Fragment-based discovery of a new family of non-peptidic small-molecule cyclophilin inhibitors with potent antiviral activities

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Cyclophilins are peptidyl-prolyl cis/trans isomerases (PPIase) that catalyse the interconversion of the peptide bond at proline residues. Several cyclophilins play a pivotal role in the life cycle of a number of viruses. The existing cyclophilin inhibitors, all derived from cyclosporine A or sanglifehrin A, have disadvantages, including their size, potential for side effects unrelated to cyclophilin inhibition and drug–drug interactions, unclear antiviral spectrum and manufacturing issues. Here we use a 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 with potent in vitro PPIase inhibitory activity and antiviral activity against hepatitis C virus, human immunodeficiency virus and coronaviruses. This family of compounds has the potential for broad-spectrum, high-barrier-to-resistance treatment of viral infections.
Immuno-modulation and cell signalling have been identified thus far. Among them, cyclophilin A (CypA) is seen as an internal proline residue. Seventeen human cyclophilins have been revealed (Fig. 1a)\textsuperscript{10}. Their PPIase catalytic groove is formed by a mixture of hydrophobic, aromatic and polar residues, including Arg55, Phe60, Met61, Gln63, Asn102, Phe113, Trp121, Leu122 and His126 (Fig. 1a,b). The existence of a deep pocket contiguous to the canonical catalytic site, called the ‘gatekeeper’ pocket, has been revealed (Fig. 1c,d)\textsuperscript{10}. The gatekeeper pocket might contribute to substrate-binding specificity, its access being determined by gatekeeper residues at its surface.

Several cyclophilins, including principally but not exclusively CypA, have been shown to play a pivotal role in the life cycle of a number of viruses, including HIV, HCV, dengue virus, Japanese encephalitis virus, yellow fever virus, coronaviruses, HBV, cytomegalovirus, influenza A virus, enteroviruses and so on\textsuperscript{3,12–14}. Cyclosporine A (CsA) and non-immunosuppressive macrocyclic analogues of CsA and of sanglifehrin A (SfA) potentantly inhibit cyclophilin PPIase activity by binding its catalytic site (Fig. 1d). They have shown in vitro effectiveness against HIV, HCV and HBV replication\textsuperscript{3}. A CsA analogue, alisporivir, showed potent anti-HCV activity in vivo\textsuperscript{15,16}, but its clinical development was halted during the Phase III programme due to severe adverse events unrelated to cyclophilin inhibition.

The existing cyclophilin inhibitors, all derived from CsA or SfA, have disadvantages, including their size resulting in poor cell permeability, the risk of drug–drug interactions, their unclear antiviral spectrum (only the effect on HCV has been reproducibly demonstrated), manufacturing issues they raise (synthesis of alisporivir is a complicated hemi-synthetic pathway starting with CsA that includes 12 steps, some of which use highly reactive and dangerous compounds) and their potential for side effects unrelated to cyclophilin inhibition. In addition to inhibiting cyclophilin PPIase activity, CsA was, for instance, shown to inhibit the mitogen-activated protein kinase pathway and affect transforming growth factor-β1 levels, whereas CsA and its non-immunosuppressive derivatives were reported to inhibit ABC transporters (for example, PgP, MRP and BCRP) and stimulate antigen presentation by enhancing major histocompatibility complex-I surface expression\textsuperscript{17–22}. Alisporivir was associated with acute pancreatitis cases, including one fatal case, when combined with pegylated interferon-α in a Phase III trial, in patients infected with HCV, through mechanisms that are unknown. Thus, new families of cyclophilin inhibitors are urgently needed.

Fragment-based drug discovery (FBDD) is based on the identification of very small molecules (fragments) that are subsequently expanded or linked together to generate drug leads with therapeutic activity\textsuperscript{23,24}. Here we use an FBDD approach using nuclear magnetic resonance (NMR) and X-ray crystallography to generate a new family of non-peptidic, small-molecule cyclophilin inhibitors, unrelated to CsA or SfA, with potent in vitro PPIase inhibitory activity and antiviral activity against several families of viruses responsible for frequent human infections.

**Results**

**Fragment screening.** In total, 34,409 fragments were computationally docked into the canonical active site and the gatekeeper pocket of CypD by means of the FlexX programme. Forty-four fragments were selected based on their mode of interaction. Their ability to interact with CypD was further studied by means of NMR spectroscopy. Ten fragment hits with low-affinity dissociation constants (millimolar range) were identified (Supplementary Fig. 1). Their scaffolds and proline-mimicking motifs were used to select in cerebro a set of 52 derivative fragments for subsequent X-ray crystallographic experiments. Apo CypD crystals were soaked with each of the 52 fragments. X-ray structures of CypD complexed with 14 fragments were obtained. Supplementary Fig. 2 shows the chemical structures of the 14 binding fragments. Four fragments (9, 11, 12 and 13) bound the catalytic site of CypD, whereas five fragments (6, 15, 16, 17 and 18) bound the gatekeeper pocket. Fragment 14 bound between the two sites. Finally, four fragments (5, 19, 20 and 21) were nonspecific multibinders. The density map of each fragment is shown in Supplementary Fig. 3 and at (https://figshare.com/articles/Stereo_views_of_cocrystal_structures_of_cyclophilin_inhibitors_with_cyclophilin_D/3490493).

The ability of each fragment to inhibit cyclophilin activity in vitro was assessed in cell-free enzyme assays for CypA, CypB and CypD. The half-maximal inhibitory concentrations (IC\textsubscript{50}) of the 14 fragments were >5 mM in all instances.

**Fragment selection for linking.** Among the 14 fragment hits, the final selection of compounds 6 and 13 for subsequent compound optimization was based on a number of criteria, including their ligand efficiency, their ability to access key regions, their synthetic tractability and the possibility to link them to generate

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**Note:** The text is a excerpt from a scientific paper focusing on the role of cyclophilins in viral infections and the development of new antiviral agents. It highlights the importance of developing new families of broad-spectrum antiviral agents to combat emerging viral infections.

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**References:**

1. Over the past decades, an increasing number of viruses causing unexpected illnesses and epidemics among humans, wildlife and livestock has emerged. These outbreaks have seriously stretched local and national resources in the economically developed world, whereas the capacity to control emerging diseases remains limited in poorer regions where many of them have their origin. A number of virus-specific antiviral agents have been developed and commercialized since the early 1980s. These agents, including drugs that specifically inhibit members of the *Herpesviridae* family, influenza viruses, human immunodeficiency virus (HIV), hepatitis B virus (HBV) and, more recently, hepatitis C virus (HCV), had a major medical impact\textsuperscript{1}. However, the development costs of specific antiviral agents are extremely high and there are many other medically important viral infections that require efficacious therapies. Thus, there is an urgent need for new families of broad-spectrum antiviral agents, that is, antiviral agents that are active against a number of different viral families\textsuperscript{2}. Such compounds should target mechanisms common to different families of viruses, such as cellular components and/or functions involved in their life cycles. The cellular proteins cyclophilins have been shown to play a key role in the life cycle of a number of different viral families. In addition, cyclophilin inhibitors were reported to inhibit the replication of different viruses, both in vitro and in vivo\textsuperscript{3,4}. Thus, cyclophilins represent an attractive target for broad-spectrum antiviral inhibition.

2. Cyclophilins are peptidyl-prolyl cis/trans isomerases (PPIase) that catalyse the interconversion of the two energetically preferred conformers (cis and trans) of the planar peptide bond preceding an internal proline residue. Seventeen human cyclophilins have been identified thus far. Among them, cyclophilin A (CypA) is present in the cytosol and involved in protein folding, trafficking, immunomodulation and cell signalling\textsuperscript{5}. The extracellular fraction of CypB is involved in cell–cell communications and inflammatory signalling. CypD is localized in the matrix of the mitochondria and acts as a key regulator of the opening of the mitochondrial permeability transition pore (mPTP), which plays a major role in calcium efflux from mitochondria to the cytosol and can lead to mitochondrial swelling and cell death\textsuperscript{6–9}. The function of most of the remaining cyclophilins is unknown\textsuperscript{10,11}.

3. Cyclophilins share a common domain of ~109 amino acids, the cyclophilin-like domain, surrounded by domains unique to each member of the family and associated with their subcellular compartamentalization and functional specialization\textsuperscript{11}. Cyclophilins adopt an eight-stranded anti-parallel β-barrel structure (Fig. 1a)\textsuperscript{10}. Their PPIase catalytic groove is formed by a mixture of hydrophobic, aromatic and polar residues, including Arg55, Phe60, Met61, Gln63, Asn102, Phe113, Trp121, Leu122 and His126 (Fig. 1a,b). The existence of a deep pocket contiguous to the canonical catalytic site, called the ‘gatekeeper’ pocket, has been revealed (Fig. 1c,d)\textsuperscript{10}. The gatekeeper pocket might contribute to substrate-binding specificity, its access being determined by gatekeeper residues at its surface.

4. Several cyclophilins, including principally but not exclusively CypA, have been shown to play a pivotal role in the life cycle of a number of viruses, including HIV, HCV, dengue virus, Japanese encephalitis virus, yellow fever virus, coronaviruses, HBV, cytomegalovirus, influenza A virus, enteroviruses and so on\textsuperscript{3,12–14}. Cyclosporine A (CsA) and non-immunosuppressive macrocyclic analogues of CsA and of sanglifehrin A (SfA) potently inhibit cyclophilin PPIase activity by binding its catalytic site (Fig. 1d). They have shown in vitro effectiveness against HIV, HCV and HBV replication\textsuperscript{3}. A CsA analogue, alisporivir, showed potent anti-HCV activity in vivo\textsuperscript{15,16}, but its clinical development was halted during the Phase III programme due to severe adverse events unrelated to cyclophilin inhibition.

5. The existing cyclophilin inhibitors, all derived from CsA or SfA, have disadvantages, including their size resulting in poor cell permeability, the risk of drug–drug interactions, their unclear antiviral spectrum (only the effect on HCV has been reproducibly demonstrated), manufacturing issues they raise (synthesis of alisporivir is a complicated hemi-synthetic pathway starting with CsA that includes 12 steps, some of which use highly reactive and dangerous compounds) and their potential for side effects unrelated to cyclophilin inhibition. In addition to inhibiting cyclophilin PPIase activity, CsA was, for instance, shown to inhibit the mitogen-activated protein kinase pathway and affect transforming growth factor-β1 levels, whereas CsA and its non-immunosuppressive derivatives were reported to inhibit ABC transporters (for example, PgP, MRP and BCRP) and stimulate antigen presentation by enhancing major histocompatibility complex-I surface expression\textsuperscript{17–22}. Alisporivir was associated with acute pancreatitis cases, including one fatal case, when combined with pegylated interferon-α in a Phase III trial, in patients infected with HCV, through mechanisms that are unknown. Thus, new families of cyclophilin inhibitors are urgently needed.

6. Fragment-based drug discovery (FBDD) is based on the identification of very small molecules (fragments) that are subsequently expanded or linked together to generate drug leads with therapeutic activity\textsuperscript{23,24}. Here we use an FBDD approach using nuclear magnetic resonance (NMR) and X-ray crystallography to generate a new family of non-peptidic, small-molecule cyclophilin inhibitors, unrelated to CsA or SfA, with potent in vitro PPIase inhibitory activity and antiviral activity against several families of viruses responsible for frequent human infections.
compounds binding both the catalytic site and the gatekeeper pocket.

The X-ray crystallographic structure of CypD complexed with fragment 6, solved at a resolution of 1.10 Å, showed that this fragment deeply buried into the gatekeeper pocket, a hydrophilic region (Fig. 2a), and its amino group displaced a water molecule present in this pocket in the apo form of CypD. Within the gatekeeper pocket, fragment 6 made one direct hydrogen bond with Thr107 and two hydrogen bonds with Ala101 and Gln111 through a water molecule (Fig. 2b).

The crystallographic structure of CypD in complex with fragment 13 was solved at a resolution of 1.35 Å (Fig. 2c). In addition to its hydrophobic contacts with Phe60, Met61, Phe113 and Leu122 within the catalytic site of CypD, fragment 13 directly interacted with Asn102 via a hydrogen bond (Fig. 2d).

**Linking strategy.** Superimposition of fragments 6 and 13 with the known structures of CsA and SFA, two cyclophilin inhibitors, suggested that a urea moiety could be used as a linker between the two fragments, because of one hydrogen bond with Gln63 and two with Asn102 (Fig. 2e). Compound 22, consisting of fragments 6 and 13 connected by a urea moiety, was thus generated (Fig. 2f). This compound inhibited CypD activity, with an IC_{50} of 6.2 ± 3.7 μM (Table 1).

The structure of CypD complexed with compound 22, obtained at a resolution of 1.93 Å, revealed binding to both the gatekeeper pocket and the catalytic site (Fig. 3a and b). Compound 22 maintained the key interactions of fragments 6 and 13 within their respective pockets, while making one, one and two additional hydrogen bonds with Arg55, Gln63 and Asn102, respectively (Fig. 3b). The binding mode of compound 22 to CypD was confirmed by means of NMR experiments. The CypD 15N-heteronuclear single quantum coherence (HSQC) spectrum revealed significant chemical shift perturbations on ligand binding only for residues located at or near the catalytic site and gatekeeper pocket, respectively (Supplementary Fig. 4). Cross-peaks of Gly151, Asn144 and Asn145 disappeared on ligand addition, likely to be due to intermediate chemical exchange, demonstrating that these residues are part of the binding site and the corresponding amide protons are located at proximity of the ligand.

Compound 22 also potently inhibited CypA and CypB (IC_{50}: 13.1 ± 5.9 and 6.1 ± 3.8 μM, respectively). These findings were confirmed by isothermal titration calorimetry experiments showing reduced conformational flexibility on binding of these proteins (Supplementary Fig. 5). Finally, superimposition of the crystallographic structures of CypA and CypD complexed with compound 22 showed identical binding modes (Fig. 3c and https://figshare.com/articles/Stereo_views_of_cocrystal_structures_of_cyclophilin_inhibitors_with_cyclophilin_D/3490493).
Structure-based lead optimization of the compounds. Structure-guided optimization was used to improve cyclophilin affinity and stability of the compounds. As ester functions are often associated with low biological stability, the first step was to replace the ester function of compound 22, which makes a key hydrogen bond with Arg55, without affecting the cyclophilin inhibitory potency. All designed compounds lacking the ester function lost their interaction with Arg55, resulting in a drastic decrease of cyclophilin inhibition (IC₅₀ > 500 μM). This was the case of compound 23 (Table 1), although this compound retained all of the other key interactions of compound 22 (Supplementary Fig. 6 and https://figshare.com/articles/Stereo_views_of_cocrystal_structures_of_cyclophilin_inhibitors_with_cyclophilin_D/3490493). Based on the observation that in the CypA–SfA

![Figure 2](link) | Cocrystal structures of fragment hits with CypD and linking strategy. (a,b) The crystal structure shows fragment 6 bound to the gatekeeper pocket, predominantly through a hydrogen bond with Thr107. The green mesh and surface represent the electron density map of fragment 6 (2Fₒ–Fₑ omit map contoured at 1.0σ). The gatekeeper pocket is zoomed in b. (c,d) The crystal structure shows fragment 13 bound to the catalytic site, predominantly through a hydrogen bond with Asn102. The green mesh and surface represent the electron density map of fragment 13 (2Fₒ–Fₑ omit map contoured at 1.0σ). The catalytic site of CypD is zoomed in d. (e) Superimposition of PDB 1CWA (CypA-CsA) and 1YND (CypA-SfA) with cocrystals CypD-fragment 6 and CypD-fragment 13. CsA is represented with yellow sticks. SfA is represented by purple sticks. Fragments 6 and 13 are represented by green sticks. The red circle shows the urea moiety used to link fragments 6 and 13. (f) Chemical structure of compound 22, generated by linking fragments 6 and 13 with a urea moiety. The orange circle shows key atoms from fragments 6 and 13.

| Compound | CypA IC₅₀ (μM) | CypB IC₅₀ (μM) | CypD IC₅₀ (μM) | LE (kcal per heavy atom) |
|----------|----------------|----------------|----------------|-------------------------|
| CsA      | 0.01 ± 0.003   | 0.01 ± 0.005   | 0.02 ± 0.003   | ND                      |
| Alisporivir | 0.07 ± 0.003   | 0.04 ± 0.012   | 0.03 ± 0.005   | ND                      |
| 13       | >5,000         | >5,000         | >5,000         | <0.45                  |
| 6        | >5,000         | >5,000         | >5,000         | <0.31                  |
| 22       | 13.1 ± 5.9     | 6.1 ± 3.8      | 6.2 ± 3.7      | 0.36                   |
| 23       | >500           | >500           | >500           | <0.29                  |
| 24       | 2.8 ± 0.6      | 1.2 ± 0.1      | 11.4 ± 3.0     | 0.27                   |
| 25       | 3.4 ± 0.7      | 3.7 ± 1.4      | 6.2 ± 2.3      | 0.28                   |
| 26       | 0.6 ± 0.2      | 0.8 ± 0.1      | 1.1 ± 0.2      | 0.31                   |
| 27       | 0.4 ± 0.1      | 0.6 ± 0.1      | 0.6 ± 0.1      | 0.31                   |
| 28       | 1.5 ± 0.5      | 1.8 ± 0.9      | 1.4 ± 0.2      | 0.26                   |
| 29       | 0.8 ± 0.1      | 0.5 ± 0.2      | 0.7 ± 0.2      | 0.26                   |
| 30       | 3.3 ± 1.4      | 1.9 ± 1.5      | 3.0 ± 0.7      | 0.23                   |
| 31       | 0.1 ± 0.07     | 0.08 ± 0.04    | 0.2 ± 0.08     | 0.28                   |
| 32       | 7.4 ± 6.8      | 8.7 ± 1.5      | 12.8 ± 2.5     | 0.34                   |
| 33       | 4.2 ± 1.6      | 2.2 ± 1.2      | 7.7 ± 0.8      | 0.22                   |

CsA, cyclosporine A; CypA, cyclophilin A; CypB, cyclophilin B; CypD, cyclophilin D; LE, ligand efficiency; ND, not defined; PPIase, peptidyl-prolyl cis/trans isomerase. CsA and the CsA analogue alisporivir are used as references.
and Fig. 4B. Interestingly, the thiomethyl groups of compounds keeping with NMR experiments shown in Supplementary of cyclophilin inhibitors with cyclophilin D/3490493), in structures of CypD in complex with compounds to be a potent inhibitor of CypA, CypB and CypD PPIase inhibitors with cyclophilin D/3490493). Compound sized, including compounds a very large number of CypB and CypD PPIase activities in enzyme assays. Table 1 shows the inhibitory effects of these compounds on CypA, CypB and CypD PPIase activities, with IC_{50s} of 2.8 ± 0.6, 1.2 ± 0.1 and 11.4 ± 3.0 μM, respectively.

A series of phenyl–pyrrolidine derivatives were then synthesized, including compounds 25–31 (Table 1). The crystal structures of CypD in complex with compounds 26, 27, 28 and 29 showed the same binding mode (Supplementary Fig. 7 and https://figshare.com/articles/Stereo_views_of_cocrystal_structures_of_cyclophilin_inhibitors_with_cyclophilin_D/3490493), Compound 24 proved to be a potent inhibitor of CypA, CypB and CypD PPIase activities, with IC_{50s} of 2.8 ± 0.6, 1.2 ± 0.1 and 11.4 ± 3.0 μM, respectively.

As compounds with an aniline motif have been reported to potentially bear toxic properties, a very large number of chemical modifications aimed at replacing compound 22’s aniline motif, while retaining its PPIase inhibitory activity, were made. These included replacement of the amino group by the following: halogens (F, Cl and Br), hydroxyl, methoxy, ester and acetamide; replacement by pyridines, aminopyridines and heterocycles that sometimes contained an amine function, and fused or non-fused 6:6 or 6:5 bicycles; and substitution with methoxy or alkyl groups. The list and structure of the compounds tested for PPIase inhibitory activity is shown in Supplementary Table 1. Among them, only compound 32, in which the aniline motif was replaced by an amino-2,3-dihydro-1H-inden-1-yl, retained the PPIase inhibitory potency of its parent compound, with IC_{50s} of 7.4 ± 6.8, 8.7 ± 1.5 and 12.8 ± 2.5 μM for CypA, CypB and CypD, respectively (Table 1). In addition, replacement of compound 31’s aniline motif by a 3-amino-pyridine led to the generation of compound 33, which, although less active than its parent compound, retained significant PPIase inhibitory activities, with IC_{50s} of 4.2 ± 1.6, 2.2 ± 1.2 and 7.7 ± 0.8 μM for CypA, CypB and CypD, respectively (Table 1).

On the other hand, macrocyclization was attempted to stabilize the bioactive conformation of the compounds. However, macrocyclic compound 569 (Supplementary Table 1) was inactive against cyclophilin PPIase activity. In contrast, the addition of a phenyl ring between the urea and carbonyl of compound 31 stabilized the bioactive conformation by a π–π interaction between the two phenyl moieties, leading to an at least threefold gain of anti-PPIase activity. Ultimately, compound 31 was the most potent cyclophilin inhibitor generated, with IC_{50s} of 0.1 ± 0.07, 0.08 ± 0.04 and 0.2 ± 0.08 μM for CypA, CypB and CypD, respectively (Table 1).

Broad-spectrum antiviral activity. Huh7 cells harbouring an HCV genotype 1b replicon were treated with increasing concentrations of compounds 26, 27, 29, 30, 31, 32 and 33. As shown in Table 2, all of them inhibited HCV replicon replication in a dose-dependent manner, with EC_{50s} ranging from 0.4 to 8.4 μM.

Figure 3 | Co-crystal structure of compounds 22 and 24 with CypA and CypD. (a) Surface representation of CypD in complex with compound 22, showing occupation of both the catalytic site (left) and the gatekeeper pocket (right) of CypD. (b) Zoom into the catalytic site of CypD showing the urea moiety linker of compound 22 making four hydrogen bonds with Arg55, Gln63 and Asn102. The green mesh and surface represents the electron density map of compound 22 (2Fo-Fc, omit map contoured at 1.0σ). (c) Superimposition of the CypD-compound 22 (pink for CypD, green sticks for compound 22) and the CypA-compound 22 (purple for CypA, white sticks for compound 22) co-crystals, showing identical binding modes. (d) Superimposition of the CypD-compound 22 cocrystal (pink for CypD, green sticks for compound 24) with the CypD-compound 22 co-crystal (purple for CypD, orange sticks for compound 22). The side chain of Arg55 is shown in stick format (pink for compound 24 and orange for compound 22). Compound 24 shares the same mode of CypD binding as compound 22 and its methoxy group pushes Arg55 to create a hydrogen bond with the urea moiety of the compound. (e) Cartoon representations of CypD in complex with compound 24, showing occupation of the gatekeeper pocket and the catalytic site of CypD. The side chain of Arg55 is represented in stick format to show the interaction with the urea moiety. The green mesh and surface represent the electron density maps (2Fo-Fc, omit map contoured at 1.0σ).
EC50s ranging from 3.6 to 15.0 µM were assessed against HIV-IIIb replication in MT4 cells. All tested compounds inhibited HCoV-229E replication with EC50s ranging from 7.2 to 34.6 µM (HCoV-229E) in MRC-5 cells. The seven tested compounds were not cytotoxic at their effective concentrations.

The antiviral activities of compounds 26, 27, 29 and 30 were also assessed against HIV-IIIb replication in MT4 cells. All tested compounds inhibited HIV replication better than CsA, with EC50s ranging from 3.6 to 15.0 µM. The compounds were not cytotoxic at their effective concentrations (Table 2).

Finally, the antiviral activities of compounds 26, 27, 29, 30, 31, 32 and 33 were assessed against human coronavirus 229E (HCoV-229E) in MRC-5 cells. The seven tested compounds inhibited HCoV-229E replication with EC50s ranging from 7.2 to 71.5 µM, without associated cytotoxicity (Table 2).

Effect of NS5A protein substitutions on anti-HCV activity. The D320E and R318H substitutions in domain II of the NS5A protein have been reported to be associated with slightly reduced HCV susceptibility to the antiviral action of CsA and its nonimmunosuppressive derivatives. The corresponding nucleotide substitutions were introduced in a genotype 1b subgenomic replicon by means of site-directed mutagenesis and the antiviral activity of compounds 27, 29, 30 and 31 was measured in the mutated replicons in comparison with a wild-type replicon. The results shown in Table 3 indicate that substitutions D320E and R318H very slightly reduced susceptibility to these compounds (1.0- to 2.8-fold change in EC50), in the same order as the change in CsA and alisporivir susceptibility they induce.

Lack of calcineurin inhibition properties. In addition to its anticycophilin activity, CsA displays potent immunosuppressive properties through the formation of a stable ternary complex in a 1:1:1 stoichiometry with calcineurin, CypA and CsA, resulting in the inhibition of calcineurin phosphatase activity. Although the new family of inhibitors is unrelated to CsA, preincubation studies were performed with the binary complex formed of CypA and compound 31, to determine whether this complex inhibits calcineurin phosphatase activity. As shown in Supplementary Fig. 9, a dose-dependent decrease of calcineurin activity was observed in the presence of increasing concentrations of the CypA–CsA complex, used as a positive control of inhibition. In contrast, no inhibition was observed in the presence of the binary complex of CypA and compound 31. In addition, CsA potently inhibited interleukin (IL)-2 production in stimulated immortalized T lymphocytes (Jurkat cells) with an EC50 of 0.005 µM, whereas compound 31 had no effect on IL-2 production (EC50 > 20 µM).

In vitro metabolism. Compounds 29 and 30 were assessed for their in vitro metabolism before compound 31 was synthesized. The results are summarized in Supplementary Table 2. The measured octanol/water partitioning (LogD) values for compounds 29 and 30 were 2.5 and 2.6, respectively. Both compounds were very soluble at pH 7.4 and at pH 1.0. They were not degraded after incubation in PBS for 24 h at 37°C and slightly

### Table 2 | In vitro antiviral activities of the cyclophilin inhibitors against HCV, HIV and HCoV-229E, and cellular toxicities in their respective cellular models.

| Compound | Huh7 cells | MT4 cells | MRC5 cells |
|----------|------------|-----------|------------|
|          | HCV genotype 1b replicon | HCV genotype 1b replicon | HIV-IIIb in MT4 cells | MT4 cells | HCoV-229E | MRC5 cells |
|          | EC50 (µM) | CC50 (µM) | EC50 (µM) | CC50 (µM) | EC50 (µM) | CC50 (µM) |
| CsA      | 0.3 ± 0.1 | 19.2 ± 4.5 | > CC50 | 7.5 ± 1.7 | > CC50 | 9.3 ± 1.2 |
| Alisporivir | 0.01 ± 0.0007 | 32.3 ± 22.0 | NT | NT | 2.6 ± 0.6 | 9.7 ± 2.2 |
| 26       | 6.0 ± 0.7 | >100 | 3.6 ± 0.8 | >53 | 66.3 ± 24.0 | >100 |
| 27       | 2.7 ± 2.5 | >100 | 6.8 ± 2.3 | >53 | 34.6 ± 18.3 | >100 |
| 29       | 1.7 ± 1.2 | >100 | 13.0 ± 2.7 | 40.5 ± 5.4 | 27.6 ± 8.6 | >100 |
| 30       | 1.4 ± 1.2 | >100 | 15.0 ± 1.2 | >53 | 7.2 ± 1.8 | >100 |
| 31       | 0.4 ± 0.3 | >100 | NT | NT | 44.7 ± 2.2 | >100 |
| 32       | 8.0 ± 1.3 | >100 | NT | NT | 71.5 ± 4.3 | >100 |
| 33       | 8.4 ± 1.0 | >100 | NT | NT | 55.3 ± 12.2 | >100 |

*CsA, cyclosporine A; HCV, hepatitis C virus; HCoV-229E, human coronavirus 229E; HIV, human immunodeficiency virus; NT, not tested.

CsA and the CsA analogue alisporivir are used as references.

### Table 3 | Effect of the D320E and R318H HCV NS5A protein substitutions on the antiviral effect of the cyclophilin inhibitors.

| Compound | HCV (WT) subgenomic replicon | HCV (D320E) subgenomic replicon | HCV (R318H) subgenomic replicon |
|----------|-------------------------------|-------------------------------|-------------------------------|
|          | EC50 (µM) | EC50 fold-increase as compared with WT | EC50 (µM) | EC50 fold-increase as compared with WT |
| CsA      | 0.1 ± 0.02 | 0.5 ± 0.06 | 3.7 | 0.4 ± 0.03 | 2.9 |
| Alisporivir | 0.04 ± 0.0005 | 0.1 ± 0.003 | 2.5 | 0.08 ± 0.02 | 2.0 |
| 27       | 5.6 ± 0.7 | 10.3 ± 2.7 | 1.8 | 4.8 ± 0.5 | 0.9 |
| 29       | 2.9 ± 0.04 | 3.9 ± 0.4 | 1.3 | 3.5 ± 0.6 | 1.2 |
| 30       | 2.1 ± 0.09 | 5.8 ± 1.1 | 2.8 | 3.2 ± 1.2 | 1.5 |
| 31       | 0.3 ± 0.16 | 0.3 ± 0.1 | 1.0 | 0.4 ± 0.01 | 1.4 |

*CsA, cyclosporine A; HCV, hepatitis C virus; WT, wild type.

CsA and its analogue alisporivir were used as references.
degraded after incubation in HCl 0.1 N at pH 1.0 for 24 h at 37 °C (98% of compound 29 and 92% of compound 30 remaining at 24 h). Permeability was high for both compounds, whereas the efflux was negligible for compound 30 and low for compound 29 in monolayers of Caco-2 cells, suggesting a high absorption potential for the family.

Binding to human plasma proteins was 85.0 and 85.1% for compounds 29 and 30, respectively. The high recovery indicated satisfactory plasma stability. Therefore, both compounds were predicted to display low shifts in biological potency in the presence of human plasma compared with cell culture medium. The metabolic stabilities were studied using human hepatic microsomal fractions in the presence of NADPH, to support oxidative metabolism. Compounds 29 and 30 were metabolized in microsomal fractions with in vitro half-lives of 21.6 and 35.5 min, respectively, yielding moderate predicted clearances.

Neither compound 29 nor compound 30 was metabolized by CYP1A2 or CYP2B6. Both compounds were metabolized at the highest rate by CYP2C19. Compound 30, but not compound 29, was metabolized by CYP3A4. The relative contributions of the individual enzymes to the metabolism of the compounds could not be determined from these experiments, but the data suggested that compounds 29 and 30 were predominantly metabolized by CYP2C19 enzymes.

Neither compound 29 nor compound 30 significantly inhibited CYP1A2, CYP2B6, CYP2C9, CYP2C19 or CYP2D6. Both compounds were moderate inhibitors of CYP2C8 and potent inhibitors of CYP3A, suggesting possible drug–drug interactions through the inhibition of CYP3A enzymes.

Discussion

In this study, we used FBDD to create a new family of non-peptidic, small-molecule cyclophilin inhibitors, unrelated to CsA or SFA, with potent PPIase inhibitory activity and with potent antiviral effectiveness against HCV, HIV and coronaviruses in vitro.

FBDD has emerged as an effective approach for drug discovery nearly 20 years ago. FBDD indeed generated a number of new drug classes, including some that reached the medical market. FBDD is based on screening of low-molecular-weight molecules with minimal chemical complexity that bind subpockets within a target site. These fragments typically bind with low affinity, in the mM range. They represent suitable starting points for structurally guided 'hit-to-lead optimisation'. The fragment-based approach offers better coverage of the chemical diversity space than high-throughput screening, while offering the advantage of structure-based chemical optimization.

The cyclophilin PPIase catalytic groove is formed by a mixture of hydrophobic, aromatic and polar residues that are highly conserved across different cyclophilins. Notably, the side-chain guanidine group of the essential Arg55 is hydrogen bonded to the prolyl nitrogen of the substrate. It promotes isomerization by weakening the double-bond properties of the peptide bond. Targeting this residue is thus crucial to achieve potent inhibition of PPIase activity. CsA, its non-immunosuppressive cyclic analogues and SFA are large macrocycle compounds (molecular weight >1,000 g mol⁻¹). They bind the cyclophilin catalytic site and potently inhibit PPIase activity. However, the shortcomings of these families of compounds in clinical practice emphasize the need for the development of small-molecule cyclophilin inhibitors. As the cyclophilin catalytic site is not a buried pocket, the design of small ligands with a molecular weight <500 g mol⁻¹ is nevertheless challenging. A deeper pocket, the ‘gatekeeper pocket’, has been identified in close vicinity to the PPIase catalytic site. This pocket could contribute to substrate binding specificity through gatekeeper residues located at its surface that restrict substrate accessibility. Thus, the gatekeeper pocket appears as an interesting target close to the catalytic site, suitable for a fragment-based approach targeting both sites combined with a linking strategy.

The most common approach for chemical optimization is the 'fragment growing strategy', which permits a multistep optimization of ligand efficiency and size within the binding site. Alternatively, the 'linking strategy' is constrained by the size of the original fragments and that of the linker, therefore resulting in a rapid buildup of atoms in a single step. Furthermore, conformational strain and flexibility mean that an energy price often needs to be paid to achieve optimal linking of the fragments.

In this study, we used FBDD to create a new family of non-peptidic, small-molecule cyclophilin inhibitor family with potent antiviral effectiveness against HCV, HIV and coronaviruses in vitro with potent antiviral effectiveness against HCV, HIV and coronaviruses in vitro. The risk related to the aniline motif must be balanced with the severity of the target disease (cost/benefit ratio) and the required duration of administration. For instance, what would be considered a major safety issue when treating a benign disease requiring long-term administration would be unimportant in case of short-term treatment of a severe, eventually life-threatening acute infection. Thus, lead compounds both with and without the aniline motif will be moved forward throughout preclinical development.

The compounds described here represent the first non-peptidic, small-molecule cyclophilin inhibitor family with broad-spectrum antiviral properties described thus far. Based on a previous work from our group, Chinese authors reported a series of thiourea-based inhibitors targeting both the HIV-1 capsid and human CypA, which inhibited assembly and uncoating of the viral capsid. However, the low CypA-binding affinity of the compounds strongly contradicted the claimed PPIase inhibitory activity and the reported effect on HIV could be exclusively related to the capsid ligand properties of the compounds in this work.

Another series of small-molecule CypA inhibitors was reported in 2009 as a result of de novo drug design. We synthesized their two ‘best’ compounds and purchased one of them (TMN-355) from Tocris Bioscience, to test their ability to inhibit PPIase activity, and HCV and coronavirus replication in vitro. As shown in Supplementary Table 3, none of these compounds exhibited any biological activity in the models. Furthermore, we could not obtain any crystallographic structure of the compounds complexed with cyclophilins. In another article in which a closely related compound was tested against enterovirus 71 (ref. 4), in vitro resistance selection experiments identified one amino acid...
substitution selected after several passages with increasing concentrations of the compound. The substitution conferred a modest fivefold reduction in EV71 susceptibility, but no resistance to CsA, further challenging the hypothesis of a common mechanism of inhibition for the two molecules. Altogether, these results cast doubt as to the reality of the anti-cyclophilin properties of this class of molecules. Other families of compounds have been reported to inhibit cyclophilin activity in vitro, but they need to be externally validated and their potential as broad-spectrum antiviral compounds has not been evaluated. These results emphasize the difficulty of developing efficacious small-molecule cyclophilin inhibitors unrelated to CsA or SfA and underline the originality and importance of our work.

Cyclophilins represent interesting targets for broad-spectrum antiviral drugs. Indeed, several cyclophilins, principally but not only CypA, have been convincingly shown to play a pivotal role in the life cycle of a number of viral families. Our results showing inhibition of HCV, HIV and HCoV-229E coronavirus by compound 31 and related compounds, together with preliminary data suggesting hepatitis B virus replication inhibition, in the life cycle of a number of viral families, support the future development of our new family of cyclophilin inhibitors as broad-spectrum antiviral compounds with a high barrier to resistance. The high barrier to resistance, demonstrated with CsA derivatives in HCV infection, results of the fact that barrier to resistance. The high barrier to resistance, demonstrated with CsA derivatives in HCV infection,16, results of the fact that cyclophilin inhibitors do not directly target a viral function but instead target a host protein involved at a key step of the viral life cycle. Thus, the likelihood to select viruses that are resistant to the action of the drug is low and, if such viruses were selected, they would be unlikely to yield high levels of resistance, as already shown.27 This was confirmed here by the demonstration that amino acid substitutions in the HCV NS5A protein known to be involved as broad-spectrum antiviral compounds has not been evaluated. These results emphasize the difficulty of developing efficacious small-molecule cyclophilin inhibitors unrelated to CsA or SfA and underline the originality and importance of our work.

In conclusion, we used FBDD combined with a linking strategy and structure-based compound optimization, to generate a new family of non-peptidic, small-molecule cyclophilin inhibitors, related to CsA or SfA, with potent PPlase inhibitory activity, antiviral effectiveness against HCV, HIV and coronaviruses in vitro, and druggable properties. This family of compounds has the potential to be useful in the high-barrier-to-resistance treatment of viral infections that use cyclophilins in their life cycle, as well as in various medical applications of CypD/mPTP opening-related cellular protection.

Methods

Expression and purification of CypD K133I. Mutant K133I of human CypD was expressed in Esherichia coli strain BL21(DE3). Bacteria were grown in Luria Broth medium at 37°C up to an optical density (OD) of 0.6 at 600 nm and induced for 2 h with isopropyl-β-D-thiogalactopyranoside. Cells were lysed by sonication in buffer A, composed of 50 mM Tris at pH 7.5, 2 mM EDTA and 2 mM β-mercaptoethanol. Then, the cell lysate was clarified by centrifugation at 40,000×g for 30 min and the supernatant was loaded on Q-Sepharose and S-Sepharose columns in series equilibrated with buffer A. The S-Sepharose column was washed with equilibrium buffer and bound proteins were eluted with a linear gradient from 0 to 1 M NaCl. The combined peak fractions were loaded on an S75 column that was gradient eluted in a second step with 0 to 1 M NaCl. The combined peak fractions were loaded on an S75 column that was gradient eluted in a second step with 0 to 1 M NaCl.

NMR experiments. NMR samples used for screening contained 100 μM 15N-labelled protein, in a buffer consisting of 50 mM KH2PO4, pH 7.3. 15N-HSQC experiments were recorded at 298 K in the presence of 5% D2O on a Bruker Avance 500 MHz spectrometer equipped with a cryoprobe. Typical acquisition time was 72 min per experiment. Mixtures of ten fragments (cocktails) at an individual concentration of 10 mM were screened. For cocktails soluble in water no DMSO-d6 was used, whereas 20% DMSO-d6 was used to solubilize cocktails insoluble in water. The attribution of the 15N-HSQC spectrum for CypD K133I deposited in the Biological Magnetic Resonance Bank (BMRB entry 7310) was used to re-attribute the 15N-HSQC spectrum of the protein in the presence of 20% DMSO-d6. Fragment binding was detected by comparing the 15N-HSQC spectra in the presence and in the absence of the fragment mixtures. At the fragment cocktails caused significant perturbations in the 15N-HSQC spectrum for some residues of the active site, one 15N-HSQC spectrum of CypD K133I was recorded in the presence of each cocktail (5% DMSO-d6) and these data were processed using Bruker software XwinNMR Version 3.0, and the analyses and comparisons were made with in-house software Cindy for fragment screening.
NMR experiments with compounds 22 and 27 were recorded at 25 °C on an Inova Agilent 600 MHz spectrometer equipped with a triple-resonance 1H, 13C, and 15N probe. 1H-15N-HSQC spectra were recorded with 50 M protein, 200 μM compound 27 and 500 μM compound 22. Resonance assignment on ligand binding was checked using three-dimensional nuclear Overhauser enhancement spectroscopy (1H-15N-HSQC) with 150 ms mixing time.

**Carlsbad, California) supplemented with 10% fetal bovine serum, 50 IU ml⁻¹ penicillin, 100 μM NaCl. Twenty to 50 μM compound was loaded into the MicroCal VP-ITC (Malvern, Orsay, France) isothermal titration calorimeter cell (microcalorimeter). Data were integrated and processed using MOSFLM and SCALA of the CCP4 suite. The crystals belong to the space group P4₁2₁2₃ with one monomer in the asymmetric unit. The structures were solved by molecular replacement using Phaser and initial phases were determined by Patterson phasing. Data collection and refinement statistics for crystal structures are presented in Supplementary Tables 4 to 11.

**Assessment of anti-HIV activity.** An HCV genotype 1b bicistronic replicon was transfected in HuH7 cells grown in DME medium Glutamax II (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum, 50 μM penicillin, 100 μM NaCl, 20 to 50 μM of protein was loaded into the MicroCal VP-ITC (Malvern, Orsay, France) isothermal titration calorimeter cell (~2 mL, cell volume ~1.4 mL). The titration syringe (250 μL volume) was filled with 1 mM ligand solution in buffer A. Titration was carried out using 40 injections of 1 μL each, injected at 5 min intervals. Stirring speed was 400 rpm. Titration were carried out at a constant temperature of 25 °C. Data were fit to a single site binding model using Origin 5.0 software.

**Solubility and chemical stability.** Kinetic solubility was determined by diluting a DMSO stock of the compound into the test media solutions (PBS pH 7.4 and 0.1 N HCl pH 1.0) to a final concentration of 100 μM with a total DMSO concentration of 1% (v/v) at 37 °C. The solutions were incubated at room temperature with shaking for 24 h and were then centrifuged, and the recovered supernatants assayed by HPLC with diode array ultraviolet/visible detection. Solubility values were calculated by comparing the amount (by chromatographic peak area) of compound detected in the defined test solution with an unextracted standard. Relative chemical stabilities of compounds after 24 h incubation in PBS at 37 °C were also determined. The solubility and chemical stability of a quality-control compound (amarenvar) were determined in parallel.
Caco-2 cell permeability. Permeability and efflux potential were determined using confluent (≥ 21 day) monolayers of Caco-2 cells grown on transwell filters. Compounds were added to donor wells at a target concentration of 10 μM and rates of appearance in receiver wells (containing 1% BSA to maintain sink conditions) were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Quality controls included transspethelial electrical resistance, lucifer yellow exclusion, low (atenolol) and high (propranolol) permeability controls and the ability to demonstrate polarized efflux of digoxin.

Protein binding. The extent of binding to pooled human plasma and to cell culture medium containing 10% fetal bovine serum was determined by equilibrium dialysis against isotonic phosphate buffer. Equilibrium dialysis was conducted at 37°C with initial concentrations of compounds of 2 μM in the non-buffer matrix. A 3 h dialysis time was used for equilibration. Following dialysis, plasma samples were drained into pre-weighed polypropylene tubes containing buffer and buffer samples were drained into pre-weighed tubes containing blank plasma. Post-dialysis plasma and buffer weights were measured and recorded for concentration and recovery calculations. After precipitation and centrifugation, LC-MS/MS assays were used for the analysis of each of the protein/phosphate buffer-mixed matrices. Relative binding to human plasma and cell culture medium was determined in a similar manner, except that the two matrices were dialysed against each other directly and the compound was spiked into both matrices. After equilibration, the plasma sample was diluted with blank cell culture medium and the cell culture medium diluted with blank plasma.

Stability with hepatic microsomal fractions. The compounds (3 μM) were incubated in duplicate for up to 1 h in the presence of human hepatic microsomal fractions (0.5 mg protein ml−1 final) at 37°C. The addition of cofactor solution (NADPH generating system) initiated the reaction and aliquots were removed at 0, 5, 15, 30, 45 and 60 min after the start. The concentrations of the compounds in each sample were determined using specific LC-MS/MS assays. The half-life for the disappearance of each was determined by fitting the concentration-time data with a monophasic exponential model. A control compound (verapamil) was run in parallel to test the enzymatic integrity of the microsomal fractions.

Metabolism by recombinant human cytochrome P450. Five micromoles of compound was incubated with individual bacterially expressed recombinant human cytochrome P450 co-expressed with human UGTs in a NADPH cytochrome P450 reductase. The reduction in substrate concentration was monitored over 45 min using specific LC-MS/MS assays and the in vitro half-life calculated in the same manner as that described for hepatic microsomal stability. Enzyme-selective positive control substrates were tested in parallel.

Cytochrome P450 enzyme inhibition assays. Up to 25 μM of compounds 29 and 30 was incubated with human hepatic microsomal fractions and NAPDH in the presence of individual probe substrates. All assays were designed so that conditions were linear with respect to time and protein concentration. Substrates were present at concentrations equal to or lower than their respective Km values. Enzyme selective products were determined by fluorometry (ethoxyresorufin O-deethylase) or by specific LC-MS/MS assays. Relative enzyme activities were determined by comparison with those assayed with DMSO vehicle instead of inhibitor. IC50 values were calculated by nonlinear curve fitting using a sigmoidal model. Positive control inhibitors for each enzyme were tested in parallel.

Data availability. Data supporting the findings of this study is available within the article and its Supplementary Information files and from the corresponding authors upon reasonable request. PDB: the complex structures for compounds 5, 6, 9, 10 to 24, 26 to 29 with CypD K133M mutant enzyme are deposited under accession codes 3RC3, 3RS9, 3RFC, 3RG4, 3RS4, 3RD9, 3RJ9, 3RS6, 3RDB, 3RCK, 3RDA, 3R57, 3RDC, 4J58, 4J5F, 4JS3, 4JS5, 4JS9 and 4JSC, respectively. The complex structure for compound 22 with CypA is deposited under accession code 3RDD. Stereo views of co-crystal structures of the compounds with cyclophilin D are available at https://figshare.com/articles/Stereo___views_of_cocrystal_structures_of_cyclophilin_inhibitors_with_cyclophilin_D_/3490493.

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Author contributions
A.A.-B., L.C., J.-M.P. and J.-F.G. designed the study and the experiments, analysed and discussed the results, and wrote the article. N.A., Q.N., M.G., Y.B., R.B., O.C., D.D., W.B. and I.K. performed the experiments and analyses and approved the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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

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