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Compensatory mutations rescue the virus replicative capacity of VIRIP-resistant HIV-1

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ABSTRACT

VIRIP has been identified as a highly specific natural inhibitor of HIV-1 that blocks HIV-1 gp41-dependent fusion by interacting with the gp41 fusion peptide. Two analogues of VIRIP (VIR-353 and VIR-576) with a few amino acid changes increase its antiretroviral potency by two orders of magnitude in cell culture. VIR-576 has been shown effective in a phase I/II clinical trial. Resistance to VIRIP and its analogue VIR-353 were generated after long-term passage in cell culture suggesting a high genetic barrier to resistance. Mutations conferring resistance to VIRIP and VIR-353 significantly reduced virus fitness. However, accumulation of additional mutations rescued the replication capacity of the virus while retaining resistance to VIR-353 and full sensitivity to T20. Combinations of VIR-353 and T20 had an additive effect on the inhibition of wild type HIV-1 replication, but only a single agent was active when combinations were tested against T20-resistant HIV-1, suggesting that both gp41 peptides do not interfere with each other in their binding to gp41. Our results provide additional support to the development of a new class of antiretroviral agents targeting gp41-dependent fusion.

1. Introduction

The process of HIV entry is a validated and relevant target for anti-HIV intervention (Esté and Telenti, 2007). Many agents targeting HIV-1 entry have been tested as putative antiretrovirals, two of them being approved (Broder, 2010) for use in HIV+ individuals: maraviroc (MVC), a CCR5 antagonist, and enfuvirtide (T20), a gp41-derived peptide that binds to the gp41 N-terminal heptad repeat (HR1) and blocks virus-cell fusion (Gulick et al., 2008; Tilton and Doms, 2010). The approval of T20 as an antiretroviral therapy has led to the development of second generation HIV fusion inhibitors with similar or improved potency. Importantly, second generation fusion inhibitors are active against T20 resistant viral strains and resistance to these new fusion inhibitors may not confer cross-resistance to T20, suggesting that new agents may be designed as alternative fusion inhibitors (Tilton and Doms, 2010).

A natural circulating 20-residue peptide, named virus-inhibitory peptide (VIRIP) was discovered as a potent inhibitor of HIV-1 replication (Münch et al., 2007). VIRIP was identified as a human hemofiltrate-purified fragment of the serine protease inhibitor α1-antitrypsin. VIRIP inhibited a wide variety of HIV-1 strains including those resistant to T20 and other antiretroviral drugs (Gonzalez et al., 2011; Münch et al., 2007). Results showed that either VIRIP or other peptidic analogues with a few amino acid changes that conferred greater anti-HIV potency (VIR-353 and VIR-576, Fig. 1), blocked HIV Env-dependent fusion through their interaction with gp41. A clinical phase I/II trial with VIR-576 showed reductions in plasma viral load of more than one order of magnitude after short-term monotherapy and without significant adverse events in patients (Forssmann et al., 2010).

The genetic barrier for HIV resistance to T20 is low, generally restricted to the amino acid motif between gp41 residues 36 and 45, that are part of the T20 binding site (Greenberg and Cammack, 2004; Menendez-Arias, 2010) and are commonly associated with a significant decrease in the replicative capacity of the virus (i.e., virus fitness). Conversely, the genetic barrier for resistance to VIRIP and its analogues appeared to be relatively high. Initially, a VIRIP-resistant virus did not emerge after 2 months of weekly passages in the presence of VIRIP (Münch et al., 2007). Long-term passage of virus in cell culture (>450 days) in the presence of a potent VIRIP analogue (VIR-353) was necessary to generate a VIRIP-resistant strain (Gonzalez et al., 2011). VIR-353-resistant HIV-1 accumulated up to seven mutations located throughout the *env* gene (Table 1), indicating a complex mechanism requiring a coordinated action of both gp41 and gp120. Site-directed mutagenesis confirmed the role of specific mutations and identified a combination of three mutations (A433T/V489I/V570I) as the most relevant to VIRIP resistance.

Here, we investigate the role of VIRIP-resistance mutations in the replicative capacity of HIV-1. We have found that mutations required for resistance significantly reduced virus fitness and

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VIRIP:   LEAIPMSIPPEVKFNKPFVF
VIR-353: LEAIPCSIPpCFLFNKPFVF
VIR-576: LEAIPCSIPPEFLFGKPFVFx2
VIR-SCR: KVINPEPIVEPFMSKPFALF
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Fig. 1. Sequence of VIRIP and its analogues VIR-353 and VIR-576. p: D-proline. Cysteine residues are linked via a disulphide bridge. Bold letters indicate changes to the VIRIP sequence. VIR-SCR: VIRIP scrambled sequence.

compensatory mutations rescued virus replicative capacity of VIRIP-resistant HIV-1, explaining the long-term required for the resistant virus to emerge.

2. Materials and methods

2.1. Cells, viruses and compounds

CD4+ lymphoid cell lines MT-4 were obtained through the Medical Research Council (MRC) Centre for AIDS Reagents, London, UK. Lymphoid cells or TZM-bl cells were grown in RPMI 1640 or DMEM (Invitrogen, Barcelona, Spain) and supplemented with 10% foetal calf serum (FCS, Cambrex, Barcelona, Spain) and antibiotics, 2 U/ml penicillin and 2 μg/ml of streptomycin (Invitrogen, Barcelona, Spain). MT-4/CCR5+ (expressing both CCR5 and CXCR4) were generated as described before (Armand-Ugón et al., 2010).

The HIV-1 strains BaL, HXB2 and NL4-3 were obtained from the MRC Centre for AIDS Reagents (London, UK). The HIV-1 NL4-3 strain, resistant to T20 has been described elsewhere (Armand-Ugón et al., 2003; Menendez-Arias and Esté, 2004). The AMD3100-resistant HIV-1 has been reported elsewhere (Armand-Ugón et al., 2010; Esté et al., 1999; Menendez-Arias and Esté, 2004). The VIR-353, VIRIP-cross resistant virus was selected in cell culture as described before (Gonzalez et al., 2011).

VIRIP, VIR-353 and a scrambled version of VIRIP (VIR-SCR) were synthesized by New England Peptide (Gardner, MA) according to the peptide sequences described by Münch et al. (2007) and He et al. (2008a, b) (Fig. 1). AMD3100, T20 and the broadly neutralising monoclonal antibodies 2F5 and 4E10 were obtained from the NIH AIDS Reagent Program. The RT inhibitor 3-azido-3-deoxythymidine (zidovudine; AZT) was purchased from Sigma–Aldrich (Madrid, Spain).

2.2. Anti-HIV and cytotoxicity assays

Anti-HIV activity and cytotoxicity measurements in MT-4 cells were based on viability of cells that had been infected or not infected with the corresponding HIV-1 strain at a multiplicity of infection (moi) of 0.003 and exposed to various concentrations of the test compound. After 5 days of incubation at 37 °C with 5% CO_2, the number of viable cells was quantified by a tetrazolium-based colorimetric method (MTT method) commonly used in the evaluation of anti-HIV active drugs (Ballana et al., 2011, 2009; Moncunill et al., 2008a, b; Pannecouque et al., 2008). Cut-off value in which a virus was considered resistant was a 5-fold increase of the EC_{50} when compared to the wild type virus due to the variability of the MTT assay in MT-4 cells. Anti-HIV activity determinations were performed in triplicates and data represents the mean of three independent experiments. For drug combinations, checkerboard 1:5 drug dilutions were prepared and evaluated as described above. Combination indexes (CI) were calculated according to the isobologram analysis of drug combinations, following the equation $CI = [(D)_1/(D_m)_1] + [(D)_2/(D_m)_2]$ where $(D)_1$ and $(D)_2$ are the doses of compounds 1 and 2 that in combination produce some specified effect and $(Dm)_1$ and $(Dm)_2$ are the doses of the chemicals that when applied singly also have the same effect (Fernandez-Piñas et al., 2010). CI values below 0.9 are synergistic, between 0.9–1.1 are additive and >1.1 are antagonistic (Férir et al., 2011).

Table 1
Amino acid changes identified in the VIR-353 resistant virus.

Order of appearance	Mutation	Gene
1	V570I	gp41
2	A433T	gp120
3	V489I	gp120
4	L545M	gp41
5	T244S	gp120
6	A612T	gp41
7	N625K	gp41

2.3. Sequence analysis of HIV strains

Genomic DNA from infected cells was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Barcelona, Spain). Extracted DNA was used to PCR amplify HIV-1 *env* gene using Expand High Fidelity PCR System (Roche) and the primers (Forward 5′-aagggccacagagggagccat-3′ and Reverse 5′-gcgtcccagaagttccacaa-3′). Before sequencing, the amplified DNA was purified by enzymatic cleanup that eliminates unincorporated primers and dNTPs (Exosap-IT™, GE Healthcare). Sequencing of amplified DNA was carried out with different primers to ensure obtaining the complete *env* sequence with the BIGDYE Terminator 3.1 Kit (Applied Biosystems, Madrid, Spain) as described before (Armand-Ugón et al., 2005; Moncunill et al., 2008a). Data were collected with the ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Madrid, Spain). Sequences were analysed with the Sequencher 4.5 software and edited with the BioEdit software. Amino acid positions were numbered according to HXB2 (Los Alamos database).

2.4. Virus replicative capacity

Virus stocks were titrated in CD4+ TZM-bl cells expressing the *LacZ* gene driven by the HIV-1 long terminal repeat (LTR) and virus infection was monitored by determination of β-galactosidase (β-gal) production as previously described (Ballana et al., 2009). The dilution of virus stocks necessary to induce 0.5 β-gal absorbance units were used to infect lymphoid MT-4 cells as described above. Virus growth kinetics were followed by the MTT colorimetric method and plotted to the wild type HIV-1 NL4-3 strain. The Student's *t* test was used to determine statistical significance between values. Values were considered significant when $^{*}p < 0.01$, $^{**}p < 0.001$.

3. Results

3.1. Anti-HIV activity of VIR-353

We have previously shown that VIR-353 was active against virus resistant to BMS-155, AMD3100, TAK-779 or nevirapine and the VIR-353-resistant virus was cross-resistant to VIRIP but remained sensitive to T20, AMD3100 or AZT (Gonzalez et al., 2011). VIRIP and VIR-353 were also active against two different T20-resistant HIV-1 strains, one derived from the HIV-1 HxB2 strain (HC43) and one derived from the HIV-1 NL4-3 strain (NT38), which is also 5-fold resistant as compared to HxB2, due to the G36V mutation in gp41 that has been associated with resistance to T20. Two monoclonal antibodies targeting gp41 (2F5 and 4E10) were similarly active against the VIR-353-resistant virus (Table 2), highlighting differences in the mode of interaction of these agents with gp41 as compared to VIRIP. We observed that the VIRIP-resistant virus was 50-fold and 10-fold hypersensitive

Table 2
Antiviral activity of VIRIP and VIR-353 against T20 resistant strains.

Compound	HIV-1 NL4-3	EC_{50}[a] (μM) [FC][b]				CC_{50}[c] (μM)
		HXB2	T20-Resistant (NT38)	T20-Resistant (HC43)	VIR-353/VIRIP-Resistant	No virus
VIRIP	22	38.14 [1]	55.22 [2]	51.44 [2]	>86.81 [N.A.]	>86.81
VIR-353	0.6	0.55 [1]	0.37 [1]	1.08 [2]	29.18 [49]	>43.8
VIR-SCR	>17.36	>17.36 [N.A.]	>17.36 [N.A.]	>17.36 [N.A.]	>17.36 [N.A.]	>17.36
T-20	0.1	0.02 [0]	1.41 [14]	>2.42 [N.A.]	0.002 [0]	>2.42
2F5	0.2	ND	ND	ND	0.2	>10
4E10	1	ND	ND	ND	1	>10

Values represent the mean of three independent experiments each one done in triplicate. ND: not determined; N.A.: not applicable.
[a] Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4 cells.
[b] FC: fold change or ratio of the corresponding EC_{50} and the EC_{50} value of the corresponding wild type HIV-1 strain.
[c] Concentration required to induce cell death by 50% as measured by the MTT method in MT-4 cells.

to T20 when compared to the NL4-3 and HxB2 strains, respectively. This effect was dependent on two mutations, D36G and T37I, found in the gp41 of the VIRIP-resistant virus that appeared after passage in cell culture for more than 400 days. Mutants D36G and T37I have been previously associated with resistance to T20 (Menendez-Arias, 2010).

3.2. Reduced replicative capacity of VIR-353/VIRIP-resistant virus

To determine the effect of mutations conferring resistance to VIRIP in virus replicative capacity (fitness), the concentration of virus required to induce 0.5 absorbance units of β-galactosidase activity after infection of HeLa TZM-bl cells was used to infect lymphoid MT-4 cells. Virus growth kinetics were followed by the MTT colorimetric method and plotted relative to the wild type HIV-1 NL4-3 strain (Fig. 2). Virus passages selected were those in which emerging mutations were first identified by sequencing of the proviral DNA of infected cells (Tables 1 and 3).

The V570I mutant had a significantly lower fitness that the parental wild-type strain. Further passages maintained a relative low fitness but increased drug-resistance without a change in T20 susceptibility (Table 3). Further passage of virus led to a significant increase in virus fitness while retaining the VIR-353/VIRIP resistant phenotype (Fig. 2). However, virus isolated after passage 62 showed a marked increase (50-fold) in susceptibility to T20 (Table 3). Taken together, these results suggest that mutations conferring resistance to VIRIP had a significant cost in virus fitness, but complementary mutations in both gp120 and gp41 allowed the recovery of the virus replicative capacity.

3.3. Combinatorial effect of VIRIP and T20

Since resistance to VIRIP may be affecting the sensitivity to T20 we evaluated the effect of combinations of both agents and AZT as a control unrelated drug (Fig. 3). Checkerboard combinations of VIR-353 with T20 or VIR-353 with AZT demonstrated an additive effect when tested against wild-type NL4-3 (Fig. 3A and B) with a mean CI of 0.99 ± 0.52 and 0.97 ± 0.27, respectively. Only VIR-353 was active when evaluating its antiviral activity in combination with T20 against a T20-resistant virus (Fig. 3D), whereas the combination of VIR-353 and AZT showed an additive effect (mean CI 1.09 ± 0.26) as with the wild-type virus (Fig. 3C). These results

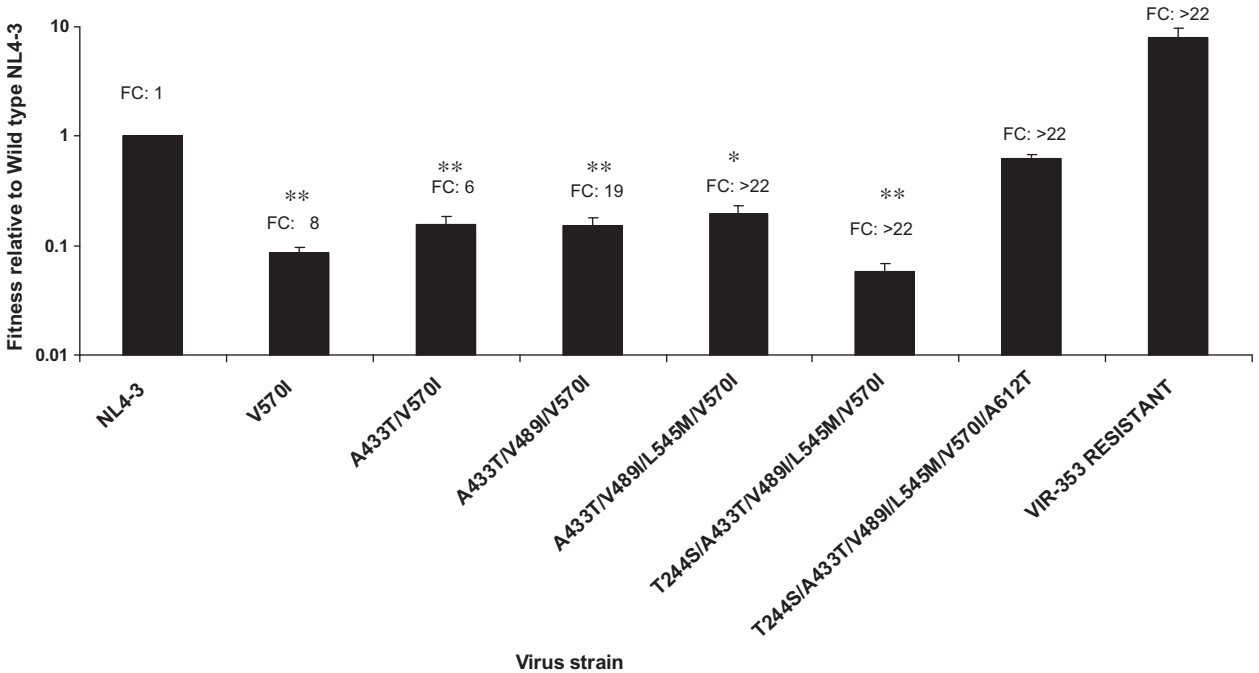

Fig. 2. Replicative capacity of the viral strains isolated at different time points during the selection of the VIRIP resistant virus. The concentration of virus required to induce 0.5 absorbance units of β-galactosidase activity after infection of HeLa TZM-bl cells was used to infect lymphoid MT-4 cells. Virus growth kinetics were followed by the MTT colorimetric method and plotted relative to the wild type HIV-1 NL4-3 strain. Bars represent the mean and SD of three independent experiments. FC: Fold-change in EC_{50} of the corresponding virus relative to the wild type NL4-3 strain. Statistical significance (Student's *t* test) compared to the parental HIV-1 NL4-3 strain is show as $p < 0.01$(*), $p < 0.001$(**).

Table 3
Antiviral activity VIRIP and VIR-353 against viral strains that emerged during the generation of resistance to VIRIP.

EC_{50}[a] (μM) [FC][b]								
Virus passage	0	25	44	51	56	62	83	90
Compound	Wild type	V570I	A433T/ V570I	A433T/V489I/ V570I	A433T/V489I/ L545M/V570I	T244S/A433T/V489I/ L545M/V570I	T244S/A433T/V489I/ L545M/V570I/A612T	VIR-353 resistant
VIR-353	0.6	4.77 [8]	3.33 [5]	11.14 [18]	>13.14 [>22]	>13.14 [>22]	>13.14 [>22]	>13.14 [>22]
VIRIP	22	42.47 [2]	29.92 [1]	>86.81 [>4]	>86.81 [>4]	>86.81 [>4]	>86.81 [>4]	>86.81 [>4]
T20	0.1	0.04 [1]	0.03 [1]	0.02 [1]	0.03 [1]	0.002 [0]	0.002 [0]	0.006 [0]
AZT	0.002	0.003 [2]	0.002 [1]	0.001 [1]	0.002 [1]	0.004 [2]	0.002 [1]	0.001 [1]
AMD3100	0.002	0.004 [2]	0.002 [1]	0.001 [1]	0.004 [2]	0.002 [1]	0.002 [1]	0.002 [1]

Values represent the mean of three independent experiments each one done in triplicate.
[a] Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4 cells.
[b] FC: fold change or ratio of the corresponding EC_{50} and the EC_{50} value of the corresponding wild type HIV-1 strain.

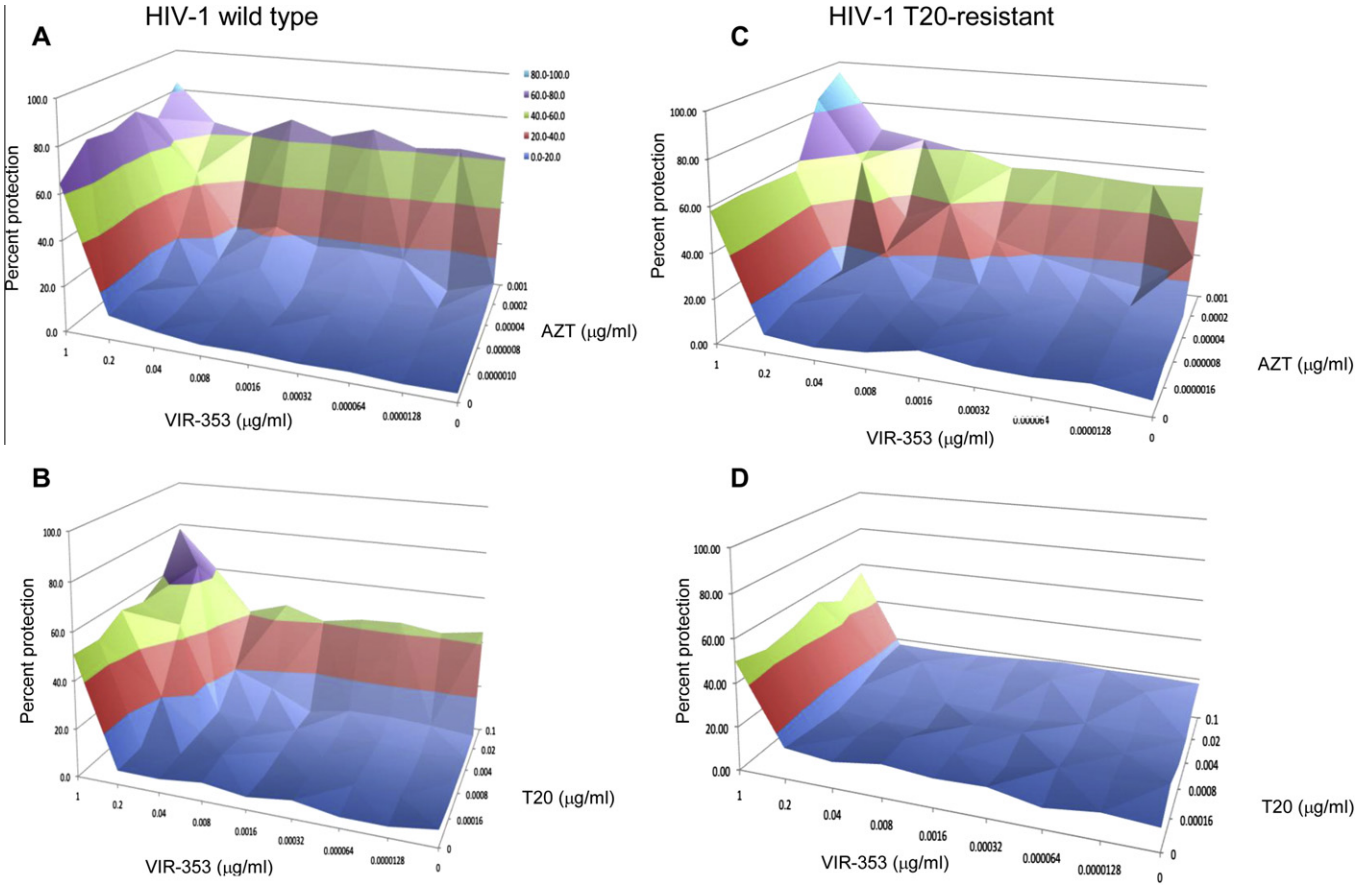

Fig. 3. Anti-HIV activity of drugs in combination with VIR-353. Checkerboard 1:5 drug dilutions starting at roughly the 50% effective concentration or below, were prepared and evaluated for anti-HIV activity in lymphoid MT-4 cells by the MTT colorimetric method. Graphs show the activity of combinations of VIR-353 with AZT (upper panels) or T20 (lower panels) in cells infected with wildtype (A, B) or T20-resistant (C, D) HIV-1 NL4-3 strains. Colours indicate the percentage of protection within a range. The figures show only one representative experiment out of three done in triplicate.

suggest that T20 and VIR-353 do not interfere with each other in their binding to gp41.

4. Discussion

The envelope gp120 has been selected by nature as a major target for neutralisation of HIV. However, through its high mutation rate, the virus fights back by easily generating neutralisation-resistant gp120 variants and decoys for the immune system. Although gp41 is also a target for neutralisation, raising antibodies that target the fusion peptide (FP) of gp41 is a difficult process (Blumenthal and Dimitrov, 2007) because it may not be readily available. Thus, the identification of natural inhibitors targeting gp41 such as VIRIP is a remarkable discovery in need of further evaluation. Importantly, VIRIP is being used as a template to generate new, more potent anti-HIV agents (Forssmann et al., 2010).

Here, we further explored the anti-HIV effect of VIRIP and its derivatives by evaluating the effect of VIRIP-resistant mutations in viral fitness. Generation of drug resistance took a long time to develop, indicating a high genetic barrier to resistance. Indeed, the first mutation selected by VIR-353 (V570I) induced a marked decrease in virus fitness. Two other mutations were also necessary to increase resistance and further 90 passages were needed to rescue virus replicative capacity. Notably, HIV-1 was able to overcome the cost of viral fitness by the incorporation of four additional mutations, leading to a fully competent VIRIP-resistant virus.

Unfortunately, our results do not allow to clearly delineate the mode of action of VIRIP, previously thought to bind to gp41 FP. We

could not discard a putative interaction between VIRIP and gp41 FP but the identified mutations are clearly located outside the fusion peptide. Moreover, mutations significantly affect virus sensitivity to both VIRIP and VIR-353, inducing a decrease in the replicative capacity of the virus, a hallmark of virus-drug resistance. The emergence of mutations throughout gp120 and gp41 suggests a general rearrangement of the virus envelope to compensate for the reduced replicative capacity.

Notably, virus passage in the presence of VIR-353 became hyper-susceptible to T20 as compared to the wild type NL4-3 virus that is naturally resistant to T20 when compared to other wild type HIV-1 strains such as HxB2. HIV-1 NL4-3 contains two polymorphisms (G36V and G36D) that are commonly associated to reduced sensitivity to T20 (Menendez-Arias, 2010). It has been shown that following discontinuation of T20, HIV-1 T20-resistant NL4-3 virus (D36) revert to the G36 sequence and therefore restoring replicative capacity associated with normal drug susceptibility (Greenberg and Cammack, 2004). The HIV-1 NL4-3 (D36/I37) and T20-resistant NL4-3 revert to the G36/I37 sequence within two weeks of growth in the absence of inhibitor. Thus, VIR-353/VIRIP appears not to exert selective pressure on the same amino acid changes that induce T20-resistance, suggesting a distinct mode of action for VIRIP and its more potent analogue. Combinations of VIR-353 and T20 showed an additive affect in blocking the replication of wild type HIV-1 but only the activity of a VIR-353 when evaluating the replication T20-resistant HIV. Taken together, these results suggest that VIRIP and its analogues may bind to gp41 in the presence of T20 and do not interfere with its anti-HIV activity.

Despite its efficacy, treatment with VIR-576 has drawbacks that include high treatment doses and intravenous injection or infusion. Furthermore, the large-scale production of peptides under GMP conditions is still relatively expensive. Nevertheless, the development of small-molecule inhibitors with an analogous mode of action that can be administered orally is ongoing (Forssmann et al., 2010). Our results shed light on the mechanism of resistance of VIRIP and its analogues and may help to identify new peptidic or non-peptidic agents with similar mode of action to VIRIP with an unique resistance profile.

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