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An ancestral HIV-2/simian immunodeficiency virus peptide with potent HIV-1 and HIV-2 fusion inhibitor activity

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Objectives: To produce new fusion inhibitor peptides for HIV-1 and HIV-2 based on ancestral envelope sequences.

Methods: HIV-2/simian immunodeficiency virus (SIV) ancestral transmembrane protein sequences were reconstructed and ancestral peptides were derived from the helical region 2 (HR2). The activity of one ancestral peptide (named P3) was examined against a panel of HIV-1 and HIV-2 primary isolates in TZM-bl cells and peripheral blood mononuclear cells and compared to T-20. Peptide secondary structure was analyzed by circular dichroism. Resistant viruses were selected and resistance mutations were identified by sequencing the *env* gene.

Results: P3 has 34 residues and overlaps the N-terminal pocket-binding region and heptad repeat core of HR2. In contrast to T-20, P3 forms a typical α-helical structure in solution, binds strongly to the transmembrane protein, and potently inhibits both HIV-2 (mean IC_{50}, 63.8 nmol/l) and HIV-1 (11 nmol/l) infection, including T-20-resistant isolates. The N43K mutation in the HR1 region of HIV-1 leads to 120-fold resistance to P3 indicating that the HR1 region in transmembrane glycoprotein is the target of P3. No HIV-2-resistant mutations could be selected by P3 suggesting that the genetic barrier to resistance is higher in HIV-2 than in HIV-1. HIV-1-infected patients presented significantly lower P3-specific antibody reactivity compared to T-20.

Conclusion: P3 is an HIV-2/SIV ancestral peptide with low antigenicity, high stability, and potent activity against both HIV-1, including variants resistant to T-20, and HIV-2. Similar evolutionary biology strategies should be explored to enhance the production of antiviral peptide drugs, microbicides, and vaccines.

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Introduction

Over the last decade, the inhibition of viral entry has become one of the most attractive fields in the research for new anti-HIV-1 molecules. Currently, there are two entry inhibitors approved for antiretroviral therapy, the fusion inhibitor peptide enfuvirtide (or T-20; Fuzeon; Roche) [1] and the CCR5 antagonist maraviroc AQ1

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(Selzentry; Pfizer) [2]. T-20 is a linear peptide composed of 36 amino acids that mimics the sequence of gp41 helical region 2 (HR2) of the HIV-1 LAI isolate [3,4]. T-20 inhibits HIV-1 entry by competitive binding to the complementary helical region 1 (HR1), thereby blocking the formation of the six-helix bundle structure and preventing viral fusion [5–7]. Despite strong anti-HIV-1 activity, the genetic barrier for T-20 resistance is low [7,8] and resistance mutations are usually found within the 36-45 positions of HR1 region [7,9]. T-20 has poor bioavailability and has to be injected subcutaneously twice daily, complicating patient adherence to treatment. Currently, T-20 is only used as a salvage therapy in HIV-1 infection [10–12]. Novel fusion inhibitor peptides have been developed in an attempt to improve antiviral potency, increase in-vivo stability, and overcome T-20 resistance [7,13]. T-1249 is a representative second generation 39-mer peptide derived from HR2 consensus sequences [14,15]. It is a strong inhibitor of HIV-1 but the elevated production costs and drug formulation difficulties associated with its long size, have hampered its clinical development beyond phase I/II trials [16]. Sifuvirtide is a third generation fusion inhibitor peptide that has shown promising results in phase II clinical studies being active against a broad range of HIV-1 isolates, including T-20-resistant strains [7,13,17].

HIV type 2 (HIV-2), the second causative agent of AIDS, is responsible for localized epidemics mainly in west Africa and in few other countries (e.g., Portugal and France), affecting an estimated 1–2 million patients worldwide [18]. HIV-2 is composed of eight groups termed A–H of which group A is by far the most disseminated worldwide [19–21]. HIV-1 and HIV-2 have different evolutionary histories [22] and share only 50% of genetic similarity [23]. Consequently, some anti-HIV-1 drugs have limited or no activity on HIV-2, namely nonnucleoside reverse transcriptase inhibitors, some protease inhibitors, and T-20 [4,24,25]. T-20 has ~~no~~ limited activity on HIV-2 possibly because its sequence divergence from HIV-2 prevents it from binding to the HR1 target in gp36 envelope glycoprotein [24,26,27]. The aim of the current work was to produce new HR2-based peptides to inhibit HIV-2 fusion and entry. To enhance the likelihood of inhibiting replication of all HIV-2 strains, the candidate peptides were derived from ancestral HIV-2 and SIVmn gp36 sequences. We found that one selected peptide, named P3, potently inhibited the fusion and entry of both HIV-1 and HIV-2.

Materials and methods

Virus stocks and titration

A total of 26 primary isolates were included in this study (seven HIV-1 and 19 HIV-2); all isolates were characterized for coreceptor usage as described previously [27]. HIV reference strains were obtained by transfection of HEK293T cells with pNL4-3 (HIV-1), pSG3.1 (HIV-1), or pROD10 (HIV-2) plasmids using Fugene 6 reagent (Roche, Switzerland) according to manufacturer's instructions. Pseudoviruses carrying the vesicular stomatitis virus (VSV) envelope were produced by cotransfection of HEK293T cells with pSG3.1Δenv plasmid and a plasmid expressing VSV envelope (pHEF-VSVG). HIV-1 variants resistant to T-20 were propagated in CEM-SS cells according to a protocol available at www.aidsreagent.org. The 50% tissue culture infectious dose ($TCID_{50}$) of all viruses was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells [27]. AQ2

Peptide design

Custom peptides were derived from ancestral gp36 HR2 sequences reconstructed from a phylogenetic tree of HIV-2 and simian immunodeficiency virus (SIV) reference sequences (see Support Digital Content 1, http://links.lww.com/QAD/A306 for a list of reference sequences used). Reconstruction of ancestral character states was performed by maximum likelihood in PAUP version 4 software [28]. MODELTEST [29] estimated best-fit models of molecular evolution for maximum likelihood analyses. The chosen model was GTR+G+I [30]. Tree searches were also performed in PAUP version 4.0 using the nearest-neighbor interchange (NNI) and tree bisection and reconnection (TBR) heuristic search strategies and bootstrap resampling.

Peptides were produced commercially by Genemed Synthesis (San Antonio, Texas, USA). They were modified with the N-terminus acetylated and the C-terminus as a carboxamide, the salt form being acetate. Reverse-phase high-pressure liquid chromatography (HPLC) was used for purification (>95%) and mass spectrometry for confirmation analysis.

Circular dichroism spectroscopy

Circular dichroism spectra were recorded for P3 and T-20 in 10 mmol/l phosphate buffer and 100 mmol/l NaF (pH 7.4) using a Jasco 810 spectropolarimeter equipped with a temperature control unit Julabo F25. Spectra were recorded at 25°C in the far ultraviolet (UV) region (185–240 nm), using a 0.1 cm pathlength cell (Helma UL, Lda), with a 50 nm/s scan speed, an 8-s response time, and 2 nm bandwidth. For each spectrum, four scans were averaged. P3 and T-20 concentration was previously determined by absorbance at 280 nm using an extinction coefficient of $\varepsilon_{280} = 13940\,M^{-1}\,cm^{-1}$ and $\varepsilon_{280} = 18350\,M^{-1}\,cm^{-1}$, respectively, in a UV-Visible spectrophotometer Jasco V-530. The final concentration used for P3 and T-20 was of 104 and 47 μmol/l, respectively. For protein secondary structure estimation, circular dichroism spectra were deconvoluted using the CDSSTR [31] deconvolution algorithm on Dichroweb [32,33]. Circular dichroism AQ3

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spectra of the appropriate buffers were recorded and subtracted from the protein spectra.

Peptide-binding assay

An ELISA assay ~~ELISA~~ was developed to study the binding specificity of peptide P3 to its predicted target in HIV-2 *env* gp36. Polystyrene immune module microwells (Maxisorp; AQ4 Nunc, Roskilde, Denmark) were coated with each peptide at a concentration of 50 μg/ml in phosphate buffered saline (PBS) solution and incubated overnight at 4°C. After two washes with PBS, microwells were blocked with 5% of AQ5 bovine serum albumin (BSA; Sigma-Aldrich, USA) in PBS for 2 h at 37°C and washed twice with PBS. A recombinant gp36 protein with a polyhistidine tag (rgp36) previously produced in our laboratory [34] was diluted in PBS containing 0.05% of Tween-20 (Bio-Rad, USA) (PBS-T) and added (100 μl) at a concentration of 2.5 μg/ml and incubated for 1 h at 37°C. After five washes with PBS-T, a 1 : 2000 dilution of mouse monoclonal antipolyhistidine antibody conjugated to alkaline phosphatase (Sigma-Aldrich) in PBS-T was added (100 μl) and incubated for 1 h at 37°C. After another five washes with PBS-T, p-nitrophenyl phosphate tablets (Sigma-Aldrich) were added as a chromogenic substrate, and the optical density was measured in a Tecan MP-500 plate reader (Tecan, Männedorf, Switzerland) at 405 nm against a reference wavelength of 620 nm. The cut-off value of the assay, calculated as the mean optical density value of negative controls + two times the standard deviation (SD), was determined for each peptide using wells in which the peptide was incubated with PBS instead of rgp36. The results of the assay are expressed quantitatively as optical $\text{density}_{\text{peptide}}$/$\text{optical density}_{\text{cut-off}}$ ratios (optical density/cut-off ratio).

Drug susceptibility assays

The antiviral activity of fusion inhibitors was evaluated using a single-round viral infectivity assay and a viral replication inhibition assay. The former was performed using the TZM-bl reporter cells as previously described [27]. Briefly, cells were infected with 200 $TCID_{50}$ of each virus. Infections were performed in the presence of serial-fold dilutions of inhibitors in growth medium, supplemented with DEAE-dextran. After 48 h of infection, luciferase expression was quantified with the One-Glow luciferase assay substrate reagent (Promega, USA) according to manufacturer's instructions. The cytotoxicity of the compounds was evaluated using control wells in the absence of the virus. Additional cell viability studies were also performed in peripheral blood mononuclear cells (PBMCs) incubated in the presence of up to 20 μmol/l of peptide and evaluated with alamarBlue reagent (Invitrogen, USA).

For the viral replication inhibition assay, phytohemagglutinin-stimulated PBMCs were infected at a multiplicity of infection of 0.01 in the presence of serial-fold dilutions of inhibitors in growth medium, followed by incubation for 7–11 days. Viral replication was measured by a p24 assay (INNOTEST HIV Antigen mAb; Innogenetics, Ghent, Belgium).

At least two independent experiments were performed for each analysis and each assay was set up in duplicate wells. Dose–response curve parameters were estimated using the sigmoidal dose–response (variable slope) equation in Prism version 4.0c for Macintosh (GrahPad Software, San Diego, California USA, www.graphpad.com).

Time-of-addition experiments

Time-of-addition experiments were set in single-round viral infectivity assays to measure the antiviral activity of P3 against HIV-1 (strain SG3.1) and HIV-2 (strain ROD) at different time-points. T-20 and AMD3100 were used as controls for inhibiting different stages of viral entry. TZM-bl cells were infected with 200 $TCID_{50}$ of HIV-1SG3.1 in the presence of P3 (129 nmol/l), T-20 (1 nmol/l), or AMD3100 (58 nmol/l) or with 200 $TCID_{50}$ of HIV-2ROD in the presence of_ P3 (876 nmol/l) or AMD3100 (32 nmol/l). These concentrations correspond to two-fold the IC_{90} value of each drug [27]. The peptide was added at various times either before or after infection. In the former, the peptide was added both to cells and viruses in separate. When added to cells only, an additional washing step with PBS (twice with 200 μl) was performed before proceeding to infection. Luciferase expression was quantified after 48 h of infection as mentioned above.

Antigenic reactivity assay

A new ELISA assay was developed to measure antigenic reactivity of peptides in HIV-infected patients using an ELISA protocol similar to the one described for the binding assay. Briefly, microwells were independently coated with each peptide at a concentration of 10 μl/ml in PBS solution and incubated overnight at 4°C. After blocking with BSA, 100 μl of a 1 : 300 dilution of plasma samples collected from 29 HIV-2 and 30 HIV-1-infected patients (all naive to T-20) in PBS-T was added and incubated for 1 h at 37°C. Wells were then washed six times with PBS containing 0.1% of Tween 20 and a 1 : 2000 dilution of goat antihuman immunoglobulin G (Fc specific) conjugated to alkaline phosphatase (Sigma-Aldrich) in PBS-T was added. Following incubation, the color was developed and optical densities were measured as described above. The clinical cut-off value of the assay, calculated as the mean optical density value of HIV-seronegative samples + two times the SD, was determined using samples from healthy HIV-seronegative individuals ($n = 10$). The results of the assay are expressed quantitatively as optical $\text{density}_{\text{clinical sample}}$/$\text{optical density}_{\text{cut-off}}$ ratios (optical density/cut-off ratio).

Selection of P3 resistance mutations

Primary HIV-1 and HIV-2 strains were used for selection of resistance mutations to P3 in PBMCs, using a standardized procedure as described elsewhere [35].

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Phytohemagglutinin-stimulated PBMCs were infected at a multiplicity of infection of 0.01 and selections started with concentrations below the IC_{50} level. Viral replication was monitored weekly by p24 antigen assay. Culture supernatants were harvested for subsequent genotypic analysis by sequencing. To this end, RNA was extracted using QIAmp viral RNA Mini Kit (Qiagen, Germany), according to manufacturer's instructions, and reverse transcribed using Titan One Tube RT-PCR System (Roche, Switzerland). The *env* gene (positions 6203–8817 in HIV-1 HXB2 and positions 6673 - 9268 in HIV-2 BEN) was amplified by nested PCR, with the Expand Long Template PCR System kit (Roche, Switzerland) and sequenced. Primers used for amplification and sequencing are described in Support Digital Content 2 and 3, http://links.lww.com/QAD/A306.

Statistical analysis

Statistical analyses were performed using Prism version 4.0c for Macintosh (GrahPad Software) with a level of significance of 5%.

Results

Design and structure of ancestral peptides

Maximum likelihood methods were used to reconstruct ancestral transmembrane protein sequences at several nodes of the envelope gene phylogenies that represent ancestors to diverse HIV and SIV virus clades (Fig. 1a). These sequences were aligned (Fig. 1b) and three peptides were derived covering different functional domains of the HR2: P1 (36 mer), P2 (42 mer), and P3 (34 mer) (Fig. 1c). Due to high hydrophobicity, P1 and P2 were very difficult to synthesize and reconstitute in an appropriate buffer suitable for cell culture assays. Therefore, only P3 was analyzed for antiviral activity. P3 overlaps the N-terminal pocket-binding region and heptad repeat core of the HR2 region (positions 628–661 of HIV-1 HXB2 Env). It differs by 21 amino acids from the consensus HIV-1 sequence and by six amino acids from consensus HIV-2. However, the positions *a* and *d* of the heptad repeat, considered critical for HIV-1 HR1/HR2 binding [36], were quite conserved. There were only four changes: I635V and Y638L, involving amino

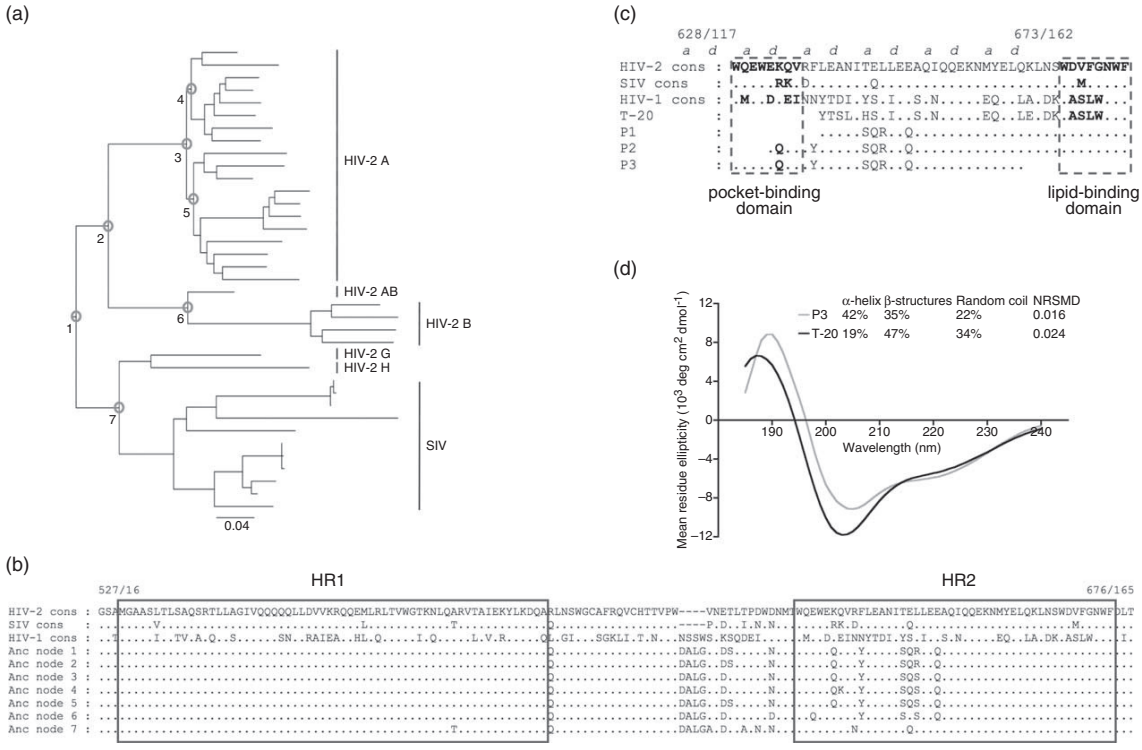

Fig. 1. Sequence and structure of ancestral peptides. (a) Ancestral reconstruction of HIV-2 gp36 sequences; the interior nodes highlighted by numbered circles in the phylogenetic tree correspond to the ancestral sequence used for peptide design. The scale bar represents evolutionary distances in substitutions per site. (b) Alignment of the gp41/gp36 HR1-HR2 segment, containing HIV-2, simian immunodeficiency virus (SIV), and HIV-1 consensus sequences, as well as the ancestral sequences. Points represent similarity relative to HIV-2 consensus and dashes represent gaps in the alignment. Sequences are numbered according to HIV-1 HXB2, Env position/gp41 position. (c) Alignment of consensus HR-2 sequences from HIV-2, SIV, and HIV-1 with T-20, P1, P2, and P3. Sequences are numbered according to HIV-1 HXB2, Env position/gp41 position. Positions *a* and *d* of HR2 represent the residues involved in HIV-1 HR1/HR2 interaction. The pocket-binding domain and lipid-binding domains are highlighted. (d) Circular dichroism spectra for P3 and T-20 peptides. β-structures include sheets and turns. NRSMD, normalized root mean square deviation.

acids with hydrophobic side chains, and L645R and S649A, involving amino acids from different chemical groups.

T-20 and P3 overlap in 24 amino acids of the heptad repeat core and diverge in 14 residues (58%). P3 is more hydrophilic than T-20 (percentage of hydrophilic residues is of 62% in P3 and 56% in T-20) [37] and its predominant secondary structure in solution is an α-helix (42%). T-20 has a lower content of regular secondary structure elements in solution with only 19% of helicity, as previously reported (Fig. 1d) [38].

P3 is a potent inhibitor of HIV-1 and HIV-2 infection

The antiviral activity of peptide P3 was first evaluated in single-round viral infectivity assays against 20 group A HIV-2 isolates, of which 19 were primary isolates, and nine HIV-1 isolates, including seven highly diverse primary isolates (Support Digital Content 4, http://links.lww.com/QAD/A306). P3 potently inhibited both HIV-2 and HIV-1 infection (Table 1). Remarkably, P3 was significantly more active against HIV-1 than against HIV-2 (IC_{50} were 63.8 nmol/l for HIV-2 vs. 11 nmol/l for HIV-1, $P < 0.0001$). Similar results were obtained in the viral replication inhibition assay in PBMCs. P3 inhibited HIV-2 and HIV-1 replication in PBMCs at IC_{50} of 109.3 and 12.1 nmol/l, respectively ($P < 0.0001$; Support Digital Content 5, http://links.lww.com/QAD/A306). No cytotoxicity was observed *in vitro* either in TZM-bl culture cells or primary PBMCs at all concentrations tested (up to 20 μmol/l). P3 did not inhibit the entry of a pseudovirus carrying the vesicular stomatitis virus envelope glycoprotein (VSV-G) indicating that its antiviral activity is HIV-envelope specific (Support Digital Content 6, http://links.lww.com/QAD/A306).

Compared with T-20, P3 was significantly more active as judged by the IC_{50} against HIV-2 and significantly less active against HIV-1 ($P < 0.0001$ for both cases; Table 1 and Fig. 2). Nonetheless, P3 and T-20 had similar IC_{90} ranges (P3, 6.2–1785.5 nmol/l; T-20, 0.5–1285.3 nmol/l) and similar dose–response curve slopes when tested against HIV-1, predicting similar antiviral activity *in vivo* [39,40]. Importantly, the antiviral activity of P3 and T-20 was null after washing cells that were preexposed to these peptides with PBS (Fig. 3a). In contrast, AMD3100 remained fully active. P3 lost almost 30% of its activity when added 3 h after virus infection but sustained its potent activity even when added 3 h prior to infection. In all, these results indicate that P3 is quite stable and that it inhibits viral fusion by binding to the virus particles and not to the cell.

P3 and T-20 bound strongly to rgp36, a recombinant HIV-2 transmembrane protein in an ELISA assay (30-fold above the cut-off for P3; Fig. 3b). This suggested that the activity of P3, like that of T-20 and other fusion inhibitor peptides, was mediated through strong binding to its putative target in the transmembrane glycoprotein of the envelope complex [41]. To investigate the binding activity of these peptides to HIV-1, we expressed the rgp41 polypeptide equivalent to the rgp36 polypeptide. However, neither P3 nor T-20 bound to rgp41 in the ELISA assay (data not shown) suggesting that unlike rgp36, rgp41 does not form the coiled-coil structure to which T-20 (and most likely P3) bind in the virus particles [42]. Why rgp41 does not assume this coiled-coil structure and rgp36 apparently does is unknown at this time.

The antigenicity of P3 and T-20 peptides was examined with plasma samples collected from HIV-2-infected and HIV-1-infected patients, all naive to T-20. The majority of HIV-2 patients (93%) had P3-specific antibodies, whereas 45% had T-20-specific antibodies (data not shown). This was expected as the amino acid sequence of P3 was derived from an ancestral HIV-2/SIV sequence. Likewise, the majority of HIV-1 patients (90%) had T-20-specific antibodies, whereas 67% had P3-specific antibodies. Of note, the mean binding affinity of the T-20-specific antibodies from HIV-1 patients was significantly higher compared to P3-specific antibodies (Fig. 3c). These results demonstrate that P3 is weakly antigenic in HIV-1 patients compared to T-20.

Table 1. Antiviral activity of P3 and T-20 against HIV-1 and HIV-2 primary isolates.

Parameter[a]	P3 (nmol/l)	T-20 (nmol/l)[b]	*P* value[c]
HIV-1 ($n = 9$)			
IC_{50} (95% CI)	11 (6.5–18.4)	1.2 (0.7–2.2)	<0.0001
IC_{90} (95% CI)	366.4 (117.5–1145.5)	95.9 (26.3–350.8)	0.107
Hill slope (95% CI)	0.6 (0.4–0.82)	0.5 (0.4–0.6)	0.263
HIV-2 ($n = 20$)			
IC_{50} (95% CI)	63.8 (51.9–78.5)	281.5 (223.2–354.9)	<0.0001
IC_{90} (95% CI)	709.6 (435.5–1158.8)	3881.5 (2393.3–6280.6)	<0.0001
Hill slope (95% CI)	0.9 (0.7–1.1)	0.8 (0.7–1)	0.492

[a]IC_{50}, IC_{90}, and Hill slope best-fit values were inferred from sigmoidal dose–response (variable slope) curves adjusted to combined results of HIV-1 and HIV-2 isolates, and represent geometric mean values; 95% CI – 95% confidence interval.
[b]T-20 susceptibilities were obtained for the same HIV-1 and HIV-2 viral panel [27].
[c]*P* value for comparison of best-fit values using the *F*-test.

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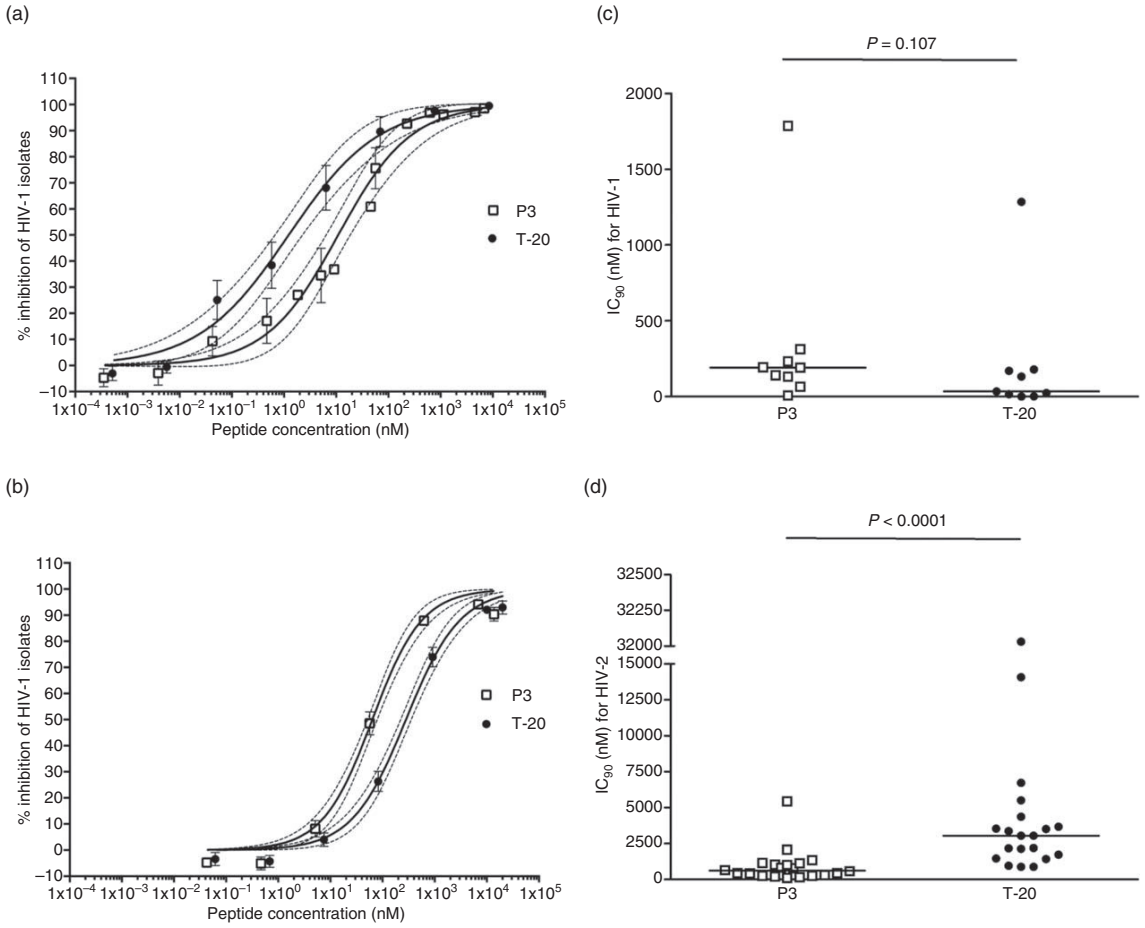

Fig. 2. Antiviral activity of P3. Representative dose–response curves for HIV-1 (a) and HIV-2 (b) with P3 and, for comparison, T-20 [27]. Data points represent the average of results obtained for HIV-1 ($N = 9$) and HIV-2 ($N = 20$) primary isolates; bars represent standard error of the mean. Sigmoidal dose–response (variable slope) curves were adjusted to these data points; dashed lines represent the 95% confidence band of the best-fit curve. Mean IC_{90} values of P3 and T-20, for HIV-1 (c) and HIV-2 (d) primary isolates. Bars represent median values. *P* values were obtained comparing best-fit values inferred from sigmoidal dose–response (variable slope) curves using the *F*-test.

P3 inhibits the replication of most T-20-resistant HIV-1 variants

To determine whether P3 is able to inhibit the infection of HIV-1 strains resistant to T-20, we measured the susceptibility of HIV-1 variants carrying well defined T-20 resistance mutations to P3 [43,44]. Notably, P3 exhibited potent activity against all but one T-20-resistant strains (IC_{50} range, 0.15–11.8 nmol/l; Table 2). The mutation N43K conferred high-level resistance to both P3 (2140-fold) and T-20 (2677-fold). Moreover, the V38A/N42D mutations conferred increased susceptibility to P3 (seven-fold lower IC_{50} compared to the wild-type). These results suggest that P3 could be used as an alternative for the treatment of patients infected with T-20-resistant HIV-1 strains.

Selection of P3-resistant variants

To investigate the mechanism of action and the pathways of resistance to P3, resistance mutations were selected *in vitro* by repeated passage of HIV-1 and HIV-2 primary isolates in PBMCs in the presence of either constant or increasing concentrations of P3, according to the viral replication capability [35]. For HIV-1, the N43K substitution in the HR1 region of gp41 was the only mutation selected in *env* after 59 days in culture (eight passages) in the presence of 212 nmol/l of P3. Inhibition of replication of this mutant virus with P3 occurred at an IC_{50} of 1.9 μmol/l and IC_{90} of 13.1 μmol/l, which represent a 120-fold and 56.4-fold decrease in susceptibility, respectively. Under the same experimental conditions and despite repeated attempts, we were not able to select P3-resistant HIV-2 isolates. Collectively, these results indicate that the HR1 region in the transmembrane glycoprotein is the target of P3 and suggest that the pathway of HIV-1 resistance to P3 differs from that of T-20 and the genetic barrier to P3 resistance is significantly higher in HIV-2 than in HIV-1.

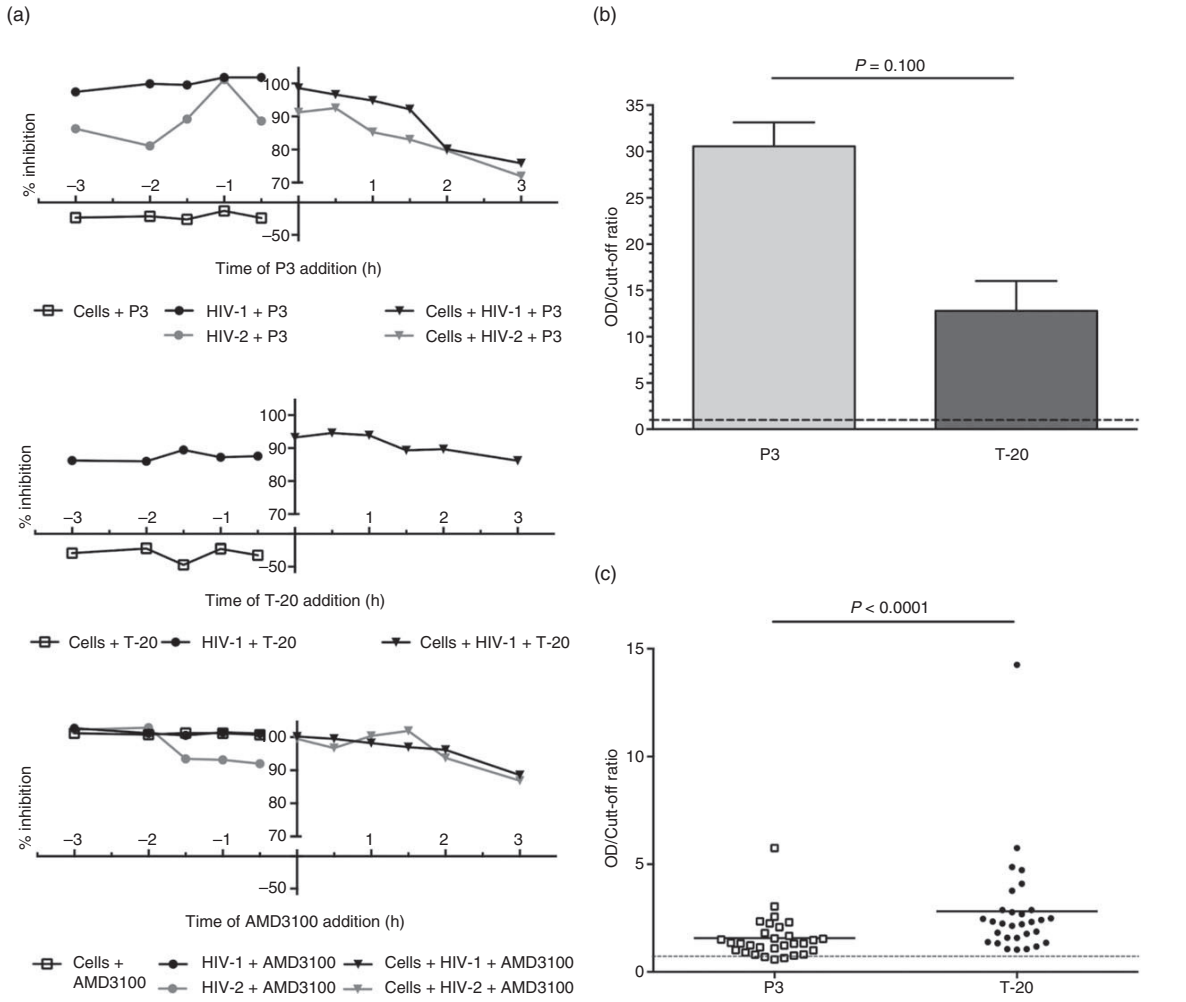

Fig. 3. Mechanism of action and antibody reactivity of P3. (a) Time-of-addition assays for P3 with HIV-1 and HIV-2. T-20 and AMD3100 were used as control drugs. In these experiments, each drug was added at different time-points either before (cells and viruses, separately) or after HIV infection. (b) Binding activity of P3 and T-20 to HIV-2 rgp36 in an ELISA assay. (c) Antibody reactivity of P3 and T-20 in HIV-1-infected patients. Bars represent mean values. Results from P3 are in open squares and from T-20 are in closed circles. *P* values were obtained with the Mann–Whitney *U*-test.

Table 2. Comparison of antiviral activity of P3 and T-20 on T-20-resistant HIV-1 variants.

		P3		T-20		
HIV-1 variant	Susceptibility to T-20[a]	IC_{50} nmol/l (95% CI)	Fold-increase[b]	IC_{50} nmol/l (95% CI)	Fold-increase[b]	*P* value[c]
NL4-3 D36G (parental)	S	0.4 (0.2–1.2)	1	0.03 (0.01–0.06)	1	0.0002
NL4-3 (D36G) V38A	R	1.5 (0.5–5.1)	3.8	43.8 (21.8–87.8)	1460	<0.0001
NL4-3 (D36G) V38A/N42D	R	0.06 (0.01–0.3)	0.15	118.2 (63.0–221.7)	3940	<0.0001
NL4-3 (D36G) V38A/N42T	R	4.7 (1.9–11.5)	11.8	482.0 (324.1–716.8)	16 066.7	<0.0001
NL4-3 (D36G) N42T/N43K	R	855.9 (628.0–1167.0)	2139.8	80.3 (61.1–105.6)	2676.7	<0.0001

CI, confidence interval.
[a]Sensitive (S) or Resistant (R).
[b]Fold-increase of IC_{50} concentration relative to NL4-3 D36G (parental).
[c]*P* value for comparison of best-fit values between P3 and T-20, using the *F*-test.

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Discussion

We show here that an ancestral peptide (named P3) derived from the HR2 domains of HIV-2 and SIV transmembrane glycoproteins potently inhibits both HIV-1 and HIV-2 cell entry and replication. The rationale for using ancestral sequences of the transmembrane envelope glycoprotein as a source for the new antiviral peptide was to minimize HIV sequence divergence by tracing the most likely evolutionary path along the phylogeny and capture more conserved structural features of the HR2 sequences [45,46]. The potent activity of P3 against divergent HIV-1 and HIV-2 primary isolates demonstrates that our strategy was highly successful. The potent activity of P3 on HIV-1 was not expected but it might be due to the conservation in P3 of the residues located in critical positions involved in the HR1/HR2 interaction (*a* and *d* residues) [36] and/or to the presence of an alanine at position 22 (corresponding to position 138 in gp41). An alanine at this position increases the binding affinity of HR2 region to HR1 and enhances the activity of HR2-based HIV-1 fusion inhibitor peptides [47].

The N43K mutation in the HR1 region of HIV-1 was sufficient to confer high-level resistance to this peptide. These results suggest that, like T-20, P3 acts by selectively binding to the HR1 region in the transmembrane glycoprotein of HIV-1. However, P3 potently inhibited the replication of T-20-resistant viruses bearing the V38A and/or the N42D resistance mutations indicating that the pathway of HIV-1 resistance to P3 differs from that of T-20. We were not able to generate HIV-2 isolates resistant to P3 even after 60 days in culture suggesting that the genetic barrier for resistance to P3 might be significantly higher in HIV-2 than in HIV-1.

Peptides with high helical content like P3 tend to be more stable and less susceptible to proteolytic degradation in biological fluids than unstructured peptides like T-20 [7,13]. In addition, they usually display high anti-HIV-1 potency due to an increased binding affinity for HR1 [48,49]. Indeed, we showed that the potent anti-HIV-1 activity of P3 was conserved for at least 3 h. Moreover, P3 bound strongly to a recombinant HIV transmembrane protein. Hence, the high helical content may also contribute to the potent antiviral activity of P3.

Antibodies present in the plasma of HIV-1-infected patients reacted poorly with P3 as compared to T-20. This was expected as HIV-1 and HIV-2 differ significantly in the HR2 region and antibodies naturally generated against this region in HIV-1 patients were unlikely to bind to a peptide derived from HIV-2/SIV. As drug-specific antibodies can compromise the clinical efficacy of therapeutic proteins either by preventing their exposure to the active site or by decreasing their half-life [50], the weaker antigenicity of P3 in HIV-1-infected patients might translate into a better bioavailability profile and durable clinical efficacy of P3 in HIV-1-infected patients.

Conclusion

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In summary, we successfully derived an ancestral peptide (P3) with low antigenicity, high stability, and potent activity against both HIV-1, including variants resistant to T-20, and HIV-2. Our findings provide proof of principle that potent antiviral peptides can be constructed using evolutionary biology strategies. Such strategies should be explored to enhance the production of antiviral peptide drugs, microbicides, and vaccines.

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The following Research and Institutes of He [55–57]; pNL4-3 [43], pSG3.1 [58], pHEF-VSVG [59] and pSG3Δenv [52,60] plasmids; T-20-resistant pNL4-3 gp41 (36G) variants [43,44]; T-20 (enfuvirtide) fusion inhibitor; bicyclam JM-2987 (hydrobromide salt of AMD-3100) coreceptor antagonist [61–63]. pROD10 plasmid was a gift from Keith Peden [64].

P.B., A.Q., and N.T. conceived and designed the experiments. P.B., R.C., J.M.M., H.B., P.P., and A.Q. performed the experiments. P.B., A.Q., and N.T. analyzed the data. P.B. and N.T. wrote the article.

Conflicts of interest

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None declared.

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