Characterization of the anti-hepatitis C virus activity of new non-peptidic small-molecule cyclophilin inhibitors with a potential for broad anti-*Flaviviridae* activity

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Running Title: SMCypI, a new family of broad-spectrum antiviral

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SUMMARY

Despite *Flaviviridae* viruses display high incidence, morbidity and mortality rates, the development of specific antiviral drugs for each virus is unlikely. Cyclophilins, a family of host peptidyl-prolyl *cis*/*trans* isomerase (PPIase), play a pivotal role in the lifecycle of many viruses and therefore represent an attractive target for broad-spectrum antiviral development. We report here the pan-genotypic anti-hepatitis C virus (HCV) activity of a small-molecule cyclophilin inhibitor (SMCypI). Mechanistic and modeling studies revealed that SMCypI bound to cyclophilin A in competition with Cyclosporin A (CsA), inhibited its PPIase activity and disrupted the CypA/NS5A interaction. Resistance selection showed that the lead SMCypI hardly selected amino acid substitutions conferring low-level or no resistance *in vitro*. Interestingly, the SMCypI selected substitutions D320E and Y321H substitutions, located in the domain II of NS5A protein. These substitutions have been previously associated with low-level resistance to cyclophilin inhibitors such as Alisporivir. Finally, the SMCypI inhibited the replication of other members of the *Flaviviridae* family with higher EC_{50} values than HCV. Thus, because of its chemical plasticity and simplicity of synthesis, our new family of SMCypIs represents a promising new class of drugs with a potential for broad-spectrum anti-*Flaviviridae* activity, as well as an invaluable tool to explore the role of cyclophilins in viral lifecycles.

INTRODUCTION

Urbanization, human migrations and climate changes facilitate emergence or re-emergence of a large number of viruses causing unexpected illnesses and epidemics, while the capacity to identify and control emerging diseases is limited in poorer regions where many of them have their origin (1). Among emerging and re-emerging viruses, members of the *Flaviviridae* family represent a global public health issue. The *Flaviviridae* family consists of four genera (Flavivirus, Hepacivirus, Pegivirus and Pestivirus) which include viruses responsible for important animal and human diseases, such as yellow fever virus (YFV), zika virus (ZIKV), Japanese encephalitis virus (JEV), West-Nile virus (WNV), hepatitis C virus (HCV) and dengue virus (DENV). YFV, ZIKV, JEV, WNV and DENV are leading causes of arthropod-borne human diseases worldwide. According to the World Health Organization (WHO), they globally infect 400 million individuals each year, causing approximately 80,000 deaths each year. HCV is a blood-borne Hepacivirus responsible for chronic liver diseases causing approximately 700,000 deaths annually (2). Approximately 71 million individuals are infected worldwide, representing 1% of the global population (Global Hepatitis Report from WHO, 2017). The recent approval of a large number of direct-acting antiviral agents (DAAs) active against HCV, including generic compounds, has revolutionized the treatment of this infection with more than 95% rates of infection cure (3). In contrast, no antiviral drugs are available so far to cure infections caused by arthropod-borne members of the *Flaviviridae* family, despite their global public health importance.

During the past 10 years, two different types of antiviral agents, including DAAs and host-targeting antiviral (HTA) agents, have been developed for the treatment of HCV infection. Among the HTAs, non-immunosuppressive derivatives of cyclosporin A (CsA) that target host cyclophilins (Cyps) yielded the most promising results. Alisporivir (ALV) was the first HTA to enter HCV clinical development and reach Phase III clinical trials (4, 5). Its development was halted following the report of seven cases of acute pancreatitis, including a lethal one (6). These events were independent from Cyp inhibition, most likely due to ALV-induced hypertriglyceridemia that potentiated the pancreatic toxicity of interferon alpha that was part of the combination regimens. Although cyclophilin inhibitor (CypI) failed to reach the market for the indication of hepatitis C treatment, they remain attractive to combat other viral infections (7-10). Indeed, CypIs have been reported to be involved in the lifecycle of viruses other than HCV (11), while having a high barrier to resistance, broad antiviral activity and possibly additive or synergistic effects with other antiviral compounds in various models.

Cyps are peptidyl-prolyl *cis*/*trans* isomerases (PPIases) that catalyze the interconversion of the two energetically preferred conformers (*cis* and *trans*) of the planar peptide bond preceding an internal proline residue. Seventeen human Cyps have been identified but more may exist. Cyps have been convincingly shown to play a pivotal role in the lifecycle of a large number of viruses from different families (11). However, there are few data available regarding the anti-*Flaviviridae* activity of CypIs, all of which were obtained with CsA and ALV (8, 12-14). The molecular mechanisms of CypI anti-HCV activity are not yet fully understood. It is believed that they exert their antiviral effect by disrupting the CypA-NS5A interaction that regulates multiple phases of HCV replication (15) (16).

92 We previously reported our rational design of a new family of small-molecule, non-peptidic
93 CypI (SMCypI) unrelated to CsA by means of a complex fragment-based drug discovery
94 approach (17). Our SMCypIs displayed antiviral effectiveness not only against HCV, but also
95 HIV and coronaviruses, suggesting, together with data reported in the literature, that they
96 could act as broad-spectrum antiviral agents, effective against a number of different viruses
97 from different viral families. The present study aims at characterizing the anti-HCV activity
98 of the new family of SMCypIs, unraveling their molecular antiviral mechanism and
99 evaluating their spectrum of anti-*Flaviviridae* activity.

MATERIALS AND METHODS

Drugs

Alisporivir (ALV) and ledipasvir (LDV) were purchased from AGV Discovery (Clapiers, France), while cyclosporine A (CsA) was purchased from Sigma-Aldrich (Saint-Louis, Missouri, USA).

Compound synthesis

SMCypI compound synthesis is described in the Supplementary Materials and Methods. Chemical reagents were obtained from Aldrich Chemical (Saint-Louis, Missouri, USA), Acros Organics (Geel, Belgium), abcr GmBh (Karlsruhe, Germany), acbblocks (Toronto, Canada) and Chembridge (San Diego, California) and used without further purification.

HCV-SGR plasmids

Plasmids *H77/SG-Feo, S52/SG-Feo* and *SA1/SG-Feo*, that contain a Firefly luciferase reporter gene and a genotype (GT) 1a, 3a or 5a HCV subgenome, respectively, were kindly provided by Dr Charles M. Rice (Rockefeller University, New York, New York) (18, 19). Plasmid *p1071-NS5A(Ni)-S2204I* contains a Firefly luciferase reporter gene and a genotype 1b HCV subgenome (Con1 strain) with an NS5A cassette from the genotype 1b HCV-N strain

(20). Plasmids *APP76-Con1-SG-Neo-(I)-hRluc2aUb* and APP*40-J6/JFH1EMCVIRES-aRlucNeo* that contain a Renilla luciferase reporter gene and genotype 1b and 2a HCV subgenomes, respectively, were purchased from Apath LLC (New York, New York, USA) (http://www.apath.com/Hepatitis_C_Virus_Technology.htm#3). Plasmid *I389-Neo/NS3-3'/5.1* that contains the Neomycin resistance gene and an HCV genotype 1b subgenome was used for resistance selection experiments and was kindly provided by Dr Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany) (21). Plasmid *DBN3acc* that contains a full-length HCV genotype 3a genome was kindly provided by Dr Jens Bukh (University of Copenhagen, Copenhagen, Denmark) (22). Finally, chimeric plasmid *fdRocco-chimeric2a/4aNS5A* consisting of a genotype 4a NS5A sequence inserted into a genotype 2a HCV subgenome from *APP40-J6/JFH1EMCVIRES-aRlucNeo* has been developed in our laboratory.

Cell cultures

Human hepatoma Huh7 cells (kindly provided by Dr Eliane Meurs) and Huh7.5 cells (Apath LLC) were cultured in complete Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, 100 µg/ml streptomycin and 0.1 µg/ml amphotericine-β (ThermoFisher Scientific).

Assessment of antiviral activity in HCV-SGR models

For transient HCV-SGR models, plasmids *p1071-NS5A(Ni)-S2204I, APP40-J6/JFH1EMCVIRES-aRlucNeo* and *fdRocco-chimeric2a/4aNS5A* were linearized with XhoI, XbaI and XbaI, respectively (FastDigest, ThermoFisher Scientific) and *in vitro* transcribed using MEGAscript T7 Transcription Kit (ThermoFisher Scientific). Then, approximately 1.5 x 10^4 Huh7.5 cells were transfected with 250 ng of HCV-SGR RNA using trans-IT mRNA transfection kit (Mirus Bio LLC, Madison, Wisconsin, USA). Four hours after transfection, compounds were added to the culture medium. Luciferase activity was monitored 96, 48 and 72 hours post-transfection for genotype 1b, 2a and chimeric 2a/4a HCV-SGR, respectively.

Huh7.5 cells stably harboring genotype 1a, 3a and 5a HCV-SGR were cultured in the presence of the compounds for 48 hours before luciferase activity measurement. Plots were fitted with a four-parameter logistic curve with SigmaPlot® v11 software (Systat Software, Inc) and the EC_{50s} were determined from the curves.

Assessment of antiviral activity in the infectious HCV model

Huh7.5 cells were seeded at the density of 1.5 x 10^4 cells and incubated 24 hours before infection with 250 µl of HCVcc (J6/JFH1 strain, GT2a/2a) in the presence of increasing concentrations of the compounds. Eight hours after infection, the cells were washed with PBS and incubated with fresh medium containing the inhibitors for 72 hours. Then, luciferase activity was measured and plotted against compound concentrations. The EC_{50s} were determined from curves fitted with a four-parameter logistic equation.

Antimicrobial Agents and Chemotherapy

Assessment of the combination of compound 31 and ledipasvir

Huh7.5 cells stably harboring a genotype 1a HCV-SGR were cultured in the presence of 10 pM LDV, 2.5 µM C31 or both drugs at the same concentrations, in the absence or presence of 500 µg/ml of G418. After 5 passages, the remaining living cells were stained with crystal violet.

Selection of clones resistant to compound 31

Huh7.5 cells stably harboring a genotype 1b HCV-SGR replicon that confers cell resistance to G418 were used for selection experiments. The cells were cultured in the presence of escalating doses of C31 (1 to 50 µM) and of 1.5 mg/ml of G418 until colonies growing in the presence of C31 appeared. Two resistant colonies were isolated after several passages and total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed with the High Capacity cDNA reverse transcription kit (ThermoFisher Scientific). The NS5A-coding region was amplified at baseline and in the resistant colonies by PCR using forward oligonucleotides 5'-GTG CAG TGG ATG AAY CGG CTG ATA GC-3', 5'-TTC CAR GAC TCT ARC ART G-3', 5'-ACT ATG TGC CTG AGA GCG ACG-3', 5'-GGR TTG TAR TCC GGS CGY GCC CAT A-3' and reverse oligonucleotides 5'-TCC CRT GYG AGC CYG AAC CG-3', 5'-GTG GTG ACG CAG CAA AGA GT-3', 5'-CCC ACA TTA CAG CAG AGA CGG C-3', and 5'-TTG ATG GGC AGC TTG GT-3'.

Phenotypic characterization of amino acid substitutions selected by compound 31

Candidate resistance-associated substitutions (RASs) were introduced into a wild-type genotype 1b HCV-SGR containing the luciferase reporter gene by means of site-directed mutagenesis (Quick Change II XL Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, California, USA). Ninety-six hours post-RNA transfection, the susceptibility to compound 31 of RAS-containing HCV-SGR was determined by measuring luciferase activity and compare to wild-type HCV-SGR susceptibility. The replication capacity of RAS-containing HCV-SGR was assessed by comparing luciferase activity 4 hours and 96 hours post-transfection and expressed as a percentage of wild-type replication capacity.

Phenotypic characterization of amino acid substitutions selected by ledipasvir

The L31V and Y93H RASs, which confer high-level resistance to LDV (23), were introduced in the NS5A-coding region of the *APP76-Con1-SG-Neo-(I)-hRluc2aUb* plasmid by means of site-directed mutagenesis. After linearization with ScaI and *in vitro* transcription, the genotype 1b HCV-SGR was transfected into Huh7.5 cells. The cells were cultured in the presence of the compounds for 84 hours and luciferase activity was measured. Then, susceptibility of HCV-SGR(L31V/Y93H) to C31, ALV and LDV was compared to that of wild-type HCV-SGR.

Competitive binding assay

215 A 96-well plate-based TR-FRET (time-resolved fluorescence energy transfer) assay
216 (Selcia, Ongar, UK) was used to determine competitive CypA binding of C31 and CsA.
217 Briefly, CypA was tagged with a polyhistidine sequence (6His) and formed a complex with
218 an anti-6His antibody labelled with a fluorescent donor F(d), while CsA was tagged with a
219 fluorescent acceptor F(a). C31 was added to the master mix containing the
220 CypA/antibody/CsA complex, with a final concentration of detergent of 0.01%. After 30 min
221 of incubation at room temperature, the plate was read on a SpectraMax M5 (Molecular
222 Devices, Sunnyvale, California, USA) at 2 wavelengths to detect F(d) and F(a) emissions. The
223 F(a)/F(d) ratio was calculated and the values were plotted against the inhibitor
224 concentration in Log_{10} molar and fitted using one-site Ki nonlinear regression to determine
225 the Kd. Non-labelled CsA and ALV were used as controls.
226
227 **Protein-protein interaction assay**
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229 An NS5A-Rluc/CypA-6his *in vitro* interaction assay was developed to measure the
230 efficiency of CypI disruption of the NS5A/CypA interaction by luminescence measurement.
231 After PCR amplification with primers 5'-AAA AAC TGC AGA TGT CCG GCT CGT GGC
232 T-3' and 5'-AAA AAC CGC GGG CAG CAG ACG ACG TCC-3' and digestion with PstI and SacII
233 (FastDigest, ThermoFischer Scientific), the genotype 1b NS5A-coding sequence was cloned
234 in fusion with the Renilla luciferase into a pRluc-N3(h) plasmid (6310009, Perkin Elmer,
235 Waltham, Massachussets, USA). NS5A-D320E was generated by site-directed mutagenesis
236 with oligonucleotides 5'-ATG GGC ACG CCC GGA ATA CAA CCC TCC ACT G-3' and 5'-CAG
237 TGG AGG GTT GTA TTC CGG GCG TGC CCA T-3' and cloned in a similar way to WT-NS5A.

Huh7.5 cells were then transfected with the pRluc-N-NS5A plasmid. Forty-eight hours later, cells were lysed with 4 thaw-freeze cycles and incubated 30 min at room temperature with 500 µg of Ni-NTA magnetic beads preloaded with 30 µg of purified CypA in an equilibration buffer (imidazole 10 mM, Tween-20 0.05% in PBS (-/-)). Increasing concentrations of the CypI were added during the incubation. The beads were then washed 3 times with a washing buffer (imidazole 20 mM, Tween-20 0.05% in PBS (-/-)) and elution was performed with imidazole 400 mM for 15 min at room temperature. Renilla luciferase activity in eluates was measured with Renilla Luciferase Assay System (Promega). Results are presented as mean ± standard deviation (SD) of at least three independent experiments. All analyses were two-sided and considered significant when $p < 0.05$.

Molecular modelling and docking of C31 into CypA

Molecular modelling and docking experiment were performed using the @TOME-2 server (24), which integrates an original interface for comparative docking of small molecules detected in the Protein Data Bank file of each template. The search for homologous sequences and alignments was performed using the @TOME-2 server and the CypD sequence (P30405), with a 75% identity. In each structural model, the active site boundaries were deduced from the vicinity of the co-crystallized ligands (compounds C32, C34, C35 and C36 were selected as templates with the corresponding Protein Data Bank (PDB) accession numbers 4J59, 4J5C, 4J5B and 4J5E, respectively) using the @TOME-2 comparative option. In addition, the same chemical entities were used to define a shape restraint to guide docking in the automatically computed models. The files for the ligands

were generated with MarvinSketch 6.2.2 for the SMILES and with Frog2 server for the mol2 (25). Figure 1A and 1B were generated using Pymol.

Assessment of compound 31 anti-*Flaviviridae* activity

Huh7 cells were infected for 48 h with the YFV strain Dakar HD 1279 (obtained from the World Reference Center for Emerging Viruses and Arborviruses – WRCEVA, Texas, USA), at an MOI of 7; or for 48 hours with DENV-4 strain Dominica 814669 (obtained from the Centro de Ingeniería Genética y Biotecnologíain, Cuba) at an MOI of 10; or for 1 h with ZIKV strain MR766 (obtained from ATCC) at an MOI of 1. Infections were performed in the presence of different concentration of C31.

For DENV and YFV experiments, total RNA was extracted from cell cultures with the NucleoSpin RNA II kit (Macherey-Nagel). First-strand complementary DNA (cDNA) synthesis was performed with the RevertAid H Minus M-MuLV Reverse Transcriptase. Quantitative real-time PCR was performed on a real-time PCR system (QuantStudio 6 Flex, Applied Biosystems) with SYBR Green PCR Master Mix (Life Technologies). The data were analyzed with the 2-ΔΔCT method, with all samples normalized to GAPDH. All experiments were performed in triplicate. Genome equivalent concentrations were determined by extrapolation from a standard curve generated from serial dilutions of the plasmid encoding a subgenomic YFV replicon. The sequences of the 17D-NS3 primers used for the RT-qPCR were the following: sense 5'-AGGTCCAGTTGATCGCGGC and antisense 5'-GAGCGACAGCCCCGATTTCT. The sequences of the pan-DENV primers were the following:

sense 5′-TTGAGTAAACYRTGCTGCCTGTAGCTC and antisense 5′-GAGACAGCAGGATCTCTGGTCTYTC.

For ZIKV experiment, total RNA was extracted from cell culture with the SV96 Total RNA isolation system (Promega). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR was performed on a real-time PCR system (Applied Biosystems 7300) with TAQMAN gene expression master mix (Applied Biosystems) and the data were normalized to GAPDH. The sequences of the primers used were the following: sense 5'ATATCGGACATGGCTTCGGA and antisense 5'GTTCTTTTGCAGACATATTGAGTG.

Statistical analysis

Statistical analyses were performed using SigmaPlot software. Statistics were calculated using t-test analysis of variance. P values below 0.05 were considered statistically significant.

RESULTS

C31 has pan-genotype anti-HCV activity

The anti-HCV activity of the new SMCypI compound C31, our most potent inhibitor of Cyp PPIase activity, was tested different HCV genotype models containing luciferase reporter genes, including an infectious chimeric J6/JFH1 (2a/2a) virus, genotype 1a, 1b, 2a, 3a, and 5a HCV-SGRs, and the chimeric 2a/4a HCV-SGR containing a genotype 4a NS5A sequence (**Supplementary Figure 1**). In addition, the anti-HCV activity of C31 was evaluated in the recently developed full-length infectious HCV genotype 3a model (DBN-3acc) (22). ALV and CsA were used as controls in all experiments.

C31 equally inhibited the replication of genotype 1a, 1b, 2a, 3a, 5a and chimeric 2a/4a HCV-SGRs, with EC_{50s} ranging from 1.20±0.83 to 7.76±1.57 μM (**Table 1**). C31 also inhibited the replication of the infectious J6/JFH1 virus, with a comparable EC_{50} of 2.80±0.40 μM. Finally, C31 inhibited DBN-3acc RNA replication in a dose-dependent manner, with a maximal 244-fold HCV RNA reduction at 10 μM (**Supplementary Figure 3**). C31 did not affect cell viability at its effective concentration (**Supplementary Figure 2**). Altogether, these results demonstrate the pan-genotype activity of the new SMCypI.

C31 anti-HCV activity is related to its binding to CypA and inhibition of CypA PPIase activity

To understand the molecular mechanism of anti-HCV action of our new SMCypI, the CypA binding modes of C31 and CsA were modelled (**Figure 1A** and **1B**, respectively). As shown in **Figure 1B,** the structure of CypA in complex with C31 revealed its expected dual binding into the "hydrophobic pocket", the CypA PPIase active site, on the one hand, and the "gatekeeper pocket" on the other hand (17, 26). Interestingly, the methoxy group of compound 31 pushes Arg55 to create a hydrogen bond with the urea moiety of the compound.

The CypA binding sites of C31 and CsA were partially overlapping, suggesting competitive binding to CypA. Thus, time-resolved fluorescence resonance energy transfer (TR-FRET) was used to assess whether C31 competes with CsA for binding to the purified CypA. Both non-labelled CsA and ALV displaced labelled CsA from its CypA binding site, with Kd values of 8.4 and <5 nM, respectively (data not shown). As shown in **Figure 1C**, C31 also displaced labelled CsA with a Kd value of 105 nM, confirming competition with CsA binding to CypA.

Finally, we assessed whether the anti-HCV effect of the SMCypIs was related to their ability to inhibit CypA PPIase enzyme activity. For this, the anti-HCV activity of 6 different chemically-related SMCypIs listed in **Supplementary Table 1**, including C31, was determined in a genotype 1b HCV-SGR and plotted against their respective inhibitory activities in a PPIase enzyme assay. As shown in **Figure 1D**, the anti-HCV activity of the SMCypIs was strongly correlated to their ability to inhibit PPIase activity, with a Pearson's correlation coefficient of 0.96 and a p value <0.0001

342 Altogether, these results demonstrate that our new family of SMCypIs inhibits HCV
343 replication by binding to both the PPIase catalytic pocket and the gatekeeper pocket of
344 CypA, thus blocking its PPIase catalytic activity.
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346 **C31 disrupts the CypA-NS5A interaction**
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348 Because CypA has been shown to play an important role in the HCV lifecycle through
349 its interaction with the non-structural NS5A protein, we measured the effect of C31 on the
350 NS5A/CypA interaction. Purified CypA tagged with a polyhistidine sequence (CypA-6his)
351 was bound to Ni-NTA (nitrilotriacetic acid) magnetic beads. A cell lysate containing WT-
352 NS5A or D320E-NS5A fused with the Renillia luciferase (NS5A-Rluc) was incubated with the
353 CypA-loaded magnetic beads in the presence or absence of C31 and of ALV, used as a
354 control. After washing and elution with imidazole, luciferase activity corresponding to
355 NS5A-Rluc/CypA-6his interacting complexes was measured. As shown in **Figure 2,** the
356 CypA/NS5A interaction was disrupted by both ALV and C31 in a dose-dependent manner.
357 The same result was observed with D320E-NS5A. Together these results indicate that
358 SMCypIs disrupt the interaction between CypA and the HCV protein NS5A through their
359 binding to CypA.
360
361 **C31 exerts at least additive antiviral effects when combined with the NS5A inhibitor**
362 **ledipasvir and is fully active against ledipasvir-resistant viruses**
363

Antimicrobial Agents and Chemotherapy

364 A combination study of C31 with the NS5A inhibitor LDV was performed in Huh7.5
365 cells stably harboring a genotype 1a HCV-SGR. As shown in **Figure 3**, under G418 selective
366 pressure, the combination of C31 and LDV at their respective EC_{50s} of 2.5 μM and 10 pM,
367 was more effective in curing cells from the replicons than each compound alone at the same
368 concentration, suggesting at least an additive effect of C31 and LDV in combination. In
369 addition, a genotype 1b HCV-SGR, containing the L31V and Y93H substitutions, which
370 confer high-level resistance to ledipasvir (714-fold increase in LDV EC_{50}, data not shown),
371 remained fully sensitive to C31 and ALV **(Table 2)**.
372 Together, these results suggest that our new SMCypI family has at least additive
373 effects with HCV DAAs targeting domain I of the NS5A protein, without cross-resistance.
374
375 **C31 hardly selects amino acid substitutions conferring low-level or no resistance *in***
376 ***vitro***
377
378 Huh7.5 cells stably harboring a genotype 1b HCV-SGR containing the Neomycin
379 resistance gene were grown under G418 selective pressure in the absence or presence of
380 increasing doses (1 μM to 50 μM) of C31. Two cellular clones growing in the presence of
381 C31 were selected after 100 days. Because CsA and its non-immunosuppressive derivatives
382 were shown to select amino acid substitutions in the NS5A region of the HCV genome (27-
383 29), the NS5A-coding region of the two C31-resistant clones was sequenced. As compared
384 to baseline, 8 amino acid changes were identified, including I133V and L183P in domain I,
385 L303P, R304W, K308I, D320E and Y321H in domain II, and E442G in domain III of the NS5A

protein (**Figure 4**). The NS5B-coding region was also sequenced and only the I585L change was observed (data not shown).

Each of the 8 amino acid substitutions selected was introduced in a WT transient genotype 1b HCV-SGR for phenotypic analysis (compound susceptibility and replication capacity) in the presence of C31, ALV and CsA **(Table 2).** Among them, only D320E and Y321H were associated with a mild increase in C31 EC_{50} (approximately 2 to 5-fold), without impairment of the replicon replication capacity. L183P, located in NS5A domain I, drastically reduced the corresponding HCV-SGR replication capacity, so its impact on compound susceptibility could not be evaluated. Thus, only D320E and Y321H in domain II of the NS5A protein, that were selected by serial passages at increasing concentrations of C31, conferred low-level resistance to the SMCypI, as already reported with CsA and ALV.

C31 inhibits the replication of other members of the *Flaviviridae* family

We assessed whether C31 could exert antiviral activities against other members of the *Flaviviridae* family. Viral replication was assessed by RT-qPCR 48 hours post-infection. A dose-dependent decrease of viral replication was observed in the presence of C31 for DENV (EC_{50}: 7.3±3.5 µM), YFV (EC_{50}: 27.2±4.7 µM) and ZIKV (EC_{50}: 48.0±5.6 µM) with a CC_{50} ≥100µM **(Figure 5)**. C31 was more potent against HCV and DENV than against YFV and ZIKV.

Although C31 is not as potent on all *Flaviviridae* than on HCV, this compound represents a good candidate for further chemical optimizations.

DISCUSSION

Anti-HCV drug research highlighted the antiviral potential of Cyp inhibition through the clinical development of non-immunosuppressive derivatives of CsA. Unfortunately, all CypIs in development for HCV were structurally-related, and the only compound that reached late-phase clinical development, ALV, was halted due to severe adverse effects unrelated to Cyp inhibition. We recently reported our use of a complex fragment-based drug discovery approach using nucleic magnetic resonance, X-ray crystallography and structure-based compound optimization to generate a new family of non-peptidic, small-molecule cyclophilin inhibitors (SMCypI) unrelated to CsA, with broad cyclophilins *in vitro* PPIase inhibitory activity and antiviral activity against HCV, HIV and coronaviruses (17).

Although ALV has been shown to potently inhibit HCV replication, its ability to block the replication of other members of the *Flaviviridae* family is unknown. The immunosuppressive CsA has been reported to bear antiviral activity against DENV-2, WNV and YFV (14), with less potency against WNV. Results regarding the susceptibility of ZIKV to CsA are limited and contradictory (30). This sparseness of data prompted us to assess the anti-*Flaviviridae* activity of our new SMCypI. Here, we showed that C31, a SMCypI inhibitor of PPIase activity with anti-HCV activity *in vitro*, also inhibits the replication of several members of the *Flaviviridae* family in cell culture models. C31 was most effective against HCV and DENV replication, with EC_{50s} in the low micromolar range. C31 inhibited YFV and ZIKV replication to a lesser extent, with EC_{50s} in the micromolar range. Although the EC_{50} was above 50 µM, the highest concentration tested, a trend towards a decrease of viral replication was also observed with WNV (data not shown). In contrast, no effect of C31

against JEV replication has been observed in our experiments (data not shown). To our knowledge, C31 is the first SMCypI with a potential for broad anti-*Flaviviridae* activity. Given the chemical plasticity and simplicity of synthesis of this new family of SMCypIs, a large number of new compounds will be synthesized in the future. It is thus likely that C31 derivatives with greater effectiveness against the different members of the *Flaviviridae* family will be generated.

The susceptibility of *Flaviviridae* family members to SMCypIs suggests that cyclophilins play a pivotal role in their lifecycles. However, the different antiviral potency of C31 across different viruses from the *Flaviviridae* family raises the question as to a similar or different mechanism of inhibition. Our SMCypIs provide a unique, easy-to-use tool to explore this role. In the present study, we used C31 to decipher the role of cyclophilins in the HCV lifecycle and understand the mechanisms of its inhibition by the inhibitors. Our results complement previous results generated with CsA or ALV in various models (27-29, 31-33).

We showed here that, like ALV (29, 32, 33), our family of SMCypIs has pan-genotypic anti-HCV activity (EC_{50s} in the low micromolar range for genotypes 1a, 1b, 2a, 3a, 4a and 5a), a high barrier to the selection of resistant viruses, and at least additive effects in combination with HCV DAAs. It has been previously demonstrated that CypA PPIase activity is required for HCV replication (34, 35). Our library of SMCypI (supplementary table 1), provides us with a unique, thus far missing pharmacological tool to dissect the molecular mechanisms of Cyp/ virus interactions and of the antiviral effects of Cyp inhibition. The library of SMCypI proved to be particularly useful to discriminate PPIase-dependent antiviral activity from effects related to other functional roles of the Cyps. Using several

SMCypI derivatives with different inhibitory potency of PPIase catalytic activity, we showed that the antiviral activity of the SMCypIs strongly correlates with their PPIase inhibitory potency, confirming that Cyp PPIase activity is required for HCV replication.

It has been shown that CypA directly interacts with the HCV NS5A protein to regulate key processes of the HCV lifecycle (16, 34, 36). Disruption of this key interaction by CsA or ALV impairs HCV replication, possibly explaining the anti-HCV effect of these compounds (33, 37). Whether similar mechanisms could be involved with other families of Cyp inhibitors remained unknown. We showed that the NS5A/CypA interaction was disrupted by C31. We also modeled the CypA binding mode of C31 and CsA. As expected, their superimposition suggested that both compounds shared a partially overlapping binding site. This result was confirmed by means of a TR-FRET assay, showing that C31 displaces CsA from the CypA catalytic site. Interestingly, we also observed that the aniline moiety of C31 was deeply buried into a pocket contiguous to the canonical catalytic site, the gatekeeper pocket, which is out of reach for CsA, suggesting that the inhibition modes of CsA and SMCypIs may partially differ. This could explain that CsA and ALV did not inhibit ZIKV replication in this study, whereas C31 did, possibly through a PPIase-independent mechanism different from that involved in HCV inhibition (data not shown).

Resistance experiments performed with ALV and CsA selected amino acid substitutions essentially clustering in domain II of the HCV NS5A protein, also suggesting that NS5A is the main viral partner of CypA (33). D320E and Y321H, both located in NS5A domain II, have been reported to confer low-level resistance to CsA and its non-immunosuppressive derivatives (28, 33, 38).

476 However, NS5A mutant proteins harboring the D320E substitution keep their
477 capacity to interact with CypA *in vitro* and this interaction remains fully sensitive to ALV
478 disruption (33). In our experiments, 100 days have been necessary to select two cellular
479 clones growing in the presence of 50 µM of C31 under selective pressure of G418. The
480 maximum selective pressure obtained with C31 (17-fold) was in keeping with data
481 previously reported with ALV and CsA (65-fold and 10-fold, respectively) (33). These
482 results confirmed the high barrier of resistance of SMCypIs.

483 Among the 8 amino acid changes identified in these clones, only D320E and Y321H
484 in domain II of the NS5A protein were associated with a modest increase in C31 EC_{50}
485 (approximately 2 to 5-fold), without impairment of the replicon replication capacity. These
486 properties were similar to those reported with CsA and ALV. The interaction of NS5A-
487 D320E with CypA remained fully sensitive to C31 disruption. Altogether, these findings
488 confirm the that the mode of antiviral action of our SMCypIs is identical to that of CsA and
489 its derivatives.

490 The fact that SMCypI do not target a viral function, but instead a host protein
491 involved in a key step of the viral lifecycle suggested that they could bear additive or
492 synergistic properties in combination with DAAs. We confirmed this hypothesis by
493 combining C31 with the potent HCV NS5A inhibitor ledipasvir. Both drugs together were
494 more efficient in curing cells from HCV-SGR than each drug alone, suggesting at least an
495 additive effect. In addition, C31 remained efficient against HCV-SGR harboring amino acid
496 substitutions known to confer high-level resistance to LDV.

In conclusion, our new family of SMCypIs presumably exhibit broad-spectrum antiviral properties against several members of the *Flaviviridae* family that represent important public health problems worldwide and remain without any therapeutic option. Their mechanism of antiviral action against one of these viruses, HCV, is related to Cyp binding, inhibition of the PPIase catalytic activity and disruption of the CypA/NS5A interaction, a mechanism common to other cyclophilin inhibitors derived from CsA. Non-immunosuppressive analogues of CsA suffer from serious caveats, including their large size resulting in poor cell permeability, their side effects unrelated to cyclophilin inhibition, their drug-drug interactions, and manufacturing issues. Thus, because of its chemical plasticity, low cellular toxicity and simplicity of synthesis, our new family of SMCypIs represents a promising new class of drugs with broad-spectrum anti-*Flaviviridae* properties, as well as an invaluable tool to explore the role of cyclophilins in viral lifecycles and the mechanisms to block them.

FIGURES

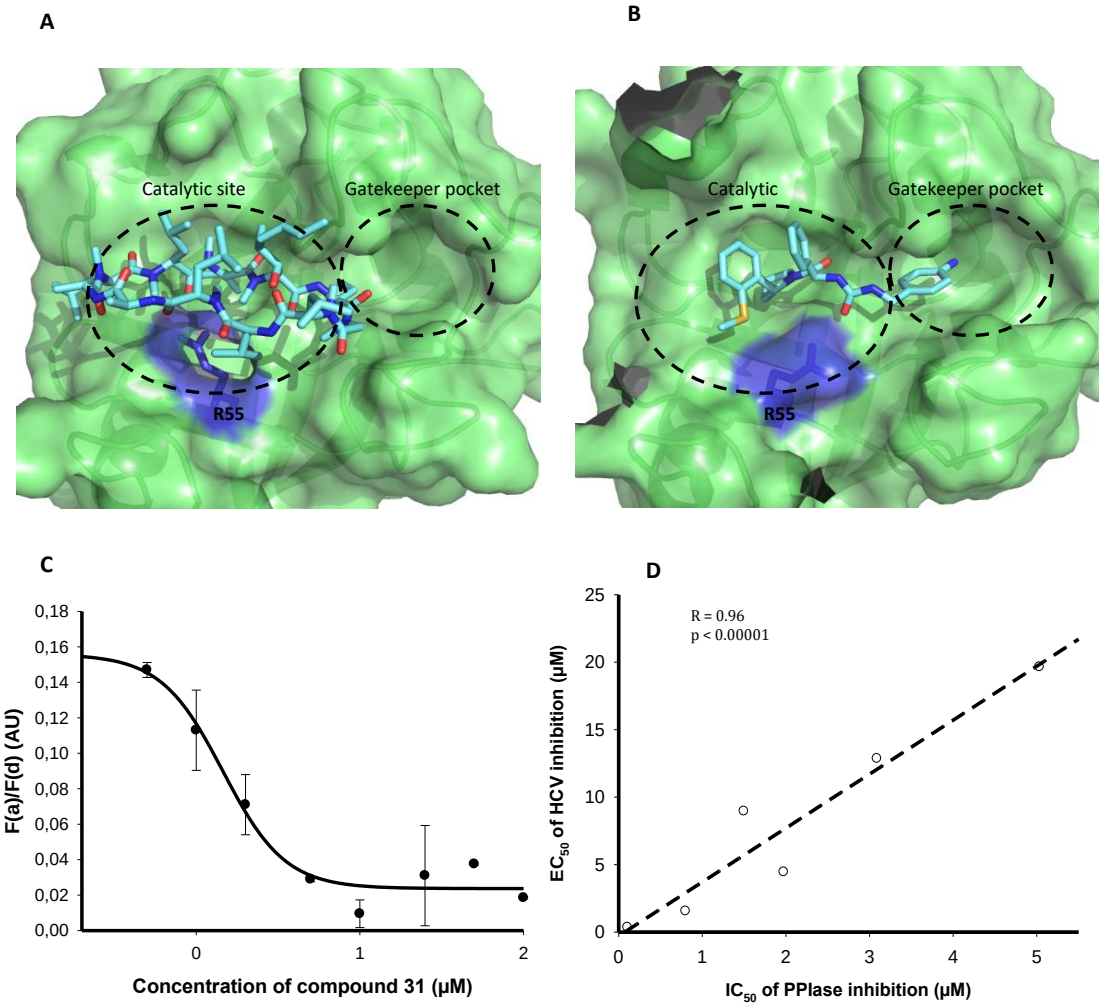

Figure 1

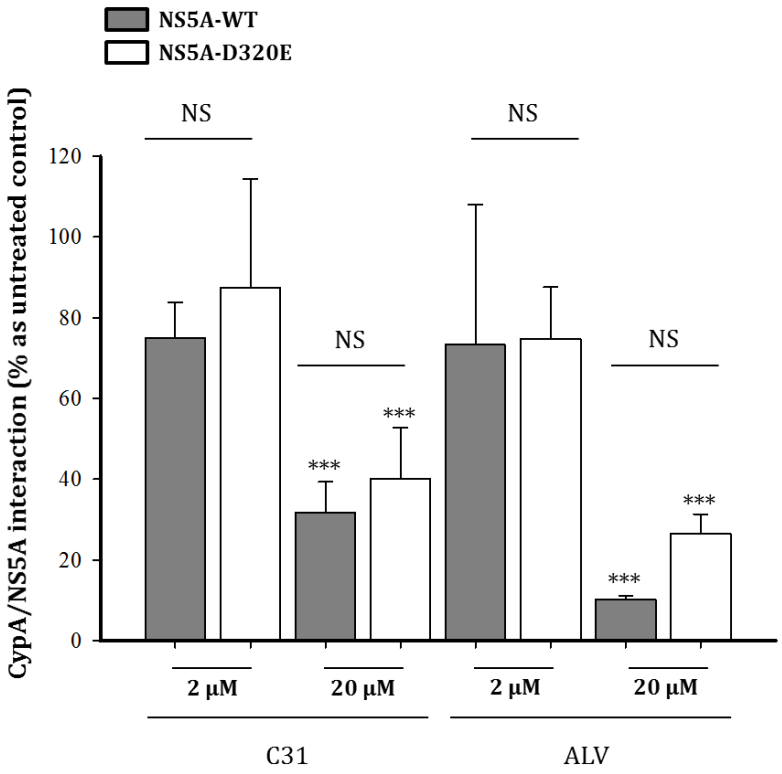

Figure 2

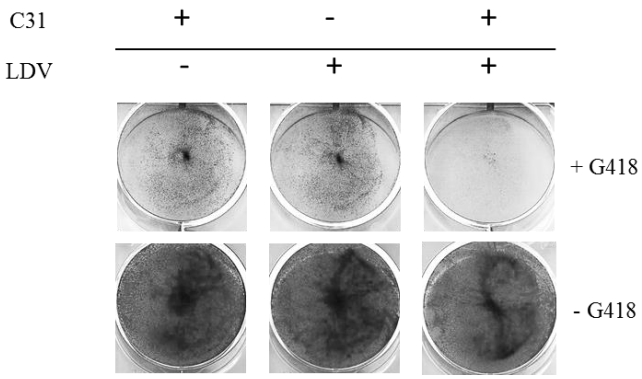

Figure 3

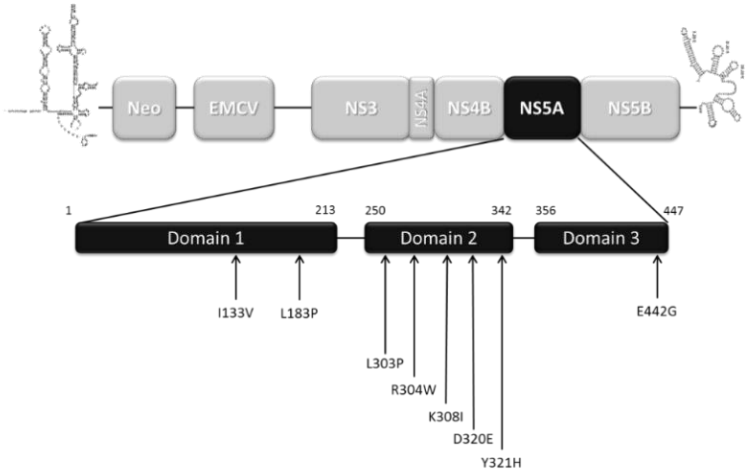

Figure 4

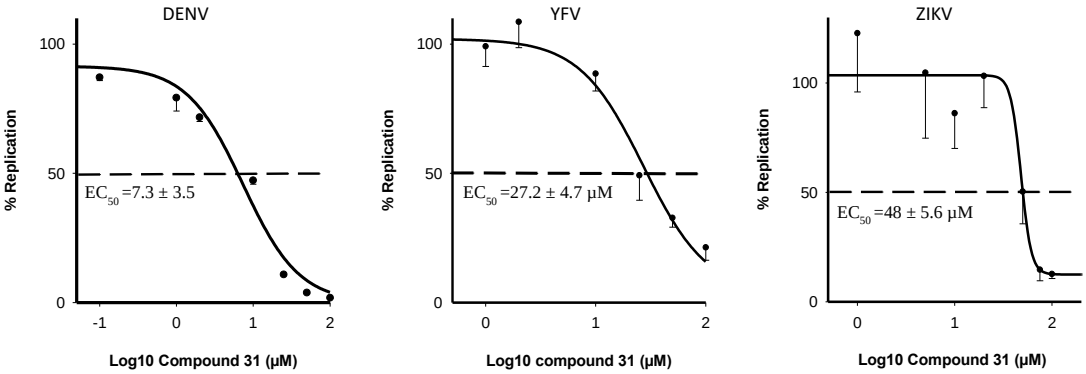

Figure 5

Keywords

Cyclophilin inhibitors, small-molecule, hepatitis C virus, *Flaviviridae*, resistance, broad-spectrum antiviral activity

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642

643 **Author contributions**

644

645 Q.N., I.R.: participated in the design of the study, performed part of the experiments, wrote
646 the article; N.A., F.D., R.B., L.S., M.C., N.J., M.G., C.B., P.B., J.-F.G., S.F.: performed part of the
647 experiments; A.A.-B., J.-M.P.: designed the study, wrote the article, led the project. All
648 authors approved the manuscript.

649

650 **Competing financial interest statements**

651

652 INSERM Transfert is the owner of patent EP 09306294.1 covering the family of cyclophilin
653 inhibitors described, for which J.-F.G., A.A.-B., and J.-M.P. are inventors. All other authors
654 declare no competing financial interests.

655

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657

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669

670 **Abbreviations**

671

672 HCV: Hepatitis C virus

673 ZIKV: Zika virus

674 YFV: Yellow fever virus

675 JEV: Japanese encephalitis virus

676 WNV: West-Nile virus

677 WHO: World Health Organization

678 SGR: Subgenomic replicon

679 GT: Genotype

680 EC_{50}: Half maximal effective concentration (i.e.: concentration which produces 50 % of the
681 maximal effect)

682 C31: Compound 31

683 ALV: Alisporivir

684 CsA: Cyclosporine A

685 CypA: Cyclophilin A

686 DAA: Direct-acting antiviral

687 RAS: Resistance-associated substitution

688 SMCypI: Small-molecule cyclophilin inhibitor

689 NS5A: Nonstructural protein 5A

690 TR-FRET: Time-resolved fluorescence resonance energy transfer

691 PPIase: Peptidyl prolyl *cis-trans* isomerase

692

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810 **NS5A and NS5B into hepatitis C virus replication complexes. J Gen Virol 91:1189-93.**

811

812 **FIGURE LEGENDS**

813

814 **Figure 1: Molecular modelling of the interaction of C31 and CsA with CypA,**
815 **competition between C31 and CsA for CypA binding and relationship between the**
816 **anti-PPIase activity of the SMCypI in an enzyme assay and their anti-HCV activity in a**
817 **genotype 1b HCV-SGR.** Surface representation of CypA in complex with CsA (**A**) and C31
818 (**B**), showing occupation of the catalytic site and the gatekeeper pocket. The side chain of
819 Arg55 is represented in stick format and highlighted in purple. (**C**) Competition between
820 C31 and CsA for CypA binding was assessed by a TR-FRET assay. The graphs represent the
821 FRET-emission ratios measured in the presence of increasing concentrations of C31.
822 Unlabeled CsA and ALV were used as internal controls. The data are shown as mean ± SD of
823 three independent experiments. (**D**) The graph represents the relationship between the IC_{50}
824 in a CypA PPIase enzyme assay and the EC_{50} in a genotype 1b HCV-SGR assay of 6 SMCypIs
825 related to C31 listed in Supplementary Table 1. The Pearson's correlation coefficient (R)
826 and p value are shown on the graph.

827

828 **Figure 2: Disruption of the CypA-NS5A interaction by the cyclophilin inhibitors.** The
829 interaction between NS5A-Rluc and CypA-6His was assessed by means of a Ni-NTA-
830 magnetic beads protein-protein interaction assay. After loading of CypA-6His, the beads
831 were incubated with a lysate of Huh7.5 cells expressing WT-NS5A-Rluc (**A**) or D320E-NS5A-
832 Rluc (**B**) and two concentrations of C31 and ALV. After washing, CypA-6His interacting
833 proteins were eluted with imidazole. The NS5A-Rluc activity was measured in the eluate.
834 Huh7.5 cells expressing Rluc were used as a negative interaction control. The data are

shown as mean ± SD of at least three independent experiments. NS: not significant; *** $p < 0.001$

Figure 3: Combination studies of C31 (2.5 μM) and LDV (10 pM) in a genotype 1a HCV-SGR in Huh7.5 cells. Huh7.5 cells stably expressing a genotype 1a HCV-SGR were cultured through 5 passages in the presence of C31 (2.5 μM), LDV (10 pM) or both drugs, in the presence (upper panel) or in the absence (lower panel) of G418. The cells were stained with crystal violet.

Figure 4: Amino acid changes selected by serial C31 passages at increasing concentrations in a genotype 1b HCV-SGR. Huh7.5 cells stably harboring a genotype 1b HCV-SGR were cultured in a medium containing 1.5mg/mL of G418 in the presence of increasing doses of C31 until resistant clones were selected. Total RNA of two resistant clones was extracted and the NS5A-coding region was sequenced. The figure shows the amino acid changes observed in the sequence of the NS5A region, domains I to III.

Figure 5: *In vitro* activity of C31 against three *flaviviruses*. Dose-dependent curves of antiviral activity against DENV, ZIKV and YFV. Viral replication was assessed by RT-qPCR 48 h post infection. The relative replication was expressed as percent of untreated control and plotted against the Log_{10} concentration of C31. The EC_{50} values are shown on the graph and represented by a dashed line. Data shown are mean ± SD of at least two independent experiments performed in triplicate.

858 **TABLES**

859

860 **Table1: Activity of C31, ALV and CsA on HCV replication**

	HCV-SGR EC_{50} (µM)						Infectious J6/JFH1 HCV EC_{50} (µM)
Compound	**Genotype 1a**	**Genotype 1b**	**Genotype 2a**	**Genotype 3a**	**Genotype 2a/4a**	**Genotype 5a**	**Genotype 2a/2a**
C31	3.80±1.90	2.95±0.60	2.30±1.20	7.76±1.57	1.40±1.10	1.20±0.83	2.80±0.40
ALV	0.04±0.03	0.03±0.01	0.02±0.01	0.02±0.01	0.01±0.002	0.01±0.01	0.03±0.002
CsA	0.60±0.20	0.17±0.02	0.20±0.04	0.19±0.12	0.04±0.03	0.13±0.04	0.06±0.01

861 EC_{50}: effective concentration 50%; C31: compound 31; ALV: alisporivir; CsA: cyclosporine A. The data are shown as mean ± SD

862 of three independent experiments.

863

864

865 **Table 2: C31, ALV and CsA susceptibility and replication capacity of a wild-type genotype 1b HCV-SGR and effect of the**

866 **introduction of amino acid substitutions selected by serial C31 passages at increasing concentrations**

	C31		ALV		CsA		Replication capacity (%)
	EC_{50} (µM)	Fold-change	EC_{50} (µM)	Fold-change	EC_{50} (µM)	Fold-change	
WT	2.95±0.60	1.0	0.03±0.01	1.0	0.17±0.02	1.0	100
I133V	2.75±0.20	0.9	0.02±0.001	0.9	0.21±0.04	1.2	110±23
L183P	NA	NA	NA	NA	NA	NA	2±1
L303P	3.05±0.40	1.0	0.04±0.01	1.3	0.23±0.04	1.4	57±7
R304W	2.47±0.20	0.8	0.03±0.004	1.1	0.21±0.004	1.2	91±4
K308I	2.59±0.20	0.9	0.04±0.004	1.3	0.17±0.03	1.0	103±3
D320E	8.73±1.10	3.0	0.12±0.05	4.4	0.51±0.04	3.0	82±16
Y321H	5.41±0.40	1.8	0.06±0.02	2.2	0.41±0.02	2.4	79±22
E442G	2.99±0.70	1.0	0.03±0.004	1.1	0.24±0.07	1.4	74±5
L31V/Y93H	1.20±0.10	0.8	0.01±0.001	0.5	ND	ND	92±4

867 EC_{50}: effective concentration 50%; C31: compound 31; ALV: alisporivir; CsA: cyclosporine A; WT: wild-type.

NA: not available, due to the lack of replication of the mutated HCV-SGR; ND: not done. The data are shown as mean ± SD of three independent experiments.

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