAC, and (D) MPA. For details, see the legend to Fig. 2.

DISCUSSION

KTR's are at increased risk for developing a severe course of COVID-19 owing to older age, comorbidities or their immunocompromised state[4, 40, 41]. The attributable effect of immunosuppression for a more severe course of COVID-19 as well as the most optimal treatment in KTRs is yet unclear[5, 9]. Different clinical observations suggested that immunosuppression did not impose an increased risk for severe COVID-19 disease or mortality[42-45]. However, increased death rates have been observed for immunocompromised COVID-19 patients[4, 46, 47]. As the efficacy of approved vaccines is yet uncertain in KTRs, gaining more insight is crucial.

In general, COVID-19 displays a triphasic course: starting with mild flu-like symptoms, followed by a second phase of viral replication and pneumonia, which in a small percentage of cases is followed by a third phase of life-threatening disease, e.g., due to a cytokine storm[48]. Antiviral drug treatment is expected to be most effective during earliest stages of

disease, while immunosuppressants (e.g. steroids, tocilizumab) may be considered a therapeutic option in later stages of disease to reduce inflammation.

An immunosuppressive regimen might ideally prevent rejection, possess antiviral properties and reduce (over)inflammation, whilst still mounting an effective antiviral response to prevent a severe disease course simultaneously. Consensus recommendations advocated to lower but not completely halt immunosuppression and some advocated steroids with CNI's based on theoretical (in vitro) advantages[49]. There is scant clinical data. Interestingly, one large single-center observational study found a clear survival benefit for patients when put on CsA compared to other experimental (off-panel) anti-inflammatory therapy for COVID-19[50] Various studies, also by our group, previously demonstrated that CNIs like CsA and TAC inhibit replication of a variety of (human) CoVs such SARS- and MERS-CoV[10, 17, 21, 51, 52]. As these *Betacoronavirus* are closely related to SARS-CoV-2 [9, 53, 54], these drugs were hypothesized to inhibit SARS-CoV-2 replication as well. In this study, we evaluated the antiviral effect of CNIs with a focus on VCS, a novel CNI with a modified amino acid–1 group on the CsA structure, which increases its affinity for calcineurin[33, 34, 55]. A potential advantage of VCS over CsA is its lower nephrotoxicity[56]. Our study demonstrates that VCS inhibits SARS-CoV-2 infection in cell culture with an EC_{50} in the sub-micromolar range, at lower concentrations than CsA or TAC. We evaluated the effect of these different CNIs on SARS-CoV-2 replication by viral load reduction assays in Calu-3 cells, a human lung epithelial cell line that was shown to be permissive to SARS-CoV-2[57, 58]. Our findings are in line with those reported in a non-peer reviewed manuscript that reported CsA inhibited SARS-CoV-2 replication in HuH7.5 and Calu-3 cells, but not in Vero cells[59]. In contrast to our finding that TAC inhibits SARS-CoV-2 replication in Vero E6 cells with an EC_{50} of ~15 μM, Dittmar et al found no activity for TAC in any of these cell lines[59], which might be due to the use of different Vero cell subclones.

While testing the pharmaceutical formulations of different immunosuppressive drugs commonly used in KTRs, we discovered that the excipients in these preparations have antiviral effects in our cell-based assays which prevented the determination of true EC_{50} values of active ingredients. Unexpectedly, this was not due to virucidal effects of surfactants that can damage the viral envelope[60-62], but rather through an effect on infected cells that could not be elucidated further in this study. Testing of highly pure powders of the various immunosuppressive compounds to circumvent the interference caused by excipients in our antiviral assays, led to much higher EC_{50} values for VCS, CsA and TAC, demonstrating that excipients that improve solubility and bioavailability of the active compound in pharmaceutical formulations also affect results in cell-based assays. VCS is known to bind to plastic, and since in general mainly plastic labware is used, we determined the available free

VCS concentrations in the medium at the beginning and end of experiments using a validated LC-MS/MS method. This revealed that VCS binding to plastic caused a>80% loss of the compound from solution. Consequently, the use of stock solutions prepared from pure VCS powder using plastic labware leads to a serious underestimation of the compound's efficacy in antiviral assays. VCS is a highly lipophilic compound, and interactions between plastic surfaces and hydrophobic drugs[63] that have a negative effect on bioavailability have been described before[64, 65]. Our attempts to prevent VCS binding to plastic by treating labware with various coatings were unsuccessful as none led to a more than ~10% recovery of the initial VCS concentration.

Since we were unable to use the oral formulation of VCS and were not able to overcome the plastic binding and loss of compound when using preparations of pure VCS, we performed experiments using glass labware only. These conditions supported growth of human Calu-3 cells and SARS-CoV-2 replication, while measurement of VCS concentrations demonstrated that there was hardly any loss of the compound. Using this setup, demonstrated that VCS reduced the production of SARS-CoV-2 infectious progeny in a dose-dependent manner in infected Calu-3 cells, and more effectively than CsA and TAC.

In summary, in this study we demonstrate that cyclophilin-dependent CNIs inhibit SARS-CoV-2 replication in cell culture more potently than other classes of immunosuppressants, like EVL and MPA. VCS inhibited SARS-CoV-2 replication at 8-fold lower concentrations than TAC. Of note, TAC concentrations that are required to inhibit SARS-CoV-2 replication likely correlate with intolerable or toxic concentrations in humans (EC_{50} of 0.2 µM equals 160 ng/ml for TAC), without taking into account that the free fraction in traffic is around one tenth of the total concentration. For CsA and VCS 0.2 µM corresponds to a concentration of 241 and 243 ng/ml respectively. Notably, VCS is also known to distribute into organs such as the lungs in higher concentrations than in blood, and higher concentrations are found in red blood cells. Consequently, higher concentrations in specific organs or cells might enable some inhibition of the virus. In conclusion, VCS has become an attractive alternative CNI for therapy that might also inhibit SARS-CoV-2 replication at concentrations that are safe in humans. Since VCS is thought to have comparable efficacy to TAC for prevention of rejection in KTRs, VCS might be an interesting option in COVID-19 patients. Based solely on the experimental data presented in this study, we do not advocate the use of VCS merely for its potential antiviral properties. However, our data suggest a potential benefit of cyclophilin-dependent CNIs, in particular VCS, among immunosuppressants commonly used in transplant medicine. This warrants further clinical evaluation and VCS is currently under investigation in SARS-CoV-2-infected KTRs [EudraCT 2020-001467-82].

MATERIALS AND METHODS

Virus and cell lines

SARS-CoV-2/Leiden-0002 (GenBank MT510999) was isolated from a nasopharyngeal sample at LUMC in March 2020. Infections were performed with a virus stock that had been passaged twice in Vero E6 cells. Vero E6 cells and Calu-3 2B4 cells [66], referred to as Calu-3 cells in this manuscript, were cultured as described previously[67]. Infections were performed in Eagle's minimal essential medium (EMEM; Lonza) with 25 mM HEPES (Lonza), 2% FCS, 2 mM L-glutamine, and antibiotics (EMEM-2% FCS). All experiments with infectious SARS-CoV-2 were performed in a biosafety level 3 facility at the LUMC.

Immunosuppressive compounds

Voclosporin (Lupkynis™), cyclosporine A (Neoral®, Novartis), tacrolimus (Prograf®, Astellas), mycophenolate mofetil (CellCept®, Roche) or everolimus (Certican®, Novartis) stock solutions were prepared by dissolving the pharmaceutical formulation of these drugs in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Placebo capsules and pure VCS powder were supplied by Aurinia Pharmaceuticals Inc., Inc. Tacrolimus (PHR1809), cyclosporin A (30024) and mycophenolic acid (M5255) were purchased from Sigma-Aldrich. Remdesivir (RDV; HY-104077) was acquired from MedChemExpress and was used as a control in all experiments. All compounds were dissolved in DMSO and single use aliquots were stored at -20°C.

Measurement of cyclosporin A, tacrolimus and voclosporin concentrations by validated LC-MS / MS

Quantification of CsA and TAC was performed by LC-MS/MS as previously described [68] by diluting samples in methanol and subsequently in blank whole blood. A Thermo Quantiva UPLC-MS/MS system (Ultimate 3000 series UHPLC system, coupled to a TSQ Quantiva triple stage quadrupole mass spectrometer) was used with an Acquity UPLC BEH C18 1.7 µm; 2.1 x 50 mm column coupled to a VanGuard BEH C18 1.7 µm precolumn for chromatographic seperation. Online solid phase extraction was performed using a Xbridge 10 µm 30 x 2.1 mm column. Before analysis, samples were diluted in methanol and subsequently whole blood to fall within the calibration line of 0-15-600 µg/L of VCS. Human whole blood was added to 10- or 20-µl samples to a final volume of 200 µl and 200 µl of 0.1 M zinc-sulphate and 500 uL of internal standard solution (32 ug/L of VCS D_4 in acetonitrile) were added. Samples were then vortexed at 2000 rpm for 5 min and centrifuged at 13000 rpm for 5 min and 20 µl was injected into the LC-MS/MS system. The method was validated according to the EMA bioanalytical method validation guideline[69].

Cytopathic effect (CPE) reduction assay

CPE reduction assays in Vero E6 cells were performed as previously described [26]. Briefly, Vero E6 cells in 96-well plates were pre-incubated with 2-fold serial compound dilutions for 30 min. Subsequently, cells were either mock-infected (to assess cytotoxicity of compounds) or infected with 300 plaque-forming units (PFU) of SARS-CoV-2 per well (multiplicity of infection (MOI) of 0.015) in a total volume of 150 µl of medium with compound. Plates were incubated for three days at 37°C, after, which cell viability was determined using the colorimetric CellTiter 96® Aqueous Non-Radioactive Cell Proliferation kit (Promega). The absorption at 495 nm was measured with an EnVision Multilabel Plate Reader (PerkinElmer) and the EC_{50} (50% effective concentration, required to inhibit virus-induced cell death by 50%), and CC_{50} (50% cytotoxic concentration, reduces the viability of uninfected cells to 50% of control), were determined using non-linear regression with GraphPad Prism v8.0. For each compound, at least two independent experiments (each in quadruplicate) were performed.

Viral yield reduction assays

Calu-3 cells were seeded in 96-well plates (3×10^4 cells per well) in 100 µl of culture medium. The next day, cells were pre-incubated for 60 min with 2-fold serial dilutions of CsA, TAC or VCS, starting at 25 µM concentration and RDV starting at 10 µM. Subsequently, cells were infected with SARS-CoV-2 (MOI of 1, based on titer determined on Vero E6 cells) in 50 µl of medium with compound. After a 1h incubation at 37°C, cells were washed three times with PBS and 100 µl of medium with compound was added. The medium was harvested from the wells at 24-hours post-infection (h p.i.). Analysis of viral progeny released from the infected Calu-3 cells was performed by plaque assay on Vero E6 cells as described[67]. VCS concentrations were measured by validated LC-MS/MS after adding 9 volumes of methanol to the harvested medium. , . A cytotoxicity assay with mock infected cells, treated in the same way, was performed in parallel, as described for the CPE reduction assay.

Viral yield reduction assays in glass bottles

Borosilicate glass reagent bottles (50-ml) were treated with glacial acetic acid to remove possible detergent residues, followed by washing twice with absolute ethanol. The bottles dried and UV-sterilized prior to use. Three times concentrated compound solutions were prepared in EMEM-2% FCS using sterile glass culture tubes, a glass 50-µl syringe (Hamilton) and glass Pasteur pipettes. One ml of each compound dilution was transferred to three different reagent bottles (triplicates). Confluent monolayers of Calu-3 cells grown in culture flasks were infected with SARS-CoV-2/Leiden-002 at an MOI of 1. After incubation for 1h at

37°C, cells were washed three times with warm PBS, trypsinized and resuspended in EMEM-2% FCS. Two ml of this cell suspension (~10^6 cells) was added to each reagent bottle that already contained 1 ml of a 3x concentrated compound solution in medium. After incubation for 24h at 37°C, the medium was collected and the infectious virus titer was determined by plaque assay on Vero E6 cells. VCS concentrations in the medium were determined by LC-MS/MS as described above.

Determination of compound cytotoxicity in glass culture tubes

Calu-3 cells were trypsinized and 1.5 x 105 cells in 1 ml of EMEM-2% FCS were divided over glass culture tubes.. Two-fold dilutions of VCS, TAC and CsA starting at 150 µM concentration (3x final concentration) were prepared in EMEM-2% FCS medium using glass labware, and 0.5 ml was added to corresponding tubes with cells (three tubes per concentration). After a 24h incubation, cell viability was determined as described above.

SUPPORTING INFORMATION

Potential virucidal activity by an excipient of pharmaceutical formulations

Since the excipients include surfactants that could destroy the viral envelope, we tested whether the contents of the placebo capsules had a virucidal effect. To determine the virucidal potential of compounds or formulations, SARS-CoV-2 virions ($5x10^4$ PFU) were incubated for 2 h at 37°C with one of the following solutions: medium, a VCS solution prepared from pure powder, the dissolved content of VCS capsules, placebo capsules or Tween solutions (present in the capsules). The effect of these treatments on SARS-CoV-2 infectivity was determined by plaque assay on Vero E6 cells (Fig. S1). Phosphate-buffered saline (PBS) was used as a negative control and 50% ethanol as a positive control for virucidal activity. The (remaining) infectious virus titer was determined by plaque assay on Vero E6 cells as described[67]. A control treatment with 50% ethanol reduced the amount of infectious SARS-CoV-2 to below the limit of detection (<100 PFU/ml), while none of the other treatments significantly affected the remaining infectivity of the virus. Therefore, we concluded that the drug product excipients had no virucidal effect, but through an uncharacterized mechanism interfered with the readout of our CPE reduction assays. This made it impossible to determine the true EC_{50} values of these compounds when they were tested in their pharmaceutical formulations.

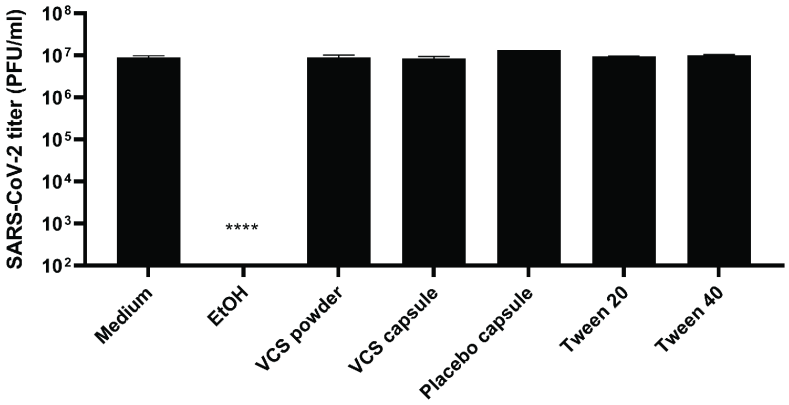

Fig. S1. Virucidal activity of VCS powder (3.2 µM), the drug formulation of VCS (3.2 µM), and content of placebo formulations (corresponding to 3.2 µM VCS), and 50% ethanol by incubation with a SARS-CoV-2 virus stock for 2 h, followed by quantification of the remaining amount of infectious virus titer by plaque assay. Statistical significance was determined by one-way ANOVA. *, p<0.1; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Effect of Coating of plastic materials

The excipients in the pharmaceutical formulation of VCS appear to be critical for its bioavailability (to prevent plastic binding) but their (non-specific) antiviral effect also interferes with the determination of the true EC50 of VCS. To enable the use of VCS solutions prepared from pure powder in antiviral assays, we attempted to prevent VCS binding to plastic by coating all plastic labware used with 3 different coating agents that were described in literature: 100 mg/ml bovine serum albumin in PBS (BSA; Sigma) [70], 1% polyethylene glycol 3350 in MilliQ water (PEG-3350; Sigma)[71, 72] and 0.2% polysorbate 40 in MilliQ water (Tween40; Fluka) [73]. In addition, we saturated the plastic materials with VCS by treating them with a 500 mM VCS solution in DMSO (Sigma). Labware, including all tubes, tips and culture plastics, was filled with blocking solution and incubated for 2 h at room temperature with rocking to homogenously coat the surfaces. After rinsing twice with MilliQ water, the items were left to dry at room temperature until further use in experiments. Solutions of 0.2 and 2 µM of VCS were prepared in EMEM-2% FCS and 100 µl of each VCS solution was incubated in coated 96-well plates. After a 2 h incubation at 37°C the remaining VCS concentration was measured by validated LC-MS/MS. None of the coating treatments were able to reduce the nonspecific binding to plastic and loss of VCS (Table S1), as only 5 to 7% of the original concentration was recovered after a 2 h incubation. Even at t=0 only ~27% of the original stock concentration could be recovered due to VCS loss in pipette tips and tubes during the preparation of dilutions. Saturation of binding sites on plastic by treatment with 500 mM of VCS prevented loss of VCS from solution, but led to non-controlled VCS leaching from the plastic. This resulted in unpredictable concentrations that were higher than those in the input solution, e.g., we measured a VCS concentration of >15 µM when a 2 µM solution was incubated in a VCS saturated plastic plate. Since none of the coating treatments prevented nonspecific binding to plastic, the problem was circumvented by using glassware instead of plastics (Table 1). We also determined whether TAC and CsA bind to plastic using the same method as for VCS. Binding to plastic was minimal for TAC (24% loss) and for CsA we observed that after a 2 h incubation the CsA concentration was still 62% of the initial concentration (Table S2)

Table 2- VCS concentration in samples incubated in plastic labware with different coatings, measured by LC-MS/MS

	Type of coating applied									
	Uncoated		500 mM VCS		100 mg/ml BSA solution		1% PEG-3350 solution		0.2% Tween-40 solution	
Incubation time	Conc. (µM)	% remaining	Conc. (µM)	% remaining	Conc. (µM)	% remaining	Conc. (µM)	% remaining	Conc. (µM)	% remaining
0 h	0.56 ±0.25	**28**	17.21 ±2.36	**861**	0.55 ±0.21	**27**	0.51 ±0.16	**26**	0.56± 0.35	**28**
2 h	0.13 ±0.07	**7**	2.73 ±1.00	**137**	0.10 ±0.04	**5**	0.09 ±0.02	**4**	0.09 ±0.04	**4**

Conc. means concentration. Note: The percentages indicate the remaining concentration relative to the concentration of the original 2 µM of VCS stock solution.

Table 3- Concentration of TAC and CsA in samples incubated in plastic labware, measured by LC-MS/MS

	TAC		CsA	
Incubation time	Conc. µM	% remaining	Conc. µM	% remaining
0 h	0.85		0.76	
2 h	0.65	**76**	0.47	**62**

Note: The percentages indicate the remaining concentration relative to the concentration of the original compound stock solution (0.8 µM).

DISCLOSURE STATEMENT

The authors of this manuscript have conflicts of interest to disclose. This is investigator-initiated research. J.L.C. is an employee of Aurinia Pharmaceuticals Inc. O.T. received a grant without restrictions from Aurinia Pharmaceuticals Inc. to support part of this project and is an investigator of Aurinia clinical trials. Aurinia Pharmaceuticals Inc. had no role in the decision (what and when) to publish. There are no other conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHORSHIP:

NO Designed experiments, performed experiments, analyzed data and wrote the paper.
EM performed LC/MS-MS measurements and analyzed data.
EJA Performed background/literature research and contributed to writing the paper.
DJM Performed experiments, analyzed data and contributed to writing the paper.
AT Performed experiments.
JLC Provided essential reagents and data and contributed to writing the paper.
EJS Provided essential materials, analyzed the data and wrote the paper.
YKOT Designed the study and wrote the paper.
APJdV Designed the study and wrote the paper.
MJvH Designed and supervised the study, analyzed data and wrote the paper.

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