A Helical Short-Peptide Fusion Inhibitor with Highly Potent Activities against HIV-1, HIV-2 and Simian Immunodeficiency Virus

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Running Title: A Short-Peptide Fusion Inhibitor on HIV-1/2 and SIV

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ABSTRACT

HIV-2 has already spread to different regions worldwide and currently about 1-2 million people have been infected, calling for new antiviral agents that are effective on both HIV-1 and HIV-2 isolates. T-20 (Enfuvirtide), a 36-mer peptide derived from the C-terminal heptad repeat region (CHR) of gp41, is the only clinically approved HIV-1 fusion inhibitor, but it easily induces drug-resistance and is not active on HIV-2. In this study, we firstly demonstrated that the M-T hook structure was also a vital strategy to enhance the binding stability and inhibitory activity of diverse CHR-based peptide inhibitors. Then, we designed a novel short-peptide (23-mer) termed 2P23 by introducing the M-T hook structure, HIV-2 sequences and 'salt-bridges'-forming residues. Promisingly, 2P23 was a highly stable helical peptide with high binding to the surrogate targets derived from HIV-1, HIV-2 and simian immunodeficiency virus (SIV). In consistence, 2P23 exhibited potent activity in inhibiting diverse subtypes of HIV-1 isolates, T-20-resistant HIV-1 mutants, and a panel of primary HIV-2 isolates, HIV-2 mutants and SIV isolates. Therefore, we conclude that 2P23 has high potential to be further developed for clinical use and it is also an ideal tool for exploring the mechanisms of HIV-1/2 and SIV-mediated membrane fusion.

IMPORTANCE

The peptide drug T-20 is the only approved HIV-1 fusion inhibitor, but it is not active on HIV-2 isolates which have currently infected 1-2 million people and continuingly spread worldwide. Recent studies have demonstrated that the M-T hook structure can greatly enhance the binding and antiviral activities of gp41 CHR-derived inhibitors, especially for short-peptides otherwise inactive. By combining the hook structure, HIV-2 sequence and 'salt-bridge'-based strategies, the short-peptide 2P23 has been successfully designed. 2P23 exhibits prominent advantages over many other peptide fusion inhibitors, including its potent and broad activity on HIV-1, HIV-2 and even SIV isolates, its stability as a helical, oligomeric peptide, and its high binding to diverse targets. The small size of 2P23 would benefit its synthesis and significantly reduce production cost. Therefore, 2P23 is an ideal candidate for further development and it also provides a novel tool for studying HIV-1/2 and SIV-mediated cell fusion.

Key words: HIV-1; HIV-2; fusion inhibitor; short-peptide

INTRODUCTION

Currently, there are approximately 34 million people worldwide living with HIV (UNAIDS, 2015). Although HIV-1 is a major causative agent of the global AIDS pandemic, about 1-2 million people have been infected with HIV-2，mostly in West Africa. In the past decades, HIV-2 has also spread to different countries in Europe, Asia and North America, resulting in the relatively high prevalence of HIV-2 infection. For example, surveillance studies in Portugal and France showed that around 2% of the new infections during 2003-2006 were caused by HIV-2 (1, 2), raising additional concerns over the control of AIDS. Therefore, preventive vaccines and therapeutic drugs that are also effective on HIV-2 should be highly appreciated.

HIV-2 was first isolated from AIDS patients in West Africa and its genome organization was determined from an isolate designated as ROD (3, 4). At present, HIV-2 strains are classified in nine groups termed A-I of which group A is by far the most disseminated worldwide (ROD is a prototypic HIV-2 group A strain) (5-7). Previous studies demonstrated that HIV-1 and HIV-2 have different evolutionary histories and share only 50% of genetic similarity (8, 9). Unfortunately, all currently available antiretroviral drugs were specifically developed to inhibit HIV-1 entry and replication, and consequently some drugs in clinical use have limited or no activity on HIV-2 including all non-nucleoside reverse transcriptase inhibitors, some protease inhibitors and the fusion inhibitor T-20 (enfuvirtide, Fuzeon) (10-13). T-20, a 36-mer linear peptide derived from the native gp41 C-terminal heptad repeat (CHR) sequence of the HIV-1 LAI isolate, was approved as the first and so far the only HIV-1 fusion

inhibitor for clinical use (14-16). Mechanically, T-20 inhibits HIV-1 entry by competitive binding to the complementary N-terminal heptad repeat (NHR) of gp41, thereby blocking the formation of the six-helical bundle structure (6-HB) that is essential for fusion of the viral and cellular membranes (17-20). Despite its strong anti-HIV-1 activity, T-20 easily induces drug-resistance through mutations within its NHR-binding sites (21, 22). Also, T-20 has poor bioavailability thus requiring large-dose injections (90 mg, twice daily), which complicates patient adherence to treatment. Furthermore, we and others demonstrated that T-20 displayed dramatically decreased activity in inhibiting HIV-2 isolates (13, 23, 24). Considerable efforts have been made to develop new fusion inhibitors with improved pharmaceutical profiles (25-28). T-1249 is a representative second-generation peptide fusion inhibitor, which has 39 amino acids derived from the consensus CHR sequences of HIV-1, HIV-2 and simian immunodeficiency virus (SIV); however, its clinical development was hampered beyond phase I/II trials due to the drug formulation difficulties associated with its long size and elevated production costs (29, 30). A number of new inhibitors were designed by using the CHR peptide C34 as a template and the resulting peptides did show increased anti-HIV-1 activity; however, in most cases they had longer sequences (>34-mer) and still limited activity against HIV-2 isolates (25, 26, 31-33). Finally, some peptides were designed using HIV-2 and/or SIV C34 as templates, generating inhibitors with somewhat improved anti-HIV-2 activity, such as $C34_{EHO}$ and P3 (23, 33).

We recently found that two residues (Met115 and Thr116) preceding the

pocket-binding domain (PBD) of CHR peptides adopt a unique M-T hook structure that can greatly enhance the binding and antiviral activities (34-38). Our crystal structures demonstrated that the residue Thr116 can redirect the peptide chain to position Met115 above the left side of the deep pocket on the trimeric coiled coil of N-terminal helices (NHR) so that its side chain caps the pocket to stabilize the inhibitor-binding (36-38). On the basis of the M-T hook structure, we generated short-peptide fusion inhibitors that mainly targeted the conserved pocket site of gp41 (36, 39, 40). For example, MTSC22 and HP23 showed dramatically improved inhibition on diverse HIV-1 isolates and high genetic barriers to the development of resistance (36, 40). In this study, we applied the M-T hook strategy to design fusion inhibitors that are also effective on HIV-2 isolates. A 23-mer helical peptide termed 2P23 was generated, which showed very potent inhibitory activities against distinct isolates of HIV-1, HIV-2 and SIV.

MATERIALS AND METHODS

Cells and reagents

HEK293T cells were purchased from American type culture collection (ATCC) (Rockville, MD). TZM-bl indicator cells stably expressing large amounts of CD4 and CCR5 along with endogenously expressed CXCR4, and plasmids for HIV-1 Env panels (subtypes A, B, B', C, G, A/C, A/E and B/C), and molecular clones of HIV reference strains (HIV-1_{NL4-3} and HIV-2_{ROD}) were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH. Two plasmids encoding SIV Env

125 (pSIVpbj-Env, pSIV239) were kindly provided by Dr. Jianqing Xu at the Shanghai
126 Public Health Clinical Center & Institutes of Biomedical Sciences, Fudan University,
127 China. Cells were cultured in complete growth medium that consists of Dulbecco's
128 minimal essential medium (DMEM) supplemented with 10% of Fetal Bovine Serum,
129 100 U/ml of Penicillin-Streptomycin, 2 mM of L-Glutamine, 1mM Sodium Pyruvate
130 and 1x of MEM Non-Essential Amino Acids (Gibco/Invitrogen, USA), and were
131 maintained at 37^{o}C in 5% of CO_2.

132 **Peptide synthesis**

133 A total of 29 CHR or NHR-derived peptides were synthesized using a standard
134 solid-phase 9-flurorenylmethoxycarbonyl (FMOC) method as described previously
135 (38). All peptides were acetylated at the N-terminus and amidated at the C-terminus.
136 Peptide concentrations were determined using ultraviolet (UV) absorbance and a
137 theoretically calculated molar-extinction coefficient ε (280 nm) of 5500 and 1490
138 mol/l per cm, based on the number of tryptophan and tyrosine residues, respectively
139 (41).

140 **Single-cycle infection assay**

141 A single-cycle infection assay was performed as described previously (42). Briefly,
142 HIV-1 or SIV pseudoviruses were generated via cotransfection of HEK293T cells
143 with an Env-expressing plasmid and a backbone plasmid pSG3Δenv that encodes
144 Env-defective, luciferase-expressing HIV-1 genome. Culture supernatants were
145 harvested 48 h after transfection, and 50% tissue culture infectious doses ($TCID_{50}$)
146 were determined in TZM-bl cells. To measure the antiviral activity of inhibitors,

147 peptides were prepared in 3-fold dilutions and mixed with 100 $TCID_{50}$ of viruses and
148 then incubated for 1 h at room temperature. The mixture was added to TZM-bl cells
149 (10^4/well) and incubated for 48 h at 37 °C. Luciferase activity was measured using
150 luciferase assay regents and a Luminescence Counter (Promega, Madison, WI). The
151 percent inhibition of viral entry by the peptides and 50% inhibitory concentration
152 (IC_{50}) values were calculated using GraphPad Prism software (GraphPad Software
153 Inc., San Diego, CA).

154 **Cell-cell fusion assay**

155 A dual split protein (DSP)-base assay was performed to determine HIV or SIV
156 Env-mediated cell-cell fusion as described previously (43, 44). Briefly, a total of 1.5 x
157 10^4 293T cells (effector cells) were seeded on a 96-well plate and a total of 8 x 10^4
158 U87-CXCR4 cells (target cells) were seeded on a 24-well plate. On the following day,
159 effector cells were transfected with a mixture of an Env-expressing plasmid and a
160 DSP_{1-7} plasmid, and target cells were transfected with a DSP_{8-11} plasmid. Forty-eight
161 hours posttransfection, the target cells were re-suspended in 300µl pre-warmed culture
162 medium, and each well was added 0.05 µl EnduRen Live Cell Substrate (Promega).
163 Then, 75 µl of the target cell suspension were transferred over each well of the
164 effector cells in the presence or absence of serially 3-fold diluted peptide fusion
165 inhibitors. The cells were then spun down to maximize cell-cell contact and incubated
166 for 1 h at 37 °C. Luciferase activity was measured by Luminescence Counter
167 (Promega).

168 **Inhibition of infectious HIV-1_{NL4-3} and HIV-2_{ROD} isolates**

169 The anti-HIV activity of peptide inhibitors was initially assessed by using molecular
170 clones of wild-type HIV-1_{NL4-3} and HIV-2_{ROD} as two indicator viruses. Briefly, viral
171 stocks were prepared by transfecting a plasmid (pNL4-3 or pROD) into HEK293T
172 cells. Culture supernatants were harvested 48 h post-transfection and quantified for
173 $TCID_{50}$ in TZM-bl cells. 100 $TCID_{50}$ viruses were used to infect TZM-bl cells in the
174 presence or absence of serially 3-fold diluted peptides. Cells were harvested 2 days
175 postinfection and lysed in reporter lysis buffer, and luciferase activity was measured
176 as described above.

177 **Inhibition of HIV-2 primary isolates**

178 A total of 9 HIV-2 primary isolates were obtained from Portuguese patients by
179 cocultivation with PBMCs from seronegative subjects (13, 23). The antiviral activity
180 of fusion inhibitor peptides was evaluated in TZM-bl cells. Firstly, 10,000 TZM-bl
181 cells were seeded in 96-well tissue culture plates and incubated overnight. Next day,
182 the growth medium was removed and replaced by 200 ul of fresh growth medium
183 supplemented with 19.7 µg/ml of DEAE-dextran. Cells were infected with 200
184 $TCID_{50}$ of each virus in the presence of three-fold dilutions of peptides. After 48 h of
185 infection, luciferase expression was quantified with the Pierce Firefly Luciferase
186 Glow Assay Kit (Thermo Fisher, USA) according to the manufacturer's instructions.
187 The cytotoxicity of the compounds was evaluated using control wells in the absence
188 of the virus. At least two independent experiments were performed for each analysis
189 and each assay was set up in duplicate wells. The 50% (IC_{50}) and 90% (IC_{90})
190 inhibitory concentrations as well as the dose-response curve slopes (Hill slope) were

191 estimated by plotting the percent inhibition of infection (y axis) against the $\log_{10}$
192 concentration of each fusion inhibitor (x axis) and using the sigmoidal dose-response
193 (variable slope) equation in GraphPad Prism software.

194 **Inhibition of HIV-2_{ROD} mutants**

195 A panel of HIV-2_{ROD} mutants carrying mutations in amino acid positions of the
196 envelope V3 loop that determine for CCR5 and/or CXCR4 usage was used for
197 evaluating the inhibitory activity of the peptides as described above. These mutants
198 were generated in the pROD10 plasmid using the QuikChange II XL Site-Directed
199 Mutagenesis Kit (Stratagene) as described (45). Mutant viruses were obtained by
200 transient transfection of HEK293T cells using the jetPrime transfection reagent
201 (Polyplus) according to manufacturer's instructions. Transfections were performed
202 with 10 µg of DNA in a 100 mm tissue culture dish. Cell culture supernatants were
203 collected 48 h post-transfection, filtered and stored at -80°C until use.

204 **Circular dichroism (CD) spectroscopy**

205 CD spectroscopy was performed according to our protocols described previously (38).
206 Briefly, a CHR peptide was incubated with an equal molar concentration of the NHR
207 peptide N36 at 37℃ for 30 min in PBS (pH 7.2). CD spectra were acquired on a
208 Jasco spectropolarimeter (model J-815) using a 1 nm bandwidth with a 1 nm step
209 resolution from 195 to 270 nm at room temperature. Spectra were corrected by
210 subtraction of a solvent blank. The α-helical content was calculated from the CD
211 signal by dividing the mean residue ellipticity [θ] at 222 nm by the value expected for
212 100% helix formation (-33,000 degree.cm^2.$dmol^{-1}$). Thermal denaturation was

213 performed by monitoring the ellipticity change at 222 nm from 20℃ to 98℃ at a
214 rate of 2 ℃/min, and T_m (melting temperature) is defined as the midpoint of the
215 thermal unfolding transition.
216
217 **RESULTS**
218 **The M-T hook structure can greatly improve the inhibitory activity of diverse**
219 **inhibitors on HIV-2**
220 To develop a fusion inhibitor that is effective for both HIV-1 and HIV-2, we
221 synthesized and characterized a large panel of CHR peptides (Table 1), including 11
222 previously reported peptides as control and 15 newly-designed M-T hook-modified
223 peptides as new inhibitors. First, we verified that most of the HIV-1 sequence-derived
224 peptides had markedly decreased activities in inhibiting HIV-2 infection, such as C34,
225 SFT and SC29 they inhibited NL4-3 replication with IC_{50} values of 1.02, 1.1 and 1.13
226 nM, respectively but they inhibited ROD with IC_{50} values of 387.93, 105.36 and
227 237.87 nM, respectively. Even specifically designed HIV-2 sequence-based peptides
228 such as $C34_{EHO}$ and P3 had much weaker anti-HIV-2 activities than anti-HIV-1
229 activities. Second, we showed that addition of the M-T hook residues to the
230 N-terminus of peptides could dramatically increase their inhibitory potency on both
231 HIV-1 and HIV-2. For example, the M-T hook-modified MTC34, MTSFT and
232 MTSC29 inhibited NL4-3 with IC_{50} of 0.5, 0.51 and 0.43 nM, while they inhibited
233 ROD with IC_{50} at 76.61, 33.93 and 22.9 nM, respectively. Therefore, these results
234 have demonstrated that the M-T hook structure is a vital strategy for optimizing an

inhibitor against both HIV-1 and HIV-2 isolates.

The M-T hook structure can greatly enhance the binding stability on HIV-2

We previously demonstrated that the M-T hook structure can dramatically enhance the binding affinity of inhibitors to the target by using HIV-1 NHR-derived peptide N36 as a target surrogate (36, 37). To get insights into the mechanism of action, we characterized the interaction between inhibitors and HIV-2 by performing CD spectroscopy. To this end, we synthesized the HIV-2 NHR-derived peptide $N36_{ROD}$ as a target and then compared seven pairs of peptides (C34/MTC34, SFT/MTSFT, SC29/MTSC29, SC22/MTSC22, $C34_{ROD}$/$MTC34_{ROD}$, $C34_{EHO}$/$MTC34_{EHO}$, P3/MTP3) for their binding stability. Interestingly, all of the M-T hook-modified peptides displayed significantly increased α-helicity as compared to their templates (Table 2 and Fig. 1). Thermal denaturation analyses showed that addition of the M-T hook structure markedly increased the T_m values of the 6-HB complexes formed between inhibitors and each of HIV-1 and HIV-2 N36 peptides (Table 2 and Fig. 2).

Design of a novel short-peptide inhibitor effective on both HIV-1 and HIV-2

Recently, we demonstrated that a short-peptide fusion inhibitor with potent anti-HIV-1 activity could be developed on the basis of M-T hook structure (36, 39, 40). HP23 and its mutant HP23L have only 23 amino acids but they possess highly potent activity in inhibiting diverse subtypes of HIV-1 isolates and T20-resistant variants. However, our results shown here indicated that both HP23 and HP23L had dramatically reduced activities on HIV-2 ROD, with IC_{50} of 0.19 v.s. 78.57 nM and 0.39 v.s. 126.33 nM, respectively (Table 1). We therefore decided to develop a short-peptide fusion

257 inhibitor that is effective on both HIV-1 and HIV-2 isolates by using the M-T hook
258 strategy and HIV-2 sequence. Disappointedly, three HIV-2-derived short-peptides
259 with the M-T hook residues ($P21_{ROD}$, $P21_{EHO}$, $P21_{P3}$) exhibited poor inhibition on
260 HIV-1 and no inhibition on HIV-2 (Table 1). Encouragingly, a 23-mer peptide named
261 2P23 was successfully designed by introducing the critical residues for HIV-2 binding,
262 the salt-bridges for peptide stability and an N-terminal capping residue. Firstly, 2P23
263 had dramatically improved binding activities on both HIV-1 and HIV-2. As shown in
264 Table 2 and Fig. 3A-D, 2P23 bound HIV-1 N36 and HIV-2 N36 with T_m values of
265 78.79 and 55.26 ℃, respectively. Secondly, 2P23 had largely increased inhibitory
266 activities. As shown in Table 1 and Fig. 3E-F, it inhibited HIV-1 and HIV-2 with IC_{50}
267 values of 0.22 and 10.57 nM, respectively, which were much better than that of HP23.
268 Taken together, these results suggested that 2P23 has promising features as a novel
269 fusion inhibitor peptide.

270 **2P23 efficiently inhibits SIV isolates**

271 We sought to determine whether 2P23 was active against SIV isolates, which are
272 believed to have crossed the species barrier into humans resulting in HIV-2 and HIV-1.
273 First, we synthesized the SIV NHR-derived peptide $N36_{SIV251}$ as a target and
274 determined its interactions with HP23 and 2P23. As shown in Fig. 4A-B, 2P23 could
275 interact with $N36_{SIV251}$ much better than HP23, with the T_m value of 47.35 v.s.
276 34.41℃. Then, we generated two SIV Env pseudotyped viruses, SIV_{pbj} and SIV_{239}
277 and used them in single-cycle infection assay to evaluate the inhibitory activity of
278 2P23 and three control peptides (T20, P3, HP23). As shown in Fig. 4C-D, 2P23

279 efficiently inhibited SIV_{pbj} and SIV_{239} with IC_{50} of 9.96 and 3.34 nM, respectively; in
280 a sharp contrast, T20, P3 and HP23 had dramatically decreased activities in inhibiting
281 both SIV isolates. Namely, T20, P3 and HP23 inhibited SIV_{pbj} with IC_{50} of 190.8,
282 121.8 and 247.7 nM, respectively, and inhibited SIV_{239} with IC_{50} of 297.67, 17.5,
283 105.65 nM, respectively.

284 **2P23 efficiently inhibits HIV- and SIV -mediated cell-cell fusion**

285 We next determined the inhibitory activity of 2P23 and three control peptides (T20,
286 HP23, P3) on viral Env-mediated cell-cell fusion by a DSP-based assay. In line with
287 its inhibition on viral infection, 2P23 exhibited the most potent activity. As shown in
288 Fig. 5A, 2P23 inhibited HIV-1_{NL4-3} Env-mediated cell fusion with a mean IC_{50} of 0.24
289 nM, whereas T20, P3 and HP23 had their mean IC_{50} values of 7.89, 2.25 and 0.33 nM,
290 respectively. Similarly, 2P23 inhibited SIV Env-mediated cell fusion efficiently, with
291 an IC_{50} of 1.8 nM on SIV_{pbj} (Fig. 5B) and an IC_{50} of 2.39 nM on SIV_{239} (Fig. 5C). In
292 sharp contrasts, three control peptides had markedly decreased inhibitory activity on
293 SIV Env. Namely, T20, P3 and HP23 inhibited SIV_{pbj} at IC_{50} of 8.35, 3.94 and 7.8 nM,
294 respectively and inhibited SIV_{239} with IC_{50} of 217.33, 6.55 and 17.68 nM,
295 respectively.

296 **2P23 is a potent inhibitor of primary HIV-1 isolates and T-20 resistant mutants**

297 As a potential inhibitor for further development, we were intrigued to know whether
298 2P23 was active like HP23 on distinct subtypes of HIV-1 isolates and those fusion
299 inhibitor-resistant mutants. Therefore, we assembled a panel of 29 HIV-1 Envs (Table
300 3), including 3 subtype A, 6 subtype B, 3 subtype B', 6 subtype C, 1 subtype G, 1

subtype A/C, 4 subtype A/E, and 5 subtype B/C. Among them, 12 Envs were recently described as a 'global panel' reference that represents the genetic and antigenic diversities of HIV-1 (46). All the corresponding pseudoviruses were generated, quantified and used in single-cycle infection assays. As shown in Table 3, 2P23 potently inhibited diverse subtypes of HIV-1 isolates with a mean IC_{50} of 5.57 nM, which was comparable with that of HP23 (4.7 nM). As control, T20 and P3 inhibited HIV-1 isolates with mean IC_{50} of 31.49 and 24.35 nM, respectively.

We also constructed a panel of 15 HIV-1_{NL4-3}-based pseudoviruses with their Envs carrying T20 or HP23-resistant mutations (47, 48). The inhibition data in Table 4 showed that (1) the long peptides T20 and P3 exhibited relatively higher resistance on T20-resistant mutants but the short-peptides HP23 and 2P23 could maintain their potency; and (2) 2P23 also displayed improved inhibition over some HP23-resistant mutants (e.g., L57R and L57R/E136G). Taken together, these results indicated that 2P23 is a highly effective fusion inhibitor against diverse subtypes of primary HIV-1 isolates and T-20-resistant mutants.

2P23 is a potent fusion inhibitor of diverse primary HIV-2 isolates

One of main purposes of this study was to create a short-peptide fusion inhibitor that is active for both HIV-1 and HIV-2 isolates. Our above data demonstrated that 2P23 had potent activities against a large panel of HIV-1 isolates, one HIV-2 isolate (ROD) and two SIV isolates (SIV_{pbj}, SIV_{239}). In order to demonstrate whether 2P23 had a broad-spectrum anti-HIV-2 activity, we further measured its inhibition on a panel of primary HIV-2 isolates and a panel of ROD-based mutants which utilize different

coreceptors (13, 23, 45). Apart from P3 and HP23, the previously reported third-generation peptide inhibitors SFT and T2635 were also included as controls. As shown in Table 5, 2P23 was able to efficiently inhibit infection of distinct primary HIV-2 isolates and ROD mutants, with mean IC_{50} values at 20.17 and 15.38 nM, respectively. T2635 also exhibited similar inhibitory activity on two panels of viruses, showing mean IC_{50} values of 17.21 and 34.83 nM, respectively. In contrast, SFT, P3 and HP23 showed significantly decreased anti-HIV-2 activity, as they inhibited primary HIV-2 isolates with mean IC_{50} of 69.96, 64.76 and 62.39 nM, respectively, and inhibited ROD mutants with mean IC_{50} of 226.12, 191.09 and 94.69 nM, respectively. We conclude therefore that 2P23 is an ideal inhibitor of diverse HIV-2 isolates.

Structural properties of 2P23 in itself

To get more insights into the mechanism underlying the binding and antiviral activities of 2P23 peptide, we determined its own secondary structure and stability by CD spectroscopy. The peptide inhibitors HP23, T20, C34, SFT, T1249, T2635 and P3 were also analyzed for comparison. As shown in Fig. 6A-B, 2P23 alone exhibited high α-helicity at different peptide concentrations and its thermal unfolding transition (T_m) was dependent on the peptide concentration, which indicated its helical and oligomeric features similar to HP23 (Fig. 6C-D); however, both the helical contents and T_m values of 2P23 at each concentration were much higher than that of HP23. In a sharp contrast, T20, C34 and SFT had no or little α-helicity suggesting their random conformation, while T1249 and P3 displayed much less helical structures. Although

345 the helical content of the electronically-constrained peptide T2635 was comparable to
346 2P23, it had a significantly lower T_m value (40.3 v.s. 48.1 ℃), as demonstrated by the
347 data in Fig. 6E-F. These results suggest that 2P23 is a helical, oligomeric
348 short-peptide fusion inhibitor having high stability.
349
350 **DISCUSSION**
351 In the present study, we have dedicated our efforts to develop a short-peptide fusion
352 inhibitor that is effective on both HIV-1 and HIV-2 isolates. Firstly, we have verified
353 that the M-T hook structure strongly boosts the binding and inhibitory activities of
354 CHR-based peptides to the NHR target of HIV-2 isolates, as it does for HIV-1 isolates.
355 Then, we have successfully designed a 23-mer helical peptide termed 2P23 by adding
356 the M-T hook structure and HIV-2 sequences, that can enhance the inhibitor binding
357 to its target, and introducing the salt-bridges that can stabilize the helical structure of
358 the peptide *per se*. Promisingly, 2P23 does show a very potent and broad-spectrum
359 antiviral activity that includes HIV-1, HIV-2 and SIV.
360 Human (HIV-1/2) and simian (SIV) immunodeficiency viruses infect host cells
361 by fusion of the viral and cellular membranes, which is mediated by viral Env
362 glycoprotein consisting of the surface subunit gp120 and the transmembrane subunit
363 gp41. Binding of gp120 to cellular receptor CD4 and a chemokine coreceptor initiates
364 the fusogenic activity of gp41, resulting in a prehairpin intermediate state in which the
365 fusion peptide of gp41 is inserted into the target membrane. Ultimately, three
366 C-terminal helices (CHR) pack antiparallelly onto the trimeric coiled coil of

367 N-terminal helices (NHR) to form a six-helix bundle (6-HB) structure, which drives
368 the apposition of the viral and cell membranes and thus concomitant cell fusion
369 occurs (17, 49, 50). Peptide fusion inhibitors can bind to the exposed NHR or CHR
370 during the prehairpin stage thereby blocking the formation of 6-HB in a
371 dominant-negative manner (17, 18, 20). However, it was found that the only clinically
372 available HIV-1 fusion inhibitor peptide T-20 and most of the newly-developed
373 next-generation peptides had significantly decreased activity in inhibiting HIV-2
374 isolates (Table 1), thus limiting their potential use for the treatment of HIV-2-infected
375 patients. As noted, the second-generation inhibitor T1249 and the third-generation
376 inhibitor T2635 did exhibit improved potency over HIV-2, but their large sizes
377 (39-mer and 38-mer, respectively) would hamper their formulation and production
378 cost. In an advance stage, the third-generation inhibitor SFT (sifuvirtide) has been
379 approved for clinical phase III trials in China and will hopefully become next HIV-1
380 fusion inhibitor in clinical use (32, 34, 42). Nonetheless, our data here indicate that
381 SFT has a dramatically decreased inhibitory activity on HIV-2 (Table 1). Additionally,
382 SFT has a similar low genetic barrier to the development of resistance, and the
383 selected HIV-1 variants display high cross-resistance to T20 (34, 51). These data
384 emphasize the importance of developing new fusion inhibitors with significantly
385 improved pharmaceutical profiles.

386 The structures of both HIV and SIV-derived 6-HBs revealed the atomic
387 interactions between the NHR and CHR sequences and identified a deep hydrophobic
388 pocket on the NHR helices, which is penetrated by the pocket-binding domain (PBD)

of the CHR helix (17, 18, 20, 52-54). Many studies demonstrated that the deep pocket critically determines the NHR/CHR interaction as well as the inhibitor binding (17, 18, 20). Our previous studies demonstrated that the M-T hook residues (Met115 and Thr116) preceding the PBD of a CHR peptide can mediate extensive hydrophobic interactions the pocket thus dramatically fortifying the binding affinity and antiviral activity of inhibitors (34, 36-38). The results shown here demonstrate that the M-T hook structure also functions well for inhibiting HIV-2 and SIV isolates and suggest that the pocket site is highly conserved among HIV-1/2 and SIV. Importantly, the results also suggest that the M-T hook structure is a general strategy for designing fusion inhibitors with a broad-spectrum activity. Obviously, the M-T hook structure is not the only one factor for the excellent performance of 2P23. The second design strategy is introducing the residues that are critical for binding HIV-2 NHR, such as valine (V), leucine (L) and glutamic acid (E) (Table 1). This is clear when comparing 2P23 and HP23 since both have the M-T hook residues but 2P23 exhibit greatly improved binding and inhibitory activities to HIV-2 and SIV isolates. The third player for 2P23 is a group of introduced 'salt-bridges' which can facilitate the helical conformation of inhibitor and also stabilize its binding to the NHR target.

In summary, 2P23 has prominent advantages over many other peptide HIV fusion inhibitors. First, it is highly effective on both HIV-1 and HIV-2 isolates; Second, it has only 23 amino acids in length which will significantly benefit its production; Third, 2P23 binds to the targets with high stability which can confer a high genetic barrier to resistance. Therefore, we conclude that 2P23 has high potential for clinical

development. Also, it provides a novel tool for exploring the mechanisms of HIV and SIV Env-mediated cell fusion.

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583 **Table 1. Inhibitory activity of peptide fusion inhibitors on HIV-1 and HIV-2 isolates**[a]

			IC_{50} (nM)	
Inhibitors	Sequence (a.a. #)	Derived from	HIV-1_{NL4-3}	HIV-2_{ROD}
Classic peptides				
T20	YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF (36)	HIV-1	111.93 ± 3.45	305.55 ± 84.01
C34	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL (34)	HIV-1	1.02 ± 0.28	387.93 ± 78.32
SFT	SWETWEREIENYTRQIYRILEESQEQQDRNERDLLE (36)	HIV-1	1.1 ± 0.52	105.36 ± 15.08
CP32M	VEWNEMTWMEWEREIENYTKLIYKILEESQEQ (32)	HIV-1	2.01 ± 0.48	370.95 ± 108.97
SC29	WEEWDKKIEEYTKKIEELIKKSEEQQKKN (29)	HIV-1	1.13 ± 0.84	237.87 ± 74.81
SC22	WEEWDKKIEEYTKKIEELIKKS (22)	HIV-1	54.58 ± 6.94	527.66 ± 80.52
T2635	TTWEAWDR AIAEYAARIEALIRAAQEQQEKNEAALREL (38)	HIV-1	0.38 ± 0.1	18.56 ± 3.21
T1249	WQEWEQKITALLEQAQIQQEKNEYELQKLDKWASLWEWF (39)	HIV-1/HIV-2/SIV	0.97 ± 0.34	10.42 ± 1.96
$C34_{ROD}$	WQEWEKQVRYLEANISKSLEQAQIQQEKNMYELQ (34)	HIV-2	5.51 ± 2.84	48.26 ± 3.7
$C34_{EHO}$	WQQWERQVRFLDANITKLLEEAQIQQEKNMYELQ (34)	HIV-2	1.88 ± 0.35	33.67 ± 4.96
P3	WQEWEQQVRYLEANISQRLEQAQIQQEKNMYELQ (34)	HIV-2/SIV	6.34 ± 1.94	83.23 ± 34.08
M-T hook modified peptides				
MTC34	MTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL (36)	HIV-1	0.5 ± 0.07	76.61 ± 2.77
MTSFT	MTWETWEREIENYTRQIYRILEESQEQQDRNERDLLE (38)	HIV-1	0.51 ± 0.21	33.93 ± 7.24
MTSC29	MTWEEWDKKIEEYTKKIEELIKKSEEQQKKN (31)	HIV-1	0.43 ± 0.16	22.9 ± 3.9
MTSC22	MTWEEWDKKIEEYTKKIEELIKKS (24)	HIV-1	1.32 ± 0.08	252.24 ± 12.48
$MTC34_{ROD}$	MTWQEWEKQVRYLEANISKSLEQAQIQQEKNMYELQ (36)	HIV-2	0.84 ± 0.29	20.57 ± 2.28
$MTC34_{EHO}$	MTWQQWERQVRFLDANITKLLEEAQIQQEKNMYELQ (36)	HIV-2	1.17 ± 0.16	34.55 ± 2.5
MTP3	MTWQEWEQQVRYLEANISQRLEQAQIQQEKNMYELQ (36)	HIV-2/SIV	1.38 ± 0.32	24.6 ± 2.37
HP23	EMTWEEWEKK IEEYTKKIEEILK (23)	HIV-1	0.19 ± 0.01	78.57 ± 3.02
HP23L	ELTWEEWEKK IEEYTKKIEEILK (23)	HIV-1	0.39 ± 0.06	126.33 ± 9
$P21_{ROD}$	MTWQEWEKQVRYLEANISKSL (21)	HIV-2	571.8 ± 41.3	>1250
$P21_{EHO}$	MTWQQWERQVRFLDANITKLL (21)	HIV-2	402.35 ± 165.1	>1250
$P21_{P3}$	MTWQEWEQQVRYLEANISQRL (21)	HIV-2/SIV	534.45 ± 295.78	>1250
2P23	EMTWEEWEKKVEELEKKIEELLK (23)	HIV-1/HIV-2	0.22 ± 0.05	10.57 ± 0.27
2P23L	ELTWEEWEKK VEELEKKIEELLK (23)	HIV-1/HIV-2	0.59 ± 0.08	18.56 ± 1.31
2P23Q	EMTWQEWEQKVEELEKKIEELLK (23)	HIV-1/HIV-2	1.39 ± 0.45	31.87 ± 7.02

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585 [a]The assay was performed in triplicate and repeated at least 3 times. Data are expressed as means

586 ± standard deviations.

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589 **Table 2. Interactions of inhibitors with HIV-1, HIV-2 and SIV-derived targets determined**
590 **by CD spectroscopy**[a]

	$N36_{NL4-3}$		$N36_{ROD}$		$N36_{SIV251}$	
Inhibitor	**% helix**	**T_m (℃)**	**% helix**	**T_m (℃)**	**% helix**	**T_m (℃)**
C34	84.5	63.62	30.88	NA	15.61	NA
MTC34	71.95	68.57	50.93	30.76	20.87	NA
SFT	81	69.18	65.64	32.96	25.48	NA
MTSFT	91.68	75.13	102.37	43.76	45.58	39.04
SC29	94.03	65.22	44.4	NA	32.33	NA
MTSC29	98.69	73.98	82.95	46.51	65.13	42.42
SC22	77.01	60.7	43.77	NA	22.09	NA
MTSC22	84.97	71.3	70.05	30.96	71.72	69.93
$C34_{ROD}$	70.87	59.06	50.52	32.91	21.82	NA
$MTC34_{ROD}$	70.22	68.39	100.36	50.4	56.61	45.84
$C34_{EHO}$	70.93	65.83	93.29	45.08	53.12	41.08
$MTC34_{EHO}$	70.18	73.52	107.34	58.24	61.65	53.95
P3	62.79	61.29	61.99	35.41	24.08	NA
MTP3	67.38	69.07	96.72	49.66	45.79	46.01
HP23	86.36	82.18	103.49	43.54	44.99	34.41
$P21_{ROD}$	70.39	48.77	40.51	NA	24.56	NA
$P21_{EHO}$	89.21	55.47	42	NA	18.08	NA
$P21_{P3}$	46.54	NA	32.92	NA	27.61	NA
2P23	102.26	78.79	117.85	55.26	62.51	47.35

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592 [a]The assay was performed 2 times and results are expressed as means.

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Table 3. Inhibitory activity of 2P23 and control inhibitors on diverse subtypes of HIV-1 isolates[a].

		IC_{50} (nM)			
Pseudovirus	**Subtype**	**T-20**	**P3**	**HP23**	**2P23**
92RW020	A	10.83 ± 1.65	21.3 ± 3.63	3.28 ± 0.55	2.71 ± 0.92
92UG037.8	A	6.68 ± 1.15	8.56 ± 0.98	2.73 ± 0.26	2.16 ± 0.28
398-F1_F6_20[b]	A	30.48 ± 10.8	24.22 ± 3.4	2.48 ± 0.37	1.6 ± 0.31
SF162	B	7.17 ± 1.3	43.95 ± 1.22	22.10 ± 0.95	15.77 ± 2.48
JRFL	B	49.38 ± 28.4	24.69 ± 2.23	24.51 ± 3.54	8.27 ± 3.24
AC10.0.29	B	3.63 ± 0.38	1.8 ± 0.07	1.13 ± 0.18	1.74 ± 0.54
SC422661.8	B	13.28 ± 1.94	5.88 ± 0.01	1.45 ± 0.06	2.56 ± 0.49
TRO.11[b]	B	18.75 ± 6.37	30.77 ± 7.67	3.68 ± 1.41	5.88 ± 2.37
X2278_C2_B6[b]	B	10.86 ± 1.9	9.85 ± 1.45	2.27 ± 0.4	1.05 ± 0.07
B01	B'	54.48 ± 21.13	68.93 ± 7.19	2.88 ± 0.12	6.35 ± 0.21
B02	B'	42 ± 14.42	83.27 ± 0.04	4.37 ± 1.48	6.56 ± 3.86
B04	B'	12.25 ± 2.52	37.45 ± 18.49	3.09 ± 1.15	6.36 ± 3.02
CAP45.2.00.G3	C	161.36 ± 28.92	87.27 ± 5.19	8.14 ± 4.69	23.59 ± 9.03
ZM109F.PB4	C	12.80 ± 0.76	3.02 ± 0.4	0.91 ± 0.07	1.18 ± 0.21
ZM53M.PB12	C	35.19 ± 2.13	4.06 ± 0.82	0.91 ± 0.18	1.22 ± 0.43
CE703010217_B6[b]	C	12.36 ± 1.66	8.3 ± 2.98	2.12 ± 0.41	4.52 ± 1.19
CE1176_A3[b]	C	13.56 ± 2.82	22.46 ± 5.54	3.59 ± 0.25	5.20 ± 0.26
HIV_25710-2.43[b]	C	23.38 ± 4.61	5.57 ± 0.44	2.38 ± 0.46	2.53 ± 0.7
X1632-S2-B10[b]	G	12.61 ± 1.77	13.71 ± 0.22	4.25 ± 0.08	4.40 ± 0.99
246_F3_C10_2[b]	A/C	28.59 ± 7.33	11.17 ± 3.15	5.15 ± 1.23	3.12 ± 1.43
AE03	A/E	5.09 ± 2.33	35.38 ± 5.15	1.43 ± 0.54	6.57 ± 0.17
AE04	A/E	9.25 ± 0.62	27.95 ± 6.13	4.33 ± 1.76	8.48 ± 0.67
CNE8[b]	A/E	40.76 ± 18.17	24.76 ± 2.17	4.99 ± 0.08	9.2 ± 1.38
CNE55[b]	A/E	30.82 ± 11.68	18.93 ± 2.04	2.26 ± 0.46	3.25 ± 0.06
CH64.20	B/C	20.15 ± 0.28	2.02 ± 0.32	0.6 ± 0.23	0.61 ± 0.14
CH070.1	B/C	176.6 ± 39.27	26.51 ± 8.71	6.05 ± 0.55	7.83 ± 1.39
CH120.6	B/C	30.25 ± 0.69	29.69 ± 7.2	7.33 ± 0.21	10.19 ± 0.71
CH119.10[b]	B/C	6.21 ± 0.86	13.69 ± 9.07	3.69 ± 0.13	7.33 ± 0.42
BJOX002000.03.2[b]	B/C	34.37 ± 17.2	10.95 ± 3.51	4.06 ± 1.21	1.37 ± 0.57
Mean IC_{50} (range)		31.49 (3.63 ~ 176.6)	24.35 (1.8 ~ 87.27)	4.7 (0.6 ~ 24.51)	5.57 (0.61 ~ 23.59)

[a]The assay was performed in triplicate and repeated at least 3 times. Data are expressed as means ± standard deviations.

[b]A global panel of HIV-1 isolates representing the genetic and antigenic diversities worldwide.

Table 4. Inhibitory activity of 2P23 and control peptides on drug-resistant HIV-1 mutants[a]

	T-20		P3		HP23		2P23	
HIV-1_{NL4-3}	IC_{50} (nM)	n-fold	IC_{50} (nM)	n-fold	IC_{50} (nM)	n-fold	IC_{50} (nM)	n-fold
WT	84.09 ± 13.84	1	7.69 ± 0.31	1	0.61 ± 0.13	1	0.69 ± 0.15	1
T20-resistants								
I37T	659.92 ± 79.83	7.85	62.64 ± 0.49	8.15	1.35 ± 0.16	2.21	1.22 ± 0.25	1.77
V38A	1514.55 ± 246.72	18.01	56.45 ± 10.52	7.34	1.07 ± 0.04	1.75	0.89 ± 0.23	1.29
V38M	689.42 ± 162.86	8.2	34.03 ± 5.27	4.43	0.99 ± 0.14	1.62	1.21 ± 0.14	1.75
Q40H	2207.22 ± 519.43	26.25	107.01 ± 21.72	13.92	1.06 ± 0.04	1.74	1 ± 0.27	1.45
N43K	681.7 ± 161.14	8.11	812.6 ± 67.36	105.67	0.79 ± 0.1	1.3	1.13 ± 0.23	1.64
D36S/V38M	471.88 ± 84.14	5.61	16.67 ± 1.55	2.17	1.39 ± 0.32	2.28	1.48 ± 0.22	2.14
I37T/N43K	6075 ± 1572.61	72.24	>2000	>260.08	1.42 ± 0.13	2.33	1.45 ± 0.32	2.1
V38A/N42T	3785.94 ± 1268.36	45.02	86.21 ± 3.43	11.21	0.57 ± 0.13	0.93	0.44 ± 0.1	0.64
HP23-resistants								
E49K	165.4 ± 19.6	1.97	87.38 ± 8.88	11.36	4.45 ± 0.71	7.3	5.2 ± 0.14	7.54
L57R	86.78 ± 4.41	1.03	38.96 ± 1.79	5.07	133.68 ± 5.84	219.15	39.49 ± 0.19	57.23
N126K	182.98 ± 38.03	2.18	12.08 ± 1.28	1.57	1.76 ± 0.04	2.89	1.59 ± 0.77	2.3
E136G	211.4 ± 18.71	2.51	22.72 ± 0.11	2.95	4.73 ± 1.1	7.75	4.66 ± 0.91	6.75
E49K/N126K	203.1 ± 18.48	2.42	134.04 ± 9.33	17.43	5.01 ± 0.45	8.21	4.38 ± 0.8	6.35
L57R/E136G	43.13 ± 14.64	0.51	65.67 ± 2.69	8.54	429.62 ± 93.64	704.3	175.12 ± 46.72	253.8

[a]The assay was performed in triplicate and repeated 3 times. Data are expressed as means ± standard deviations.

Table 5. Inhibitory activity of 2P23 and control peptides on diverse HIV-2 isolates[a]

	IC_{50} (nM)				
HIV-2	**SFT**	**T2635**	**P3**	**HP23**	**2P23**
Primary isolates					
00PTHDECT (R5)	30.71 ± 8.86	7.35 ± 3.2	48.89 ± 4.28	99.82 ± 8.29	21.96 ± 5.05
03PTHCC6 (R5)	63.69 ± 0.08	30.11 ± 9.11	114.65 ± 13.45	44.47 ± 14.13	14.51 ± 1.12
03PTHCC19 (R5)	25.7 ± 15.76	43.38 ± 37.01	121.0 ± 11.9	9.14 ± 8.72	7.35 ± 1.57
03PTHCC1 (R5)	16.49 ± 1.47	4.79 ± 1.15	19.36 ± 8.37	2.22 ± 2.08	2.99 ± 0.76
00PTHCC20 (X4)	15.02 ± 13.61	6.79 ± 1.41	13.28 ± 0.65	27.19 ± 5.14	8.74 ± 2
10PTHSMNC (R5)	55.87 ± 2.58	5.33 ± 0.21	86.7 ± 19.3	32.85 ± 8.14	16.4 ±0.31
03PTHCC12 (R5)	58.85 ± 2.71	16.51 ± 0.12	72.85 ± 19.42	29.3 ± 2.08	24.84 ± 0.53
03PTHSM2 (R5)	98.91 ± 12.99	22.33 ± 0.41	44.02 ± 2.98	98.39 ± 11.22	25.72 ± 2.15
03PTHSM9 (X4)	264.45 ± 32.15	18.29 ± 3.3	62.12 ± 2.07	218.15 ± 37.45	59.04 ± 0.83
Mean IC_{50} (range)	69.96 (15.02 ~ 264.45)	17.21 (4.79 ~ 43.38)	64.76 (13.28 ~ 114.65)	62.39 (2.22 ~ 218.15)	20.17 (2.99 ~ 59.04)
ROD and its mutants					
ROD10 WT (X4)	188 ± 13.5	24.77 ± 14.48	80.59 ± 1.86	57.08 ± 18.31	13.22 ± 2.67
H18L (R5/X4)	312.3 ± 9	49.44 ± 2.54	228.35 ± 8.75	178.8 ± 63.9	16.99 ± 0.56
d23d24 (R5/X4)	174.7 ± 0.3	19.49 ± 0.36	172.65 ± 30.05	82.21 ± 6.35	10.35 ± 0.23
K29T (X4)	179.5 ± 12.7	42.24 ± 0.82	197.15 ± 15.65	52.87 ± 3.88	11.8 ± 1.04
H18L+d23d24 (R5)	274.05 ± 3.35	39.22 ± 1.28	262.3 ± 29.8	113.65 ± 14.66	21.69 ± 1.41
H18L+K29T (R5/X4)	219.55 ± 5.75	24.65 ± 0.52	163.95 ± 1.05	78.41 ± 6.47	15.14 ± 0.47
H18L+d23d24+K29T (R5)	234.75 ± 14.55	44 ± 0.19	232.65 ± 33.35	99.84 ± 5.27	18.46 ± 0.2
Mean IC_{50} (range)	226.12 (174.7 ~ 312.3)	34.83 (19.49 ~ 49.44)	191.09 (80.59 ~ 262.3)	94.69 (52.87 ~ 178.8)	15.38 (10.35 ~ 21.69)

[a]The assays were performed in duplicate and repeated at least 2 times. Data are expressed as means ± standard deviations.

Figure legends

Fig. 1. The α-helicity of peptide inhibitors with $N36_{ROD}$ determined by CD spectroscopy.

(A) SFT and MTSFT; **(B)** SC29 and MTSC29; **(C)** SC22 and MTSC22; **(D)** $C34_{ROD}$ and $MTC34_{ROD}$; **(E)** $C34_{EHO}$ and $MT\text{-}C34_{EHO}$; **(F)** P3 and MTP3. Final concentration of each peptide in PBS was 10μM.

Fig. 2. Binding stability of peptide inhibitors with $N36_{ROD}$ determined by CD spectroscopy.

(A) SFT and MTSFT; **(B)** SC29 and MTSC29; **(C)** SC22 and MTSC22; **(D)** $C34_{ROD}$ and $MTC34_{ROD}$; **(E)** $C34_{EHO}$ and $MT\text{-}C34_{EHO}$; **(F)** P3 and MTP3. Final concentration of each peptide in PBS was 10μM.

Fig. 3. Biophysical properties and anti-HIV activity of 2P23 and control peptides.

(A) The α-helicity of HP23 and 2P23 in complexes with $N36_{NL4\text{-}3}$; **(B)** The thermostability of HP23 and 2P23 in complexes with $N36_{NL4\text{-}3}$; **(C)** The α-helicity of HP23 and 2P23 in complexes with $N36_{ROD}$; **(D)** The thermostability of HP23 and 2P23 in complexes with $N36_{ROD}$; **(E)** Inhibition of 2P23 and control peptides (T20, P3, HP23) on infection of HIV-$1_{NL4\text{-}3}$; **(F)** Inhibition of 2P23 and control peptides (T20, P3, HP23) on infection of HIV-2_{ROD}. CD experiments were performed with a final concentration of each peptide at 10μM. The inhibition assays were performed in triplicate and repeated 3 times. Percentage inhibition of the peptides and IC_{50} values

were calculated as described. Data are expressed as means ± standard deviations (SD).

Fig. 4. Biophysical properties and anti-SIV activity of 2P23 and control peptides. **(A)** The α-helicity of HP23 and 2P23 in complexes with $N36_{SIV251}$; **(B)** The thermostability of HP23 and 2P23 in complexes with $N36_{SIV251}$. **(C)** Inhibition of 2P23 and control peptides (T20, P3, HP23) on SIV_{pbj} Env-pseudotyped virus in single-cycle assay; **(D)** Inhibition of 2P23 and control peptides (T20, P3, HP23) on SIV_{239} Env-pseudotyped virus in single-cycle assay. CD experiments were performed with a final concentration of each peptide at 10 μM. Single-cycle infection assays were performed in triplicate and repeated 3 times. Percentage inhibition of the peptides and IC_{50} values were calculated. Data are expressed as means ± SD.

Fig. 5. Inhibitory activity of 2P23 and control peptides on Env-mediatedcell fusion.

Inhibition of 2P23 and control peptides on HIV-1_{NL4-3} Env **(A),** SIV_{pbj} Env **(B)** and SIV_{239} Env-mediated cell-cell fusion was measured by DSP-based assays. The experiments were performed in triplicate and repeated at least 2 times. Percentage inhibition of the peptides and IC_{50} values were calculated. Data are expressed as means ± SD.

Fig. 6. The secondary structure and stability of 2P23 and control peptides determined by CD spectroscopy.

661 The α-helicity **(A)** and thermostability **(B)** of 2P23 in itself and the α-helicity **(C)** and
662 thermostability **(D)** of HP23 in itself were measured at different concentrations in
663 PBS. The α-helicity **(E)** and thermostability **(F)** of control peptides (T20, C34, SFT,
664 T1249, T2635, P3) were measured at a final concentration of 20 μM in PBS. The
665 helical contents and T_m values were shown in parentheses. NA means no applicable
666 for precise calculation. The experiments were repeated at least two times and
667 representative data are shown.
668

Fig. 1

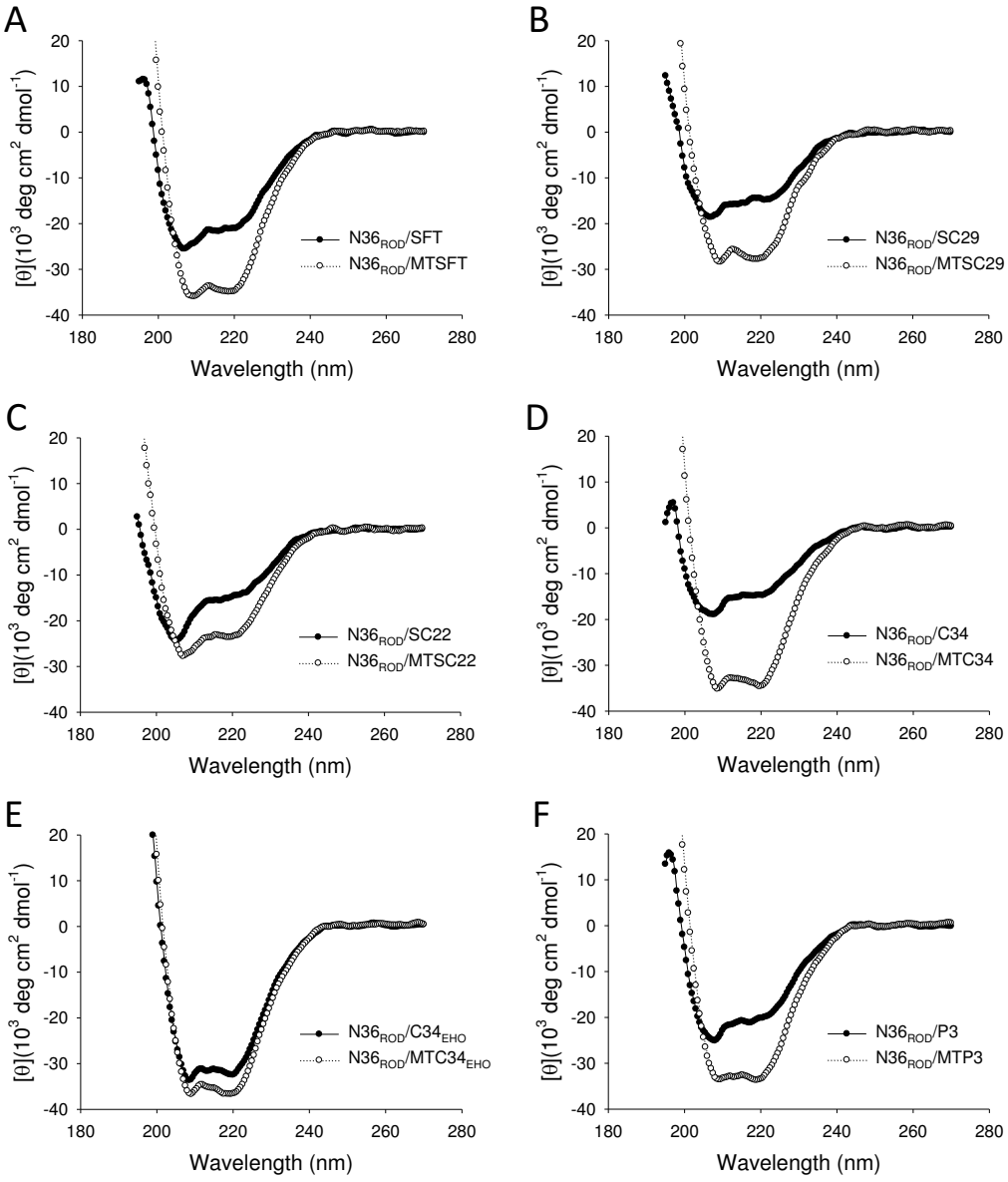

Fig. 2

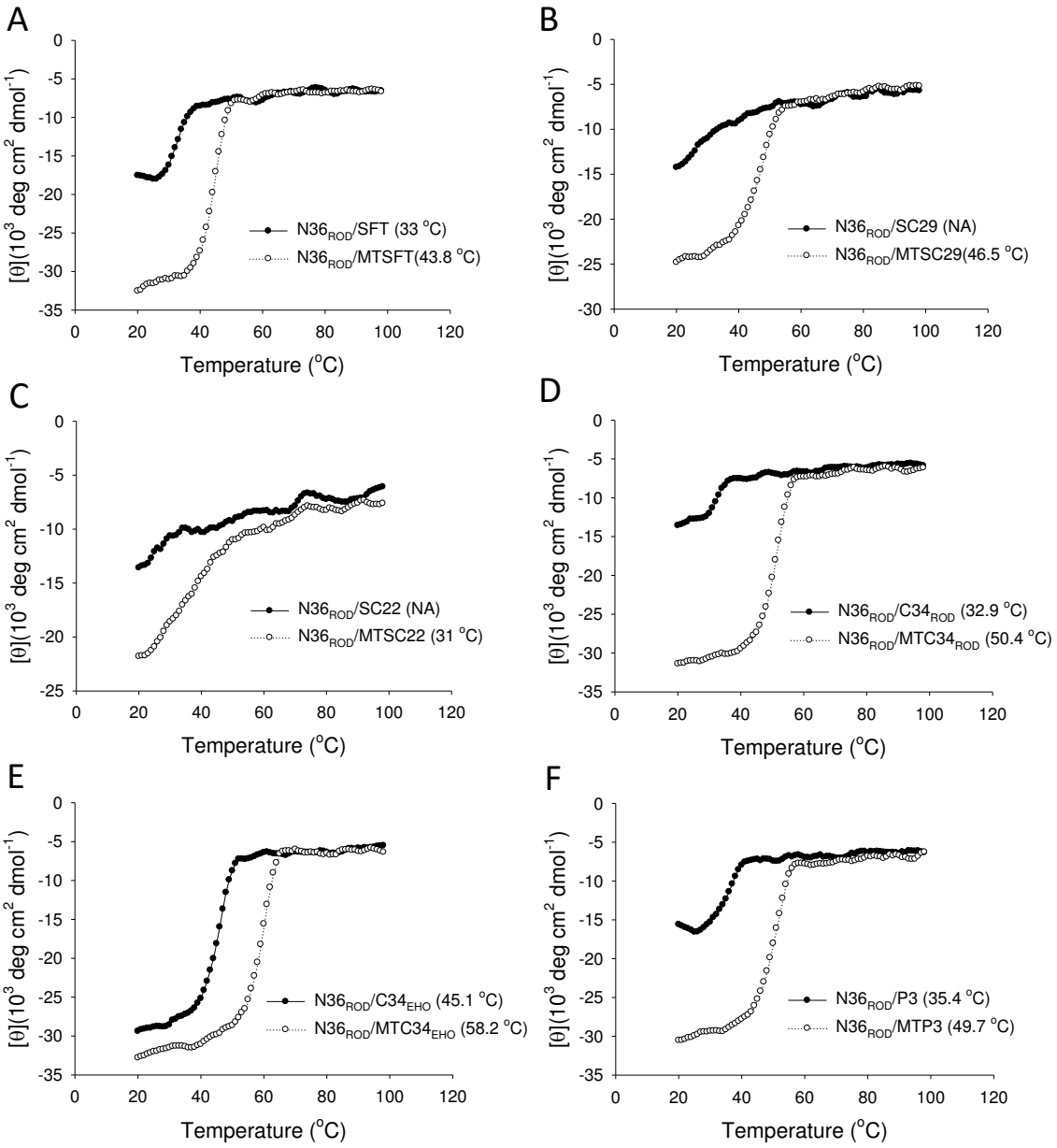

Fig. 3

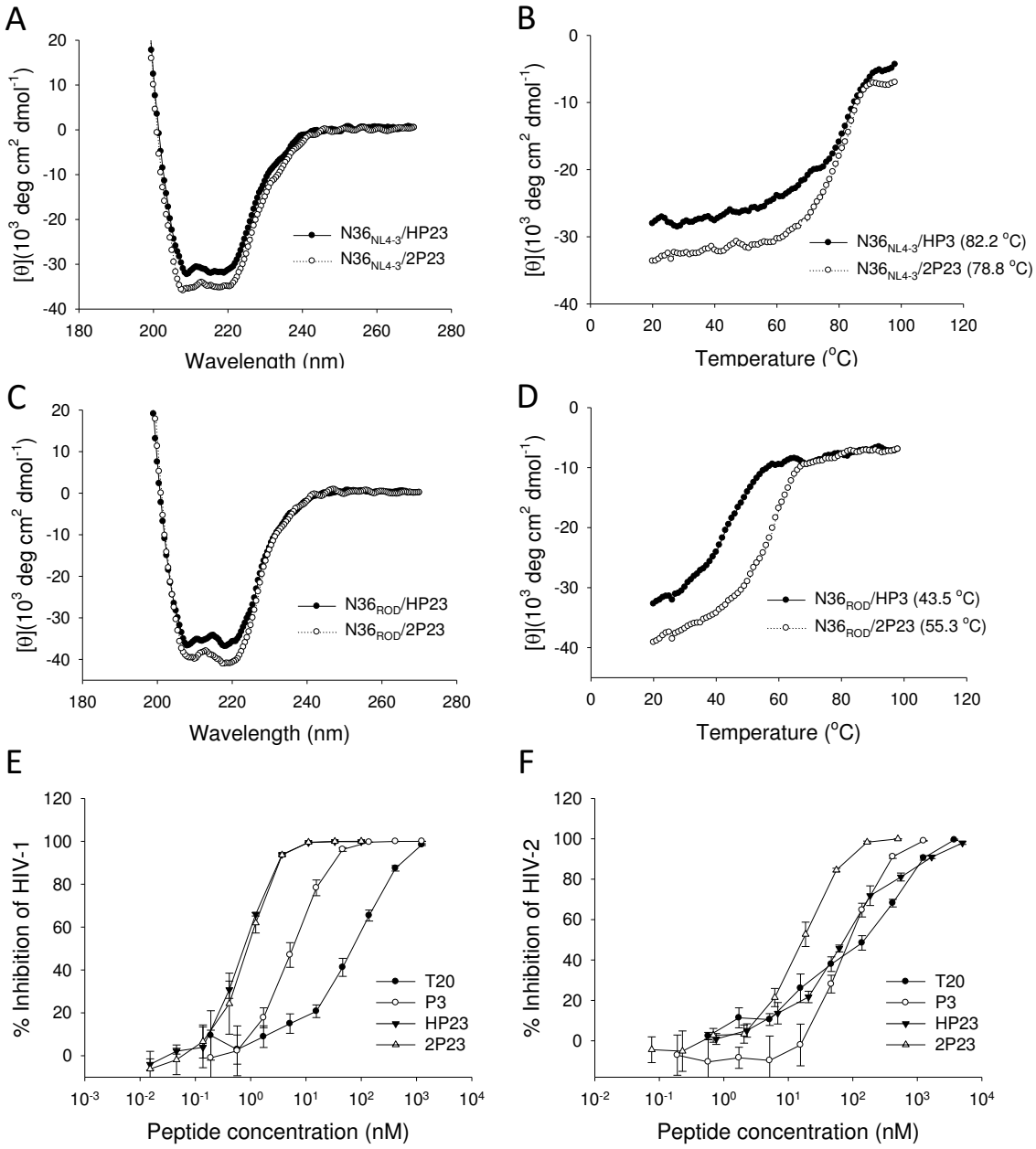

Fig. 4

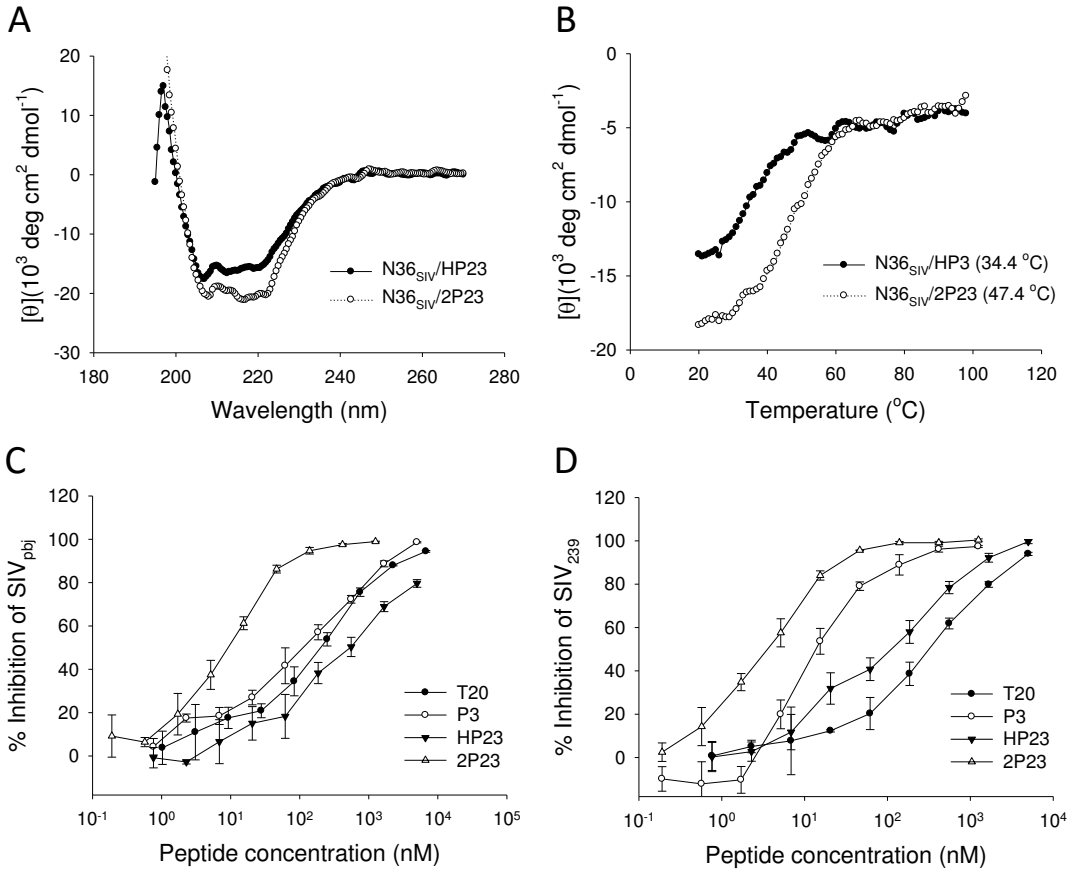

Fig. 5

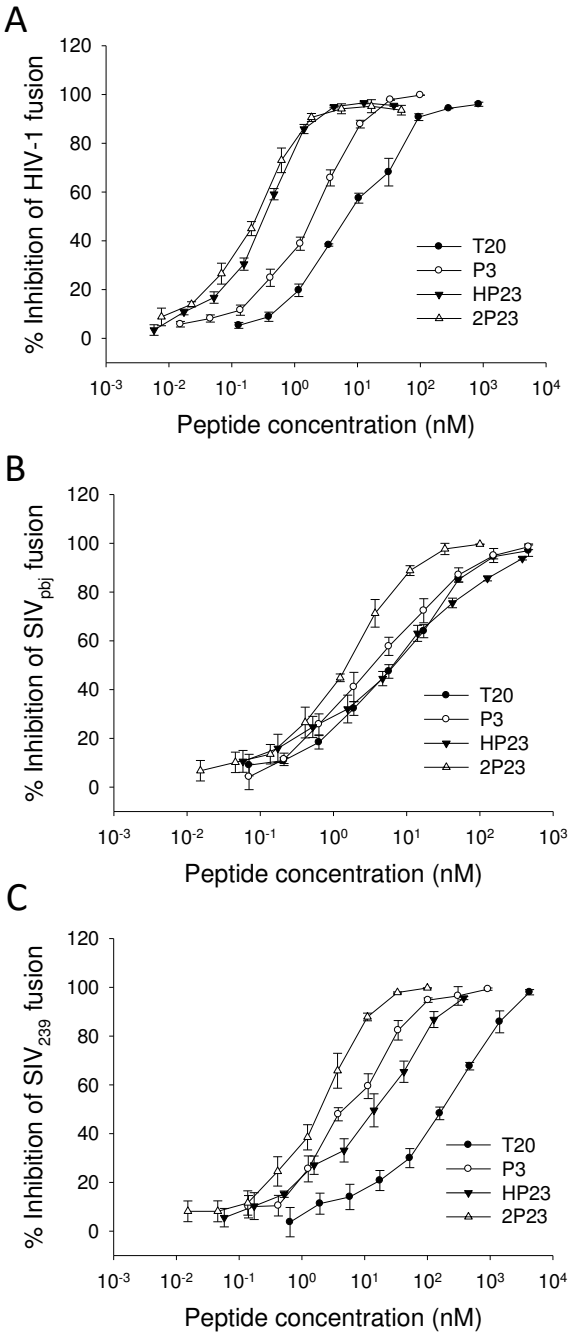

Fig. 6