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Template-constrained cyclic sulfopeptide HIV-1 entry inhibitors†

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Abstract

Template-constrained cyclic sulfopeptides that inhibit HIV-1 entry were rationally designed based on a loop from monoclonal antibody (mAb) 412d. A focused set of sulfopeptides was synthesized using Fmoc-Tyr(SO_3DCV)-OH (DCV = 2,2-dichlorovinyl). Three cyclic sulfopeptides that inhibit entry of HIV-1 and complement the activity of known CCR5 antagonists were identified.

Protein–protein interactions that mediate entry of human immunodeficiency virus type 1 (HIV-1) into host cells have been increasingly exploited in recent years as targets for new antiviral agents.[1,2] The HIV-1 envelope glycoprotein (Env), which assembles as a trimer before being cleaved into gp120 and gp41 subunits, is able to interact with several host cell surface proteins including CD4.[1] Upon binding of CD4, the conformation of gp120 changes to expose an additional binding site[3] for a co-receptor, either CCR5 or CXCR4.[4] Strains of HIV-1 that use CCR5 for entry, so-called R-tropic viruses, utilize two separate domains of CCR5 to mediate fusion and entry: the *N*-terminus (CCR5*Nt*), and the extracellular loops. The degree to which HIV-1 entry relies upon each of these two domains in CCR5 varies among viral strains.[5] Although it is well accepted that the CCR5*Nt* and the extracellular loop 2 (ECL2) play key roles in HIV-1 entry, all strains do not share a common set of interactions with CCR5.6

A conserved site on gp120 that binds *O*-sulfonated tyrosine (Tys) residues presents a structural target[7] against which to design novel entry inhibitors. The Tys residues in the CCR5*Nt* facilitate HIV-1 entry through interactions with gp120,[8] and CCR5*Nt* peptides that include Tys10 and Tys14 bind gp120 and inhibit entry of viruses pseudotyped with R-tropic Envs.[9,10] Several monoclonal antibodies, including the neutralizing antibody 412d, also require *O*-sulfonation of tyrosine residues to bind to a CD4-induced epitope on HIV-1 gp120,[11] which further illustrates the potential to inhibit HIV-1 entry by exploiting Tys recognition by CD4-gp120. *In silico* screening of small molecule libraries against the conserved Tys-binding site identified two entry inhibitors that are effective against engineered HIV-1 strains and weakly neutralize primary HIV-1 isolates. [12] Herein, we describe an alternative strategy to identify HIV-1 entry inhibitors that disrupt interaction between gp120 and the CCR5*Nt*. Template-constrained cyclic sulfopeptides were rationally

†Electronic supplementary information (ESI) available: Schemes of the synthesis strategy and structures of all of the peptides screened, figures reporting the screening results, ELISA experiments to determine the binding site of peptides **1–3**, experimental procedures, and characterization. See DOI: 10.1039/c3ob41395k.

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designed, synthesized, and screened against an engineered HIV-1 strain (TA1)[13] that is highly dependent on residues in the CCR5*Nt* for entry into host cells. TA1 pseudotypes are R-tropic, and require CCR5 ECL2 as well as the CCR5*Nt* for entry into target cells. From a focused set of cyclic sulfopeptides we identified three entry inhibitors with high specificity for TA1, and that complement CCR5 antagonists.

Based on a crystal structure of mAb 412d complexed with CD4-gp120,[7] cyclic peptides were designed to mimic residues from the complementarity determining region (CDR) H3 of mAb 412d that interact directly with the conserved binding site on gp120. Tyrosines 100 and 100c of mAb 412d are functionally *O*-sulfonated, and bind to the same residues on CD4- gp120 as the Tys residues of CCR5*Nt* peptide,[7] which suggested that mimics of the CDR H3 region of mAb 412d including Tys100 and Tys100c might act as competitive inhibitors for the interaction between CD4-gp120 and CCR5. We, therefore, identified Tys100, Asn100a, Asp100b, and Tys100c as the minimum residues for binding CD4-gp120. The dihedral angles of Tys100 and Asn100a closely agree with the canonical values for a type I β-turn,[14] so we envisioned that small, cyclic peptide β-turn mimetics might provide suitable scaffolds for the design of entry inhibitors.

Molecular models of template-constrained cyclic peptides were built in Macromodel, energy minimized with the Amber force field, and compared to the target residues in the crystal structure of CD4-gp120-412d[7] (PDB: 2QAD). Cyclic pentapeptides constrained by *m*-aminomethylbenzoic acid (Mamb) have been thoroughly characterized in solution and solid state, and have been shown to adopt well-defined conformations that mimic types I and II′ β-turns.[15–18] The conformation about the β-turn is sensitive to the stereochemistry of the residue at the *i*+ 1 position,[15,19] but not the nature of the side chains.[15–18] We, therefore, investigated models in which Tys100–Tys100c from mAb 412d were constrained by Mamb. To validate the Amber force field, we constructed a model of cyclo(D-Abu-NMeArg-Gly-Asp-Mamb) whose solution and crystal structures are reported to be quite similar to each other.[15] Because the energy minimized model matched the backbone conformation and C_β positions in the crystal structure of cyclo(D-Abu-NMeArg-Gly-Asp-Mamb),[15] we applied the same procedure to four model sequences: cyclo(Tys-Asn-Asp-Tys-Mamb), cyclo(D-Tys-Asn-Asp-Tys-Mamb), cyclo(Tys-D-Asn-Asp-Tys-Mamb), and cyclo(D-Tys-D-Asn-Asp-Tys-Mamb). Cyclo(Tys-Asn-Asp-Tys-Mamb) (**1**), which formed a type I β-turn, overlaid well with residues Asn100a, Asp100b, and Tys100c of mAb 412d in the crystal structure.[7] However, cyclo(D-Tys-Asn-Asp-Tys-Mamb) overlaid well with all four of the target residues from mAb 412d. A D-amino acid at position *i*+ 1 of the β-turn mimetic favors a type II′ β-turn.[15,19] Both models, which are shown in Fig. 1 overlaid with the target sequence from 2QAD,[7] gave us confidence that peptides constrained by the Mamb linker could adopt reasonable conformations that mimic features of the CDR H3 when mAb 412d is bound to CD4-gp120.[7]

A focused set of cyclic sulfopeptides was prepared by cyclization of side chain-protected linear peptides in solution followed by elimination of the side chain protecting groups. Because the conserved Tys-binding site on gp120 was previously shown to be selective for monoaryl sulfates over benzyl sulfonates, aryl sulfonates, and monoaryl phosphates,[9, 20] we restricted the series to Tys-containing peptides. The Tys residues present a challenge for chemical synthesis of peptides[21–25] due to acid-catalyzed elimination of SO_3 from aryl sulfonic acids.[26,27] Side chain-protecting groups for sulfotyrosine residues have begun to address this challenge.[28–33] The linear peptide intermediates were prepared on a solidphase resin following Fmoc/^{t} Bu-based protocols for amino acid coupling and elimination of the Fmoc protecting group. Fmoc-Tyr(OSO_3DCV)-OH (DCV = 2,2-dichlorovinyl)[32,33] was incorporated during synthesis of the linear, side chain-protected peptides. 2,2-Dichlorovinylsulfate esters are susceptible to nucleophilic addition by secondary amines, so

the more sterically hindered base 2-methylpiperidine[32,33] was employed in the Fmoc deprotection steps. A 2-chlorotrityl resin was used as the solid support for the linear peptide synthesis, which allowed for cleavage of the side chain-protected peptide from the resin under mildly acidic conditions. The linear peptides were cyclized in dilute DMF solution[18] using HBTU as the condensation reagent. Side chain-protecting groups were removed from the cyclic peptides in a two-step sequence. First, acid-sensitive side chain-protecting groups were removed with trifluoroacetic acid without affecting the sulfate esters. In the second stage, the 2,2-dichlorovinyl groups were removed by hydrogenolysis. [32,33] The cyclic sulfopeptides were purified by reversed phase HPLC in H_2O–MeCN buffered with 20 mM NH_4OAc to minimize hydrolysis of the aryl sulfonic acid groups. Table 1 reports the structures of cyclic sulfopeptides **1**–**4**.

Type I β-turn mimetics **1**–**3** were identified as inhibitors of HIV-1 entry in a cell-based assay for entry of an HIV vector that expresses luciferase and is pseudotyped with the TA1 Env.[13] The pseudotypes were pre-incubated with cyclic peptide at two different concentrations (*i.e.*, 10 μM and 100 μM) to indicate whether the apparent hits were active in a dose-dependent manner. Among eleven peptides that were screened in the pseudotype inhibition assay, compounds **1**–**3** met our criteria of <80% infection by TA1 at either concentration, and showed greater activity at 100 μM than 10 μM in this initial screening (see ESI†). The type II′ β-turn mimetic **4** was inactive in the initial screening, which may be due to the fact that the design strategy considered only a single minimum energy conformation of the peptides. When the β-turn is centered on a secondary amide, cyclic peptides constrained by the Mamb linker can adopt several closely related conformations.[15,17] However, there is a family of conformations found for type I β-turn mimetics constrained by the Mamb linker, like **1**–**3**, in which the macrocycle twists in the opposite direction[15,17,34] compared to the other conformations and may be important for the activity of peptides **1**–**3**.

Dose-dependent inhibition of TA1 infection was confirmed by a second stage of evaluation. The dose–response profile for compound **1** is presented in Fig. 2, and similar dose–response profiles were obtained for peptides **2** and **3** (see ESI†). The dose–response profiles of **1**–**3** validated the criteria set for identifying active entry inhibitors as well as the minimal design strategy we employed. The IC_{50} values for peptides **1**–**3** were estimated from fits to the dose–response data and are reported in Table 1. Peptide **3** matches the amino acid sequence of mAb 412d CDR H3 in a type I β-turn mimetic, and we found an IC_{50} of 159 (±30) μM for peptide **3**. Replacing the Asn residue of **3** with Asp improved the IC_{50} to 38 (±3) μM for **1**. Peptide **1** tolerates replacement of Asp at the *i*+ 2 position of the β-turn mimetic with Leu (*i.e.*, **2**). We estimate the IC_{50} of **2** to be 31 (±5) μM. Peptides **1**–**3** appear to have similar affinities as linear peptides derived from the CCR5*Nt*,[9,10] which are much larger than **1**–**3**.

While several CCR5 antagonists have broad activity against primary and lab-adapted HIV strains,[35] resistance to these inhibitors may develop *in vivo* and *in vitro*,[36–39] and baseline resistance to CCR5 antagonists in an untreated patient has been described.[40,41] The CCR5 antagonists aplaviroc (APL), maraviroc (MVC), and TAK-779 (TAK) are thought to inhibit HIV-1 infection by altering the conformation of the co-receptor extracellular loops in subtle but distinct ways, but these CCR5 antagonists do not block gp120 interactions with the CCR5*Nt*.[42] We hypothesized that combinations of CCR5 antagonists with inhibitors of the Tys-binding site would decrease infection by members of the virus population that are partially or completely resistant to CCR5 antagonists. To test this hypothesis, we compared inhibition of viral entry by **1** alone and in combination with APL, MVC, or TAK. We included several different pseudotyped viruses in our screening assay: TA1, which exhibits broad resistance to CCR5 antagonists; pre- 5.2, which is partially resistant to APL, MVC, and TAK-779; and R3, which exhibits narrow resistance to MVC. We also included the

primary Envs R3A[43] and YU2, which are inhibited >99% by each of the CCR5 antagonists. Only R3A and TA1 were inhibited by more than 50% at 100 μM of **1** alone (Fig. 3).

Cyclic peptide **1** enhanced inhibition of TA1 pseudotype entry in the presence of APL, MVC and TAK (Fig. 3), reducing infection by 56%, 63% and 74% respectively compared to CCR5 antagonist alone. It is interesting to note that although **1** alone had no effect on pre-5.2 entry, the presence of CCR5 antagonist plus **1** increased inhibition of pre-5.2 pseudotypes, although the reduction in infection was only ~35%. Peptide **1** had no effect on R3 infection in the presence of MVC, which is consistent with the finding that this Env very efficiently uses the MVC-bound co-receptor for entry.[42] Although R3 uses CCR5 ECLs and CCR5*Nt* for entry, we interpret this result to mean that the R3 gp120–ECL2 interaction is very efficient at triggering fusion. However, **1** modestly increased inhibition in the presence of APL or TAK by 43% and 23% respectively, supporting previous data[41] that interactions between R3 and the CCR5*Nt* are also important for entry. Infection by R3A and YU2 pseudotyped viruses was only modestly inhibited when **1** was added to cells pre-treated with one of the three CCR5 antagonists. It was difficult to quantify any additive effect of **1** since the CCR5 antagonists alone reduced infection by >99%, and we were near the lower limit of detection for the luciferase assay.

Conclusions

Template-constrained cyclic peptides that recapitulate features of the CDR H3 of mAb 412d bound to gp120[7] can inhibit viral entry. By comparing models of template-constrained β-turn mimetics to the crystal structure of mAb 412d bound to gp120 (PDB: 2QAD),[7] we selected a suitable linker (*i.e.*, Mamb[15]) for the cyclic peptides. The sulfate-protected amino acid derivative Fmoc-Tyr(OSO_3DCV)-OH[32,33] enabled the synthesis of a focused set of peptides from which three inhibitors of HIV-1 entry were identified. The peptides are active against a primary Env (R3A) and its drug-resistant derivative (TA1) at micromolar concentrations. We observe greater reduction of viral entry when a template-constrained cyclic sulfopeptide is used together with CCR5 antagonists. Peptides **1**–**3** are new tools with which to interrogate HIV-1 entry and a starting point further optimization of their efficacy and selectivity.

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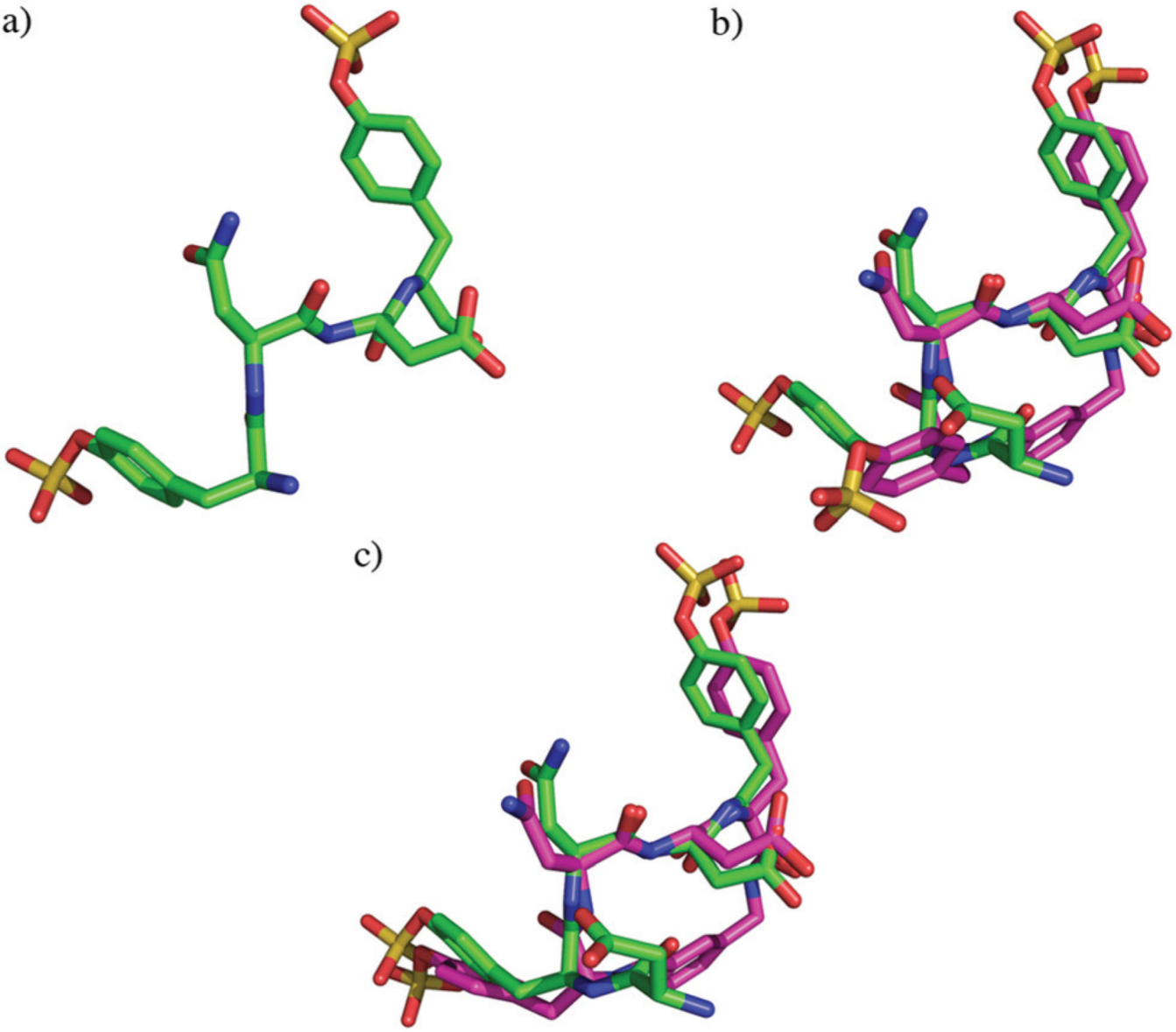

Fig. 1.

(a) Complementarity determining region of mAb 412d including residues Tys100–Tys100c. Energy minimized models for template-constrained cyclic sulfopeptides adopting (b) type I, and (c) type II' β-turn conformations overlaid with the mAb 412b residues shown in (a). Coordinates for mAb 412d and gp120 are from PDB: 2QAD.[7]

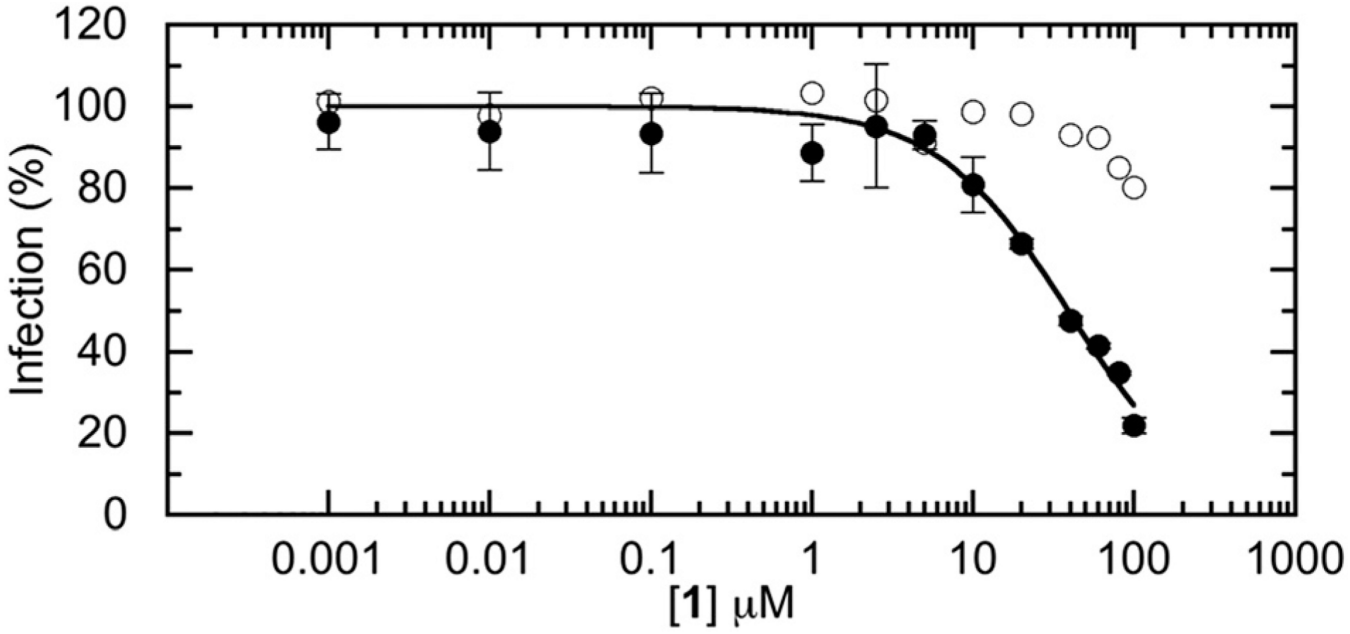

Fig. 2.
Inhibition of TA1 (●) or VSVG (○) entry into NP2.CD4.CCR5 cells by peptide 1. The IC_{50} values are calculated from a fit (solid line) to the TA1 data.

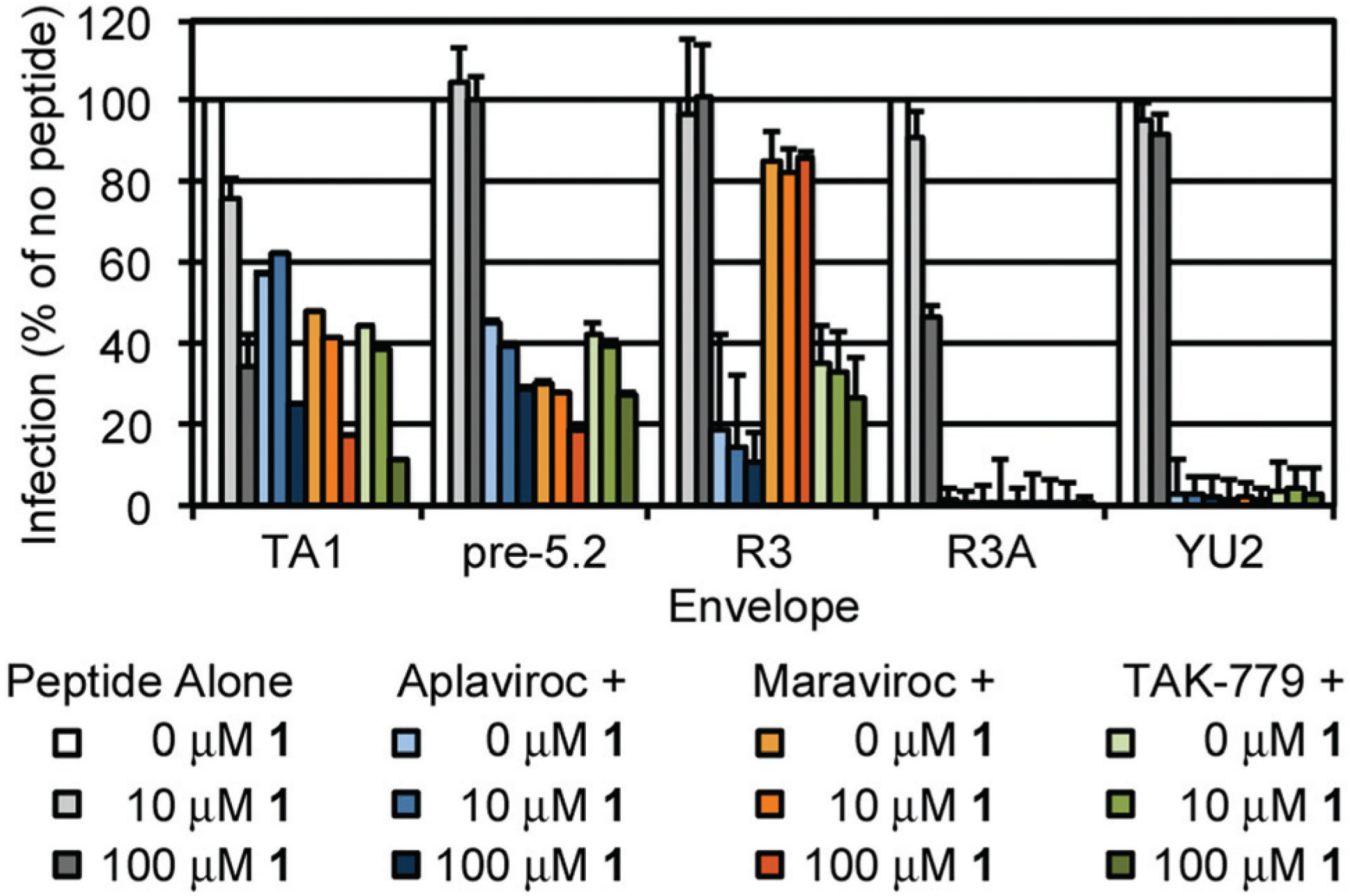

Fig. 3.
Inhibition of viral entry into NP2.CD4.CCR5 cells by peptide **1** alone or in the presence of saturating concentrations of APL, MVC or TAK-779.

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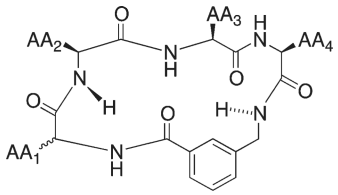
Table 1

Structure and IC50 values of selected cyclic sulfopeptides for inhibition of TA1 viral entry



Peptide	AA_1	AA_2	AA_3	AA_4	IC_{50} (μM)
1	L-Tys	Asp	Asp	Tys	38 ± 3
2	L-Tys	Asp	Leu	Tys	31 ± 5
3	L-Tys	Asn	Asp	Tys	159 ± 30
4	D-Tys	Asp	Asp	Tys	n.d.[a]

[a]The peptide was not active at 10 μM or 100 μM in the initial screen.