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Original article

# Discovery of a potent peptidic cyclophilin A inhibitor Trp-Gly-Pro

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ABSTRACT

Through virtual screening of a rationally built database consisting of 40 peptides, we identified three short peptides. After testing these three synthetic peptides, we found that the peptide Trp-Gly-Pro (WGP) showed comparable inhibitory ability as positive control cyclosporine A (CsA) on CypA-mediated PPIase activity with $IC_{50}$ values of 33.11 nM and 10.25 nM, respectively. The peptide WGP had same order of CypA-binding affinity as CsA with dissociation equilibrium constant $K_D$ of $3.41 \times 10^{-6}$ and $6.42 \times 10^{-6}$ M, respectively. This peptide could also inhibit HIV-$1_{IIIB}$ infection. This study provides a novel strategy for rational design and development of peptidic drugs.



## 1. Introduction

Cyclophilin A (CypA) is an important drug target for cyclosporine A (CsA), which is an immunosuppressant drug widely used in post-allogeneic organ transplant for the prevention of graft rejection [1–3]. CypA can also interact with human immunodeficiency virus type 1 (HIV-1) gag protein and enhance viral infectivity. Therefore, CsA is able to inhibit HIV-1 replication *in vitro* [4] and may have a potential to be developed as an anti-HIV drug.

CsA is a cyclic polypeptide consisting of 11 *amino acids with* a molecular mass of 1202 Da. Because it exhibits very poor solubility in water, CsA has to be formulated in suspension and emulsion forms, such as Sandimmune (http://www.rxlist.com/sandimmune-drug.htm) for oral administration and for injection. Besides, CsA may have some serious side effects [5]. Therefore, it is essential to identify a short peptide with better oral availability, solubility and less toxicity to replace CsA in clinical application. Indeed, several groups have reported that like CsA, some peptides or pseudopeptides can effectively inhibit the PPIase activity of CypA. However, their $IC_{50}$ values are in tens to hundreds micromolar range, while that of the CsA is in low nanomolar level [3,6,7].

Our previous studies on the electronic structures of the complex CypA/CsA (PDB entry of 1CWA) demonstrated that there was an intrinsic relationship between the frontier orbitals and the biological activity of proteins [8]. We found that interactions occurred between the HOMO (Highest Occupied Molecular Orbital) of CsA and the LUMOs (Lowest Unoccupied Molecular Orbitals) of CypA, and residue Phe60 and Phe113 were critical for expression of the biological activity of CypA. Besides, residue Pro was found to have the lowest contact energy with residue Phe (−4.25 RT) [9,10]. In other words, residue Pro is the preferred residue to interact with residue Phe. The ligand binding pocket of CypA is mainly composed of hydrophobic or non-polar residues. Therefore, the active part of a peptide fitting into the binding pocket should also be hydrophobic or non-polar. Pro happens to be a hydrophobic and non-polar residue. Electronic structure calculations of peptides suggested that the HOMO of peptides always located on the C terminals. Hence,

*Abbreviations:* CypA, cyclophilin A; CsA, cyclosporine A; LBE, lowest binding energy; LUMOs, Lowest Unoccupied Molecular Orbitals; HOMO, Highest Occupied Molecular Orbital.

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a peptide with residue Pro as its C terminal may be a good inhibitor of CypA. Considering the shape and volume of the ligand binding site of CypA, peptides consisting of three or four residues may be preferable to fit into the binding site. Therefore, we hypothesized that some of the peptides with the sequences of X-Gly-Pro or X-Ala-Gly-Pro might be effective in inhibiting the biological activity of CypA.

In this study, we intended to design a focused peptide library, to screen the peptide library using a molecular docking program and to test the selected peptides for their inhibitory activity on CypA-mediated PPIase activity and HIV-1 replication. The aim of this study is to identify a peptide with smaller molecular weight than CsA, but has similar CypA-binding affinity as CsA. This peptide will be used as a lead compound for developing CypA inhibitors with less toxicity and better oral availability and solubility to replace CsA in clinical application.

## 2. Results and discussion

### 2.1. Identification of virtual hits by docking

Based on our previous findings on the electronic structures of the complex CypA/CsA (PDB entry of 1CWA), we designed a focused peptide library consisting of 20 X-Gly-Pros peptides and 20 X-Ala-Gly-Pros peptides (Table 1). Using a molecular docking program (Autodock 4.2) [11,12], we virtually screened this peptide database. We found that three peptides, Trp-Gly-Pro (WGP), Trp-Ala-Gly-Pro (WAGP) and Tyr-Gly-Pro (YGP) had lower LBE (the lowest binding energy) than the positive control peptide CsA (WGP = −9.50; WAGP = −9.23, YGP = −8.94, and CsA = −8.83 kcal/mol). Besides, these three peptides also had lower ligand efficiency than CsA (WGP = −0.37; WAGP = −0.30, YGP = −0.37, and CsA = −0.10) (Table 1), which allowed enough room for further lead optimization.

It was worth noting that in Autodock 4.2 calculations for these three peptides and CsA, the best ranked cluster of conformations coincided with the most populated cluster as shown in Supplementary Fig. 1 (for CsA the exceptional result of 90 conformations was obtained for the best ranked cluster, while 47, 45 and 21 for the peptide YGP, WGP and WAGP, respectively). The number of conformations in the best ranked cluster was much larger than that in the second ranked cluster. It implied that there was only a single binding model for each compound. The binding models of three peptides and CsA predicted by Autodock 4.2 were shown in Fig. 1. All the three selected peptides and the active residues of CsA fitted perfectly into the binding site of CypA respectively. These three peptides were also found to overlap well with the active part of CsA (Supplementary Fig. 2).

**Table 1**
The rank of the 40 peptides and positive control CsA by the lowest free energy of binding. Peptides WGP, WAGP and YGP have lower LBE (the lowest binding energy) than the positive control CsA.

| Peptides | Runs | NC[a] | NLC[b] | LBE | LE[c] | Cal. MW[d] |
|---|---|---|---|---|---|---|
| Trp-Gly-Pro (WGP) | 100 | 37 | 24 | −9.50 | −0.37 | 358 |
| Trp-Ala-Gly-Pro (WAGP) | 100 | 64 | 9 | −9.23 | −0.30 | 430 |
| Tyr-Gly-Pro (YGP) | 100 | 28 | 34 | −8.94 | −0.37 | 335 |
| CsA | 100 | 9 | 90 | −8.83 | −0.10 | 1,202 |
| Gln-Gly-Pro (QGP) | 100 | 44 | 27 | −8.80 | −0.42 | 300 |
| Phe-Gly-Pro (FGP) | 100 | 22 | 57 | −8.67 | −0.38 | 319 |
| Phe-Ala-Gly-Pro (FAGP) | 100 | 48 | 24 | −8.19 | −0.29 | 390 |
| Glu-Gly-Pro (EGP) | 100 | 46 | 12 | −8.06 | −.038 | 301 |
| Leu-Ala-Gly-Pro (LAGP) | 100 | 55 | 13 | −7.99 | −0.32 | 356 |
| Tyr-Ala-Gly-Pro (YAGP) | 100 | 56 | 12 | −7.88 | −0.27 | 406 |
| Asn-Ala-Gly-Pro (NAGP) | 100 | 66 | 9 | −7.83 | −0.31 | 357 |
| Arg-Ala-Gly-Pro (RAGP) | 100 | 89 | 7 | −7.79 | −0.28 | 399 |
| Gln-Ala-Gly-Pro (QAGP) | 100 | 75 | 5 | −7.78 | −0.30 | 371 |
| Leu-Gly-Pro (LGP) | 100 | 28 | 43 | −7.64 | −0.38 | 285 |
| Cys-Ala-Gly-Pro (CAGP) | 100 | 52 | 7 | −7.64 | −0.33 | 346 |
| His-Gly-Pro (HGP) | 100 | 36 | 31 | −7.61 | −0.35 | 309 |
| Lys-Gly-Pro (KGP) | 100 | 46 | 14 | −7.49 | −0.36 | 300 |
| Met-Gly-Pro (MGP) | 100 | 28 | 55 | −7.39 | −0.37 | 303 |
| Asn-Gly-Pro (NGP) | 100 | 49 | 11 | −7.38 | −0.37 | 286 |
| Pro-Ala-Gly-Pro (PAGP) | 100 | 26 | 32 | −7.34 | −0.31 | 340 |
| Gly-Gly-Pro (GGP) | 100 | 12 | 40 | −7.32 | −0.46 | 229 |
| Ile-Ala-Gly-Pro (IAGP) | 100 | 54 | 10 | −7.22 | 0.28 | 356 |
| Ile-Gly-Pro (IGP) | 100 | 29 | 48 | −7.19 | −0.36 | 285 |
| Met-Ala-Gly-Pro (MAGP) | 100 | 64 | 14 | −7.06 | 0.28 | 374 |
| Arg-Gly-Pro (RGP) | 100 | 66 | 10 | −7.00 | 0.30 | 328 |
| Val-Ala-Gly-Pro (VAGP) | 100 | 48 | 11 | −6.98 | −0.29 | 342 |
| His-Ala-Gly-Pro (HAGP) | 100 | 59 | 7 | −6.87 | −0.25 | 380 |
| Thr-Ala-Gly-Pro (TAGP) | 100 | 48 | 10 | −6.87 | −0.29 | 344 |
| Val-Gly-Pro (VGP) | 100 | 23 | 40 | −6.82 | −0.36 | 271 |
| Asp-Ala-Gly-Pro (DAGP) | 100 | 67 | 7 | −6.80 | −0.27 | 358 |
| Glu-Ala-Gly-Pro (EAGP) | 100 | 65 | 9 | −6.77 | −0.26 | 372 |
| Gly-Ala-Gly-Pro (GAGP) | 100 | 40 | 12 | −6.62 | −0.32 | 300 |
| Pro-Gly-Pro (PGP) | 100 | 10 | 51 | −6.50 | −0.34 | 269 |
| Ala-Gly-Pro (AGP) | 100 | 16 | 66 | −6.49 | −0.38 | 243 |
| Cys-Gly-Pro (CGP) | 100 | 22 | 41 | −6.49 | −0.36 | 275 |
| Asp-Gly-Pro (DGP) | 100 | 44 | 10 | −6.38 | −0.32 | 287 |
| Ala-Ala-Gly-Pro (AAGP) | 100 | 37 | 14 | −6.30 | −0.29 | 314 |
| Thr-Gly-Pro (TGP) | 100 | 28 | 29 | −6.11 | −0.32 | 273 |
| Ser-Ala-Gly-Pro (SAGP) | 100 | 34 | 14 | −5.95 | −0.26 | 330 |
| Ser-Gly-Pro (SGP) | 100 | 27 | 32 | −5.91 | −0.33 | 259 |
| Lys-Ala-Gly-Pro (KAGP) | 100 | 79 | 3 | −5.63 | 0.22 | 371 |

[a] Number of clusters.
[b] Number of conformations in the lowest energy cluster.
[c] Ligand efficiency.
[d] Molecular weight.

### 2.2. Binding model analysis

A detailed inspection of the complex system CypA/WGP showed that the C terminal (residue Pro) of peptide WGP fitted deeply inside the hydrophobic binding site of CypA (Fig. 2A) and six hydrogen bonds formed between them (Fig. 2B). As expected at the beginning of the peptide sequence design, the C terminal (residue Pro) fitted well into the hydrophobic binding pocket of the CypA (Fig. 2A). Fig. 2B showed that the peptide WGP was held tightly by these six hydrogen bonds (two on up side and four on down side). Details of the six hydrogen bonds were shown in Table 2. Both the two oxygen atoms of the residue Pro were involved in formation of hydrogen bonds as acceptors (Table 2). Moreover, the HOMO of the WGP was found to localize on the residue Pro through electronic structure calculations. These atoms localized by the HOMO of the WGP were considered as the active atoms of the WGP as shown in Supplementary Fig. 3A. The two oxygen atoms of the residue Pro involved in the hydrogen bonds interactions were found to have particularly large orbital coefficients. The active atoms covered right above the active residue Phe113 of the CypA, which verified that the interactions occurred between the HOMO of the WGP and the LUMOs of the CypA. As shown in Supplementary Fig. 3B, a typical edge-to-face (T-shaped) interactions geometry formed between aromatic rings of the residue Pro and the residue Phe113 of the CypA. The T-shaped orientation is the most favorable interactions with aromatic rings in chemical and biological recognition [13].

### 2.3. Biological assays of virtual hits

Subsequently, we had these three selected peptides synthesized and tested for their inhibitory activity on the PPIase activity of CypA using the method as previously described [14,15]. The inhibition results were shown in Table 3 and Supplementary Fig. 4. Of the three peptides tested, only the peptide WGP showed inhibition on the PPIase activity of CypA in a concentration-dependent manner and had the lowest $IC_{50}$ value (33.11 nM), which was close to the $IC_{50}$ level of the positive control peptide CsA (10.25 nM).

The surface plasmon resonance analysis (see Supporting Information for more experimental details) confirmed that the

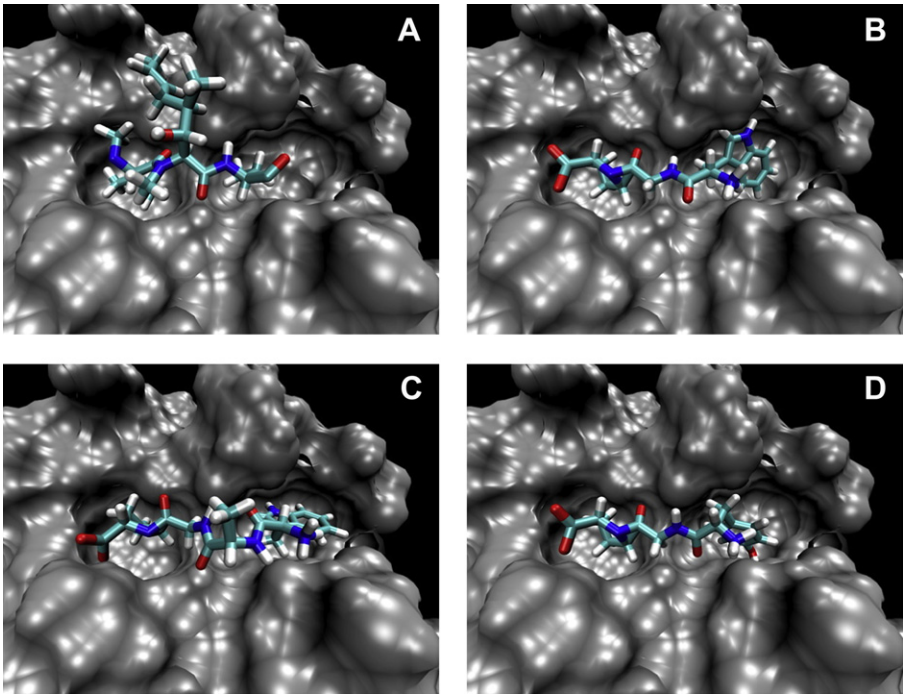


**Fig. 1.** Predicted binding models for CsA (A) and hits WGP (B), WAGP (C) and YGP (D). For CsA, only three active residues (Mva11, Bmt1 and Aba2) that fitting into the ligand binding site were shown here. For the three peptides, their C terminals (residue Pro) were fitting well into the binding pocket (on the left hand side) of CypA, while their N terminals fitting well into the other pocket on the right hand side.

peptide WGP could directly interact with CypA, with a $K_D$ value of $3.41 \times 10^{-6}$ M, in a similar range of interaction between CsA and CypA ($K_D = 6.42 \times 10^{-6}$ M) (Supplementary Fig. 5). In contrast, the peptides YGP and WAGP had very weak interactions with CypA (data not shown).

Vajdos et al. crystallized the residues 87–92 of HIV-1 capsid (87HAGPIA92, a hexapeptides) in complex with CypA, and compared with structures of CypA in complexes with larger fragments of HIV-1 capsid [16]. They found that CypA recognition of the hexapeptides involved contacts with peptide residues Ala88, Gly89 and Pro90, and was independent of the context of longer sequences. Moreover, the directed mutagenesis further identified capsid residues Gly89 and Pro90 as critical for CypA binding [17,18]. Here we intended to test the potential inhibitory activity of the peptide WGP against HIV-$1_{IIIB}$ replication with a previously described flow cytometric method [14]. As shown in Fig. 3, WGP (1 mM) exhibited 75.5% inhibition of HIV-$1_{IIIB}$ infection, while CsA (10 μM) showed 100% inhibition of the HIV-1 infection. The low anti-HIV-1 activity of the short peptide WGP in the *in vitro* testing system may be attributed to its instability in the cell culture which contains a variety of proteolytic enzymes. Appropriate chemical modification, such as the substitution of the natural amino acids with unnatural amino acids or D-amino acids [19,20], to increase the peptide stability may greatly improve its anti-HIV-1 efficiency.

## 3. Conclusions

We have rationally built a focused peptide library consisting of 40 peptides for screening hits that bind to the protein CypA. Three peptides were suggested by molecular docking calculations with their LBE lower than that of the positive control peptide CsA. These three peptides were synthesized and tested for their inhibitory activity on the PPIase activity of CypA and HIV-1 infectivity. We found that only WGP exhibited comparable inhibitory activity on

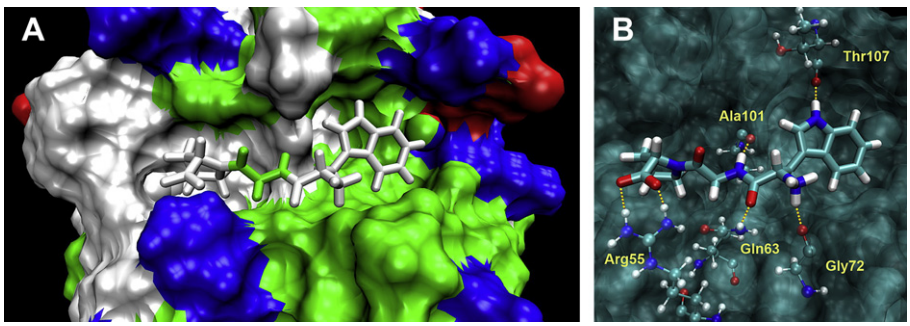


**Fig. 2.** Binding model of CypA/WGP. (A) Peptide WGP fitted well into the binding site of CypA. Residues are colored based on their hydrophobicity and acidity: hydrophobic (white), hydrophilic (green) and charged (blue and red). The C terminal (left hand side) of the WGP is the hydrophobic residue proline, which is embraced by the hydrophobic pocket of the CypA. (B) Six hydrogen bonds formed between WGP and CypA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Details of the six hydrogen bonds formed between WGP and CypA.

| Donor | Acceptor | DA[a] distance (Å) | DHA[b] angle (degree) |
|---|---|---|---|
| WGP:Trp2:HN2 | CypA:Gly72:O | 1.7 | 167.6 |
| WGP:Trp2:HE1 | CypA:Thr107:O | 1.7 | 168.3 |
| WGP:Gly3:HN | CypA:Ala101:O | 1.9 | 156.8 |
| CypA:Arg55:HH11 | WGP:Pro4:OT2 | 1.8 | 157.6 |
| CypA:Arg55:HE21 | WGP:Pro4:OT1 | 1.8 | 171.6 |
| CypA:Gln63:HH21 | WGP:Trp2:O | 2.2 | 147.8 |

[a] Donor–acceptor.
[b] Donor-hydrogen-acceptor.

**Table 3**
Inhibition of PPIase activity of CypA and the equilibrium constants of the most promising peptides identified by virtual screening and the positive control CsA determined by enzymatic activity assay and surface plasmon resonance analysis, respectively.

| Peptide | $IC_{50}$ for inhibiting PPIase activity (nM) | Binding to CypA, $K_D$ (M) |
|---|---|---|
| WGP | 33.11 | $3.41 \times 10^{-6}$ |
| WAGP | Inactive | Inactive |
| YGP | Inactive | Inactive |
| CsA | 10.25 | $6.42 \times 10^{-6}$ |

the CypA-mediated PPIase activity and similar CypA-binding affinity as CsA. Like CsA, the peptide WGP could also inhibit HIV-$1_{IIIB}$ infection, although the potency of the former is much higher than the latter. Considering its low cytotoxicity (Supplementary Fig. 6) and low molecular size, its analogous peptides consisting of unnatural amino acids or D-amino acids may be used as leads for developing orally available CypA inhibitors to replace CsA for clinical application.

## 4. Experimental section

### 4.1. Construct the 3D structures of peptides

We used the program Arguslab 4.0 [21] to build the 3D structures for all 40 peptides. In the polypeptide builder of Arguslab, we chose beta strand with $\Psi$ and $\Phi$ angles of $-120.00^\circ$ and $120.00^\circ$, respectively (Actually, any angle would be no problem since the docking program allows full ligand flexibility). We built the 3D structures of 20 X-Gly-Pros and 20 X-Ala-Gly-Pros in turn. We added hydrogen to each peptide and saved the 3D structures in PDB file format.

### 4.2. Molecular docking

Docking is an approach to rational drug design that seeks to "predict the structure and binding free energy of ligand–receptor complex given only the structure of free ligand and receptor" [11,22]. Autodock is one of the most popular docking programs and is free of charge. It takes into account full ligand flexibility and has a well-tested scoring function. For simplicity, here we only allowed the full flexibility of the ligand, as opposed to the flexibility of the protein, during the docking experiments. We employed the latest version of Autodock (Autodock 4.2) for screening. The optimized parameters concerned the grid spacing (0.375 Å, with a grid of 65 Å × 56 Å × 60 Å), the maximum number of energy evaluation (2,500,000) and the number of hybrid Larmarckian genetic algorithm local search runs (100). The number of energy evaluation was big enough to test whether the complex system converged or not. At the end of docking, a cluster analysis was performed on the results of docking conformations.

### 4.3. Proteins and compounds

The full-length coding sequence of *CypA* was inserted in frame into plasmid pET28a (Clontech) to generate recombinant human

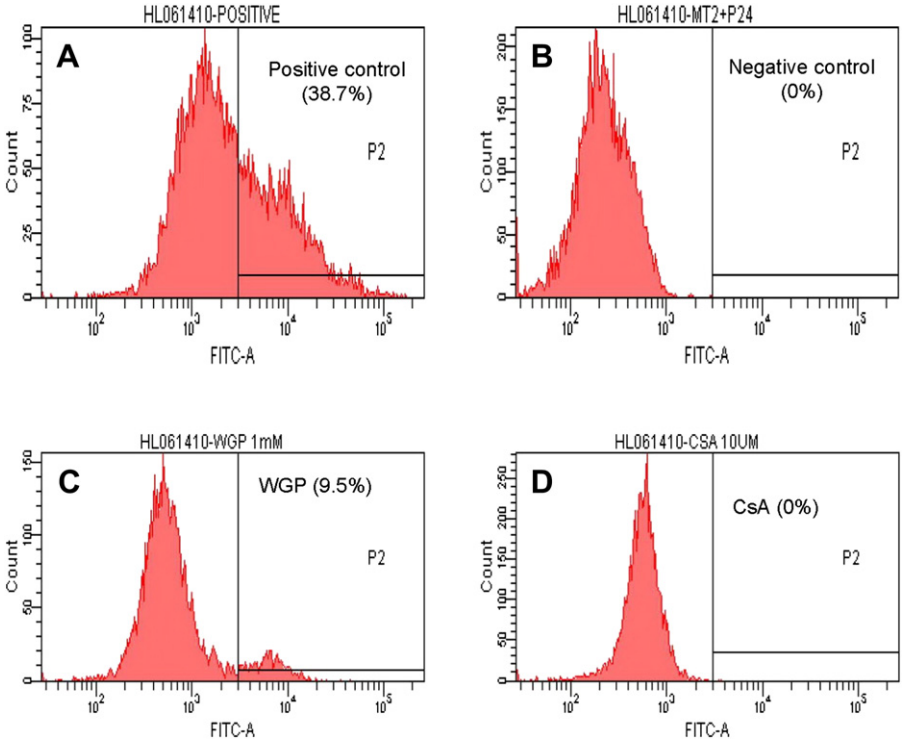


**Fig. 3.** The inhibitory activity of the peptide WGP on HIV-$1_{IIIB}$ replication as detected by flow cytometry. The ratio of p24-positive cells recognized by FITC-conjugated anti-p24 antibody KC57 represents HIV-$1_{IIIB}$ infectivity in MT-2 cells. When no inhibitor was added, the ratio of $p24^+$ cells was 38.7%, while addition of 1 mM of WGP and 10 μM of CsA resulted in reduction of the ratio to 9.5% and 0% (*i.e.*, 75.7% and 100% inhibition of HIV-1 infection), respectively.

CypA protein. His-CypA protein was expressed and purified (Supplementary Fig. 7). CsA, the substrate N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanlilide (Suc-AAPF-pNA), α-chymotrypsin, and 2,2,2-trifluorethanal (TFE) were purchased from Sigma (St. Louis, MO).

The suggested peptides WGP, YGP and WAGP were synthesized by Shanghai Science Peptide Biological Technology Co., LTD (Shanghai, China) using the Fmoc (9-fluorenylmethoxycarbonyl) strategy. The peptides were cleaved from the Fmoc-Pro-resin by a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water at a ratio of 94:3:3 at room temperature. All the peptides were purified by reverse phase HPLC using a C-18 column to >98% homogeneity. The molecular weight of the peptides was confirmed by using a Shimadzu LCMS-2010 System (Shimadzu, Tokyo, Japan).

### 4.4. Assay for inhibition of PPIase activity of CypA

The inhibitory activity of the peptides on CypA-mediated PPIase activity was determined as previously described [14]. In brief, Suc-AAPF-pNA and α-chymotrypsin were dissolved in TFE containing 480 mM of LiCl and 1 mM HCl, respectively. Each test peptide was diluted in 94 μl of assay buffer (50 mM HEPES, 100 mM NaCl; pH 8.0 at 0 °C), and then mixed with 2 μl of CypA solution (5 μM). After pre-equilibrating for 3 h on ice, 2 μl of α-chymotrypsin solution (6 mg/ml) and 2 μl of Suc-AAPF-pNA substrate (3 mM) were added to the assay mixture. Absorbance at the wavelength of 390 nm was recorded on a Jasco V-550 spectrophotometer (Jasco, Inc., Easton, MD) for 20 s. The rate constants for the *cis–trans* conversion were evaluated by fitting the data to the integrated first-order rate equation through nonlinear least-square analysis as previously described [14]. Three independent experiments were performed for each test compound and the respective half-maximal inhibitory concentration ($IC_{50}$) was calculated with OriginPro 8.0 software (OriginLab, http://www.originlab.com/).

### 4.5. Inhibition of WGP on HIV-1 replication

The inhibitory activity of the peptide WGP on HIV-$1_{IIIB}$ replication was determined using the flow cytometry method as previously described [4,14]. Briefly, MT-2 cells ($5 \times 10^5$/well) were infected by HIV-$1_{IIIB}$ at 0.01 MOI (multiplicity of infection) in 24-wells cell culture plates at 37 °C for 1 h, followed by washing with PBS and addition of the peptides WGP and CsA at indicated concentration. After culture at 37 °C for 48 h, cells were fixed with 1% paraformadehyde-PBS and assayed for intracellular p24 by using a FITC-conjugated anti-p24 antibody (KC57, Beckman Coulter, Inc.) and flow cytometry (Dickinson FACSCalibur, Becton, Dickinson, and Company, San Jose, CA). The percent inhibition of HIV-1 replication was calculated as previously described [14].

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## Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2011.02.023.

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