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Inhibition of infectious human immunodeficiency virus type 1 particle formation by Gag protein-derived peptides

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Sequential overlapping Gag protein-derived oligopeptides of human immunodeficiency virus type 1 (HIV-1) 22 to 24 amino acids long, were synthesized and tested *in vitro* for antiviral activity. Two synthetic peptides, one derived from the matrix protein p17 (NPGLLETSEGCRQ, amino acids 47 to 59) and one located in the capsid protein p24 (PAATLEEMMTA, amino acids 339 to 349) inhibited the production of infectious virus when added to HIV-1-infected cultures when used in the range of 20 to 200 μg/ml. As shown by thin section electron microscopy, peptide treatment resulted in the release of immature, deformed virus particles suggesting that the two peptides interfered with assembly and maturation. Other Gag protein-derived oligopeptides had little or no influence on virus production. To characterize further the functionally active regions we synthesized peptide derivatives with three consecutive amino acids substituted by alanine; they did not cause inhibition. Therefore the regions responsible for inhibition were located between amino acids 50 to 61 in p17, and 342 to 350 in p24. These observations might lead to the development of a new antiviral strategy affecting the late stage of virus replication.

Human immunodeficiency virus type 1 (HIV-1), like many other complex enveloped viruses, is formed and released in a budding process, which involves a number of critical steps (Gelderblom, 1991; Wills & Craven, 1991). Distinct elements essential for particle formation have been localized on the structural proteins of HIV-1 using different expression systems and electron microscopy (Gheysen *et al.*, 1989; Hu *et al.*, 1990; Vernon *et al.*, 1991). These essential functions for particle formation are potential targets for inhibiting virus formation and maturation. The Gag protein is synthesized in the form of a polyprotein precursor ($Pr55^{gag}$), post-translationally modified at the amino-terminal end by myristoylation and endoproteolytically cleaved into four large (p17, p24, p7 and p6) and two small (p2 and p1) proteins by the virus-encoded protease (Henderson *et al.*, 1990). A variety of functions have been shown for the Gag proteins. Through the myristic acid modification the $Pr55^{gag}$ molecule is targeted to the inner leaflet of the cell membrane (Göttlinger *et al.*, 1988). The interaction of the viral genomic RNA with the potential 'zinc finger' motif of p7 is essential for packaging the RNA into the particle (Gorelick *et al.*, 1990; South *et al.*, 1991). Cleavage of $Pr55^{gag}$ at defined sites by the protease is required for maturation and infectivity of the virus (Tritch *et al.*, 1991); p6 seems to form the core–envelope link facilitating the closure of the core sphere (Göttlinger *et al.*, 1991; Höglund *et al.*, 1991). Furthermore, protein–protein interactions are required during the self-assembly process involving the accumulation of $Pr55^{gag}$ at the membrane and the viral core formation. We postulated that oligopeptides derived from domains participating in protein–protein interaction during the oligomerization of $Pr55^{gag}$ molecules might inhibit virus assembly and maturation.

In this work we defined amino acid sequences within the $Pr55^{gag}$ protein functionally involved in virus assembly. For this approach we used a library of synthetic peptides, 22 to 24 amino acids in length spanning the entire sequence of $Pr55^{gag}$ (Haist *et al.*, 1992), and analysed them for their respective potential to influence the assembly process of viral Gag proteins by defining their ability to inhibit the release of infectious virus particles. The purified peptides were dissolved in PBS (pH 7·0) and used at concentrations of 40 to 200 μg/ml. CD4-positive, HIV-1-negative Jurkat cells were incubated with various amounts (100 to 1000 $TCID_{50}$) of HIV-1 (strain HTLV-IIIB; Ratner *et al.*, 1985) in microtitre

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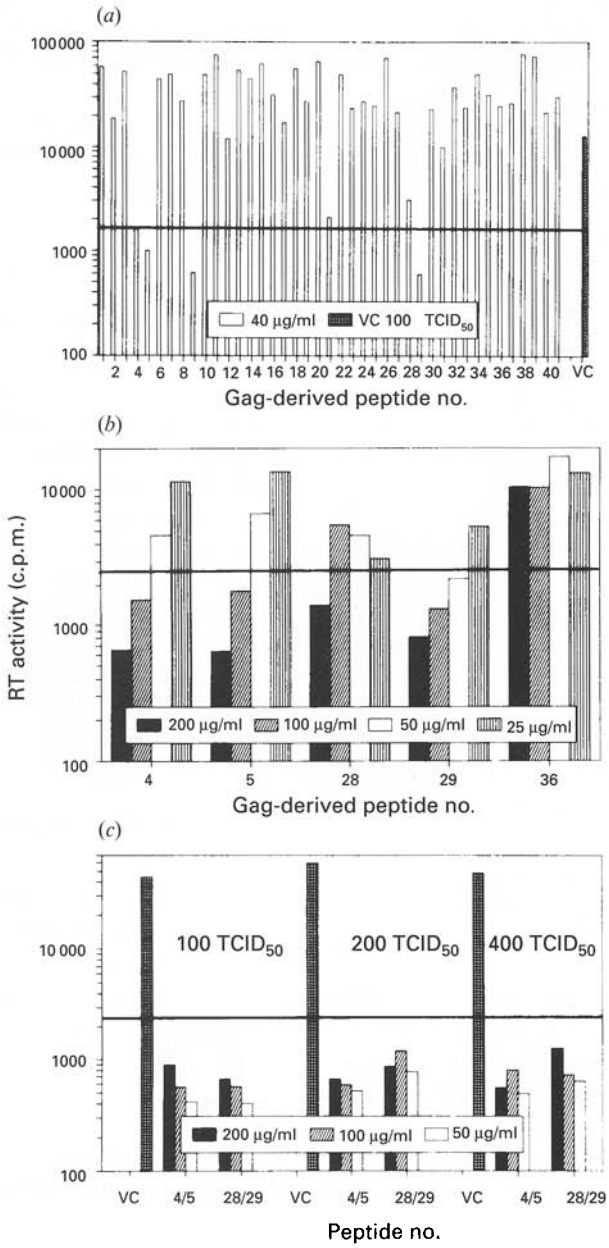

Fig. 1. Inhibition of HIV production by Gag-specific oligopeptides. (*a*) RT activity in the supernatants of the indicator cell cultures. Jurkat cells were infected with 100 $TCID_{50}$ and treated with the 41 Gag-derived oligopeptides at concentrations of 40 µg/ml. After 3 days, 100 µl cell-free supernatant was added to fresh Jurkat cells. After 2 weeks the supernatant of these cells was analysed for RT activity. Numbering refers to the peptide used for treatment of the cell culture. RT values of untreated HIV-infected Jurkat cell cultures were used as a positive control (virus control, VC), those of uninfected cell cultures as a negative control. RT activity with values below twice the negative control value was considered negative; this value is indicated by the horizontal line. (*b*) RT activity in supernatants of HIV-1-infected Jurkat cells treated with various concentrations (25, 50, 100 and 200 µg/ml) of peptides 4, 5, 28, 29 and 36 (control peptide without effect). (*c*) RT activity in supernatants of Jurkat cells infected with various amounts of HIV-1 (100, 200 and 400 $TCID_{50}$) and treated with various concentrations of peptides 4/5 and 28/29.

plates (1×10^5 cells per well). After 2 h of adsorption the sample was removed and replaced by medium supplemented with the different peptides. In a similar way chronically HIV-1-infected H9 cells were mixed with HIV-negative H9 cells at a 1:10 ratio and treated with the peptide dilutions at the time point of mixing. HIV-infected, peptide-treated cells were screened for toxic effects. Uninfected, peptide-treated control cell cultures were analysed separately for viability by trypan blue exclusion. Three to 4 days after infection 100 µl of cell-free culture supernatants was added to fresh Jurkat cells and assayed for their infectious virus content. The indicator cultures were analysed daily for HIV-induced syncytium formation. Reverse transcriptase (RT) activity in the cell supernatants was measured after 2 weeks of incubation using a microassay according to Gregersen *et al.* (1988). Briefly 100 µl of cell culture supernatant was concentrated by polyethylene glycol treatment to 20 µl. Enzyme activity was determined by incorporation of [^{3}H]thymidine using poly(A) as template/primer.

Four out of 41 peptides present at concentrations of 40 µg/ml in HIV-1-infected Jurkat cell supernatants reduced infectivity. Two of the peptides (4 and 5) comprised sequences of the p17 matrix protein and the two other peptides (28 and 29), comprised sequences of the p24 capsid protein (Fig. 1*a*, Table 1). The 'indicator cultures' were monitored daily for c.p.e. and [^{3}H]thymidine incorporation was used for determination of the levels of RT in the supernatants after 2 weeks of incubation. This long period of incubation of the supernatants from peptide-treated cells with new cell cultures was chosen to allow detection of traces of infectious HIV due to replication. Levels of HIV-specific RT reflect the concentration of infectious virus in biological samples (Land *et al.*, 1989); these were reduced in the peptide-treated cells by about 100-fold in comparison to the untreated control (Table 1). The highest reduction of infectivity in the cell culture supernatants was observed with peptides 5 and 29. The other 37 Gag-derived peptides showed no effect in this assay except peptide 9, which had toxic effects on cell cultures. In addition, peptide 21 was found to reduce the concentration of infectious virus; however, this effect was not seen when the neighbouring overlapping peptides were tested and thus could not be correlated with a defined sequence. Therefore in all further experiments we focused our interest on peptides 4, 5, 28 and 29. Comparable results in the reduction of infectious virus were obtained when infected H9 cells were treated with the Gag-derived peptides. Only peptides 4, 5, 28 and 29 were able to reduce the release of infectious virus. During treatment with the inhibiting peptides syncytium formation in primary infected cultures was only slightly reduced compared to the untreated controls indicating

Table 1. *Amino acid sequences of peptides active in inhibition of HIV production in cell culture supernatants**

Peptide	Amino acid sequence	HIV infectivity in peptide-treated cell culture supernatants RT activity (% of infectivity)		Inhibitory activity†
		Peptide concentration (μg/ml)		
		50	200	
4	WASRELERFAVNPGLLETSEGCRQIL	588 (0·0%)	555 (0·0%)	+++
5	NPGLLETSEGCRQILGQLQPSLQT	439 (0·0%)	489 (0·0%)	+++
4/5l	FAVNPGLLETSEGCRQIL	659 (0·0%)	446 (0·0%)	+++
4/5m	NPGLLETSEGCRQ	615 (0·0%)	1045 (0·5%)	+++
4/5s	LLETSEG	20482 (54·0%)	4474 (9·6%)	+
4/5a	AAANPGLLETSEGCRQIL	1114 (0·6%)	1067 (0·5%)	+++
4/5b‡	FAVAAALLETSEGCRQIL	–‡	–‡	
4/5c	FAVNPGAAATSEGCRQIL	43493 (100·0%)	1132 (0·7%)	–
4/5d	FAVNPGLLEAAAGCRQIL	24088 (62·0%)	916 (0·1%)	+
4/5e	FAVNPGLLETSEAAAQIL	11386 (8·0%)	7434 (17·5%)	++
4/5f	FAVNPGLLETSEGCRAAA	21663 (55·0%)	847 (0·0%)	+
28	ANPDCKTILKALGPAATLEEMMTAC	399 (0·0%)	648 (0·0%)	+++
29	AATLEEMMTACQGVGGPGHKARVLA	476 (0·0%)	667 (0·0%)	+++
28/29l	ALGPAATLEEMMTACQGV	455 (0·0%)	371 (0·0%)	+++
28/29m	PAATLEEMMTA	537 (0·0%)	390 (0·0%)	+++
28/29s	ATLEEMM	7546 (17·8%)	3278 (6·4%)	++
28/29a	AAAPAATLEEMMTACQGV	847 (0·0%)	1335 (1·2%)	+++
28/29b	ALGAAATLEEMMTACQGV	1141 (0·7%)	951 (0·1%)	+++
28/29c	ALGPAAAAAEMMTACQGV	20103 (51·4%)	7917 (18·8%)	+
28/29d	ALGPAATLEAAATACQGV	52059 (100·0%)	20917 (53·9%)	–
28/29e	ALGPAATLEEMMAAAQGV	8498 (20·3%)	14753 (37·1%)	++
28/29f	ALGPAATLEEMMTACAAA	1353 (1·3%)	427 (0·0%)	+++
	Positive control (HIV-infected, not treated)	37418 (100%)		
	Negative control (not infected, not treated)	868 (0%)		

* The amount of infectious HIV was determined by analysis of RT activity ([^{3}H]thymidine incorporation). The corresponding amount of infectious HIV was calculated using 100% as equivalent to positive control (untreated, HIV-infected cultures, 0 μg peptide) and 0% as equivalent to uninfected, untreated culture supernatant.

† Inhibitory activity was estimated in the following way: +++, 0 to 5% infectious HIV (in relation to input virus concentration); ++, 5 to 30%; +, 30 to 60%; –, > 60%.

‡ Not tested, not soluble in PBS.

that viral envelope proteins are synthesized and the CD4–gp120 interaction mediating syncytium formation is not affected (data not shown).

To characterize further the minimal number of amino acids responsible for the inhibitory activity we synthesized peptides spanning the overlapping parts of the reactive compounds. Peptides were shortened from their amino and carboxy termini down to seven residues for peptides 4/5 and 28/29. Except for the smallest derivatives all peptides were able to inhibit the release of infectious HIV particles (Table 1). Hence the minimal length of the effective peptides could be mapped to amino acids 47 to 59 (NPGLLETSEGCRQ) and 339 to 349 (PAATLEEMMTA) of the Gag sequence.

After defining the minimal amino acid sequences necessary for inhibition of the production of infectious HIV-1 we attempted to assign this effect to individual residues and functional amino acid side-chains. Therefore peptide derivatives of the shortened active compounds (4/5l and 28/29l) were synthesized in which triplets of the original amino acid sequence were replaced by alanine. Alanine was chosen as it is a relatively small amino acid without functionally active groups in side-chains and therefore not known to induce major structural changes. Again the activity of these peptide compounds was measured by determination of RT activity in supernatants of indicator cell cultures. In all supernatants we found a reduction of RT activity using 100 μg/ml or more of the peptide derivatives as compared to untreated controls. Some derivatives of peptide 4/5 showed a total loss of inhibitory activity (peptide 4/5c) at 50 μg/ml, when positions 50 to 61 (LLETSEGCRQIL) were altered (Table 1). Peptide 4/5b could not be tested in this assay owing to problems with its solubility at the concentrations used. The data were even more consistent when the alanine-substituted peptide 28/29 were used in cell culture systems: substitution of residues 342 to 350 (TLEEMMTAC) resulted in the loss of inhibitory activity at all concentrations (Table 1). Therefore we conclude that the region responsible for inhibition is located between amino acids 50 and 61 in the p17 and between 342 and 350 in the p24 sequences.

To determine whether the inhibitory effect could be overcome by the use of high virus concentrations we incubated the shortened forms of the active peptides 4/5m and 28/29m at various concentrations with cells infected with up to 400 $TCID_{50}$ of HIV-1. Fifty μg/ml peptide concentrations were sufficient to inhibit the infectivity of high virus loads even when applied for a long period of 20 days (Fig. 1*c*). In addition, this demonstrated that the peptides did not exert major toxic effects on the cell cultures and that the inhibitory effect of the peptides could not be washed out. Slight variations observed in RT activity in cells treated with different amounts of peptides are in the range of error intrinsic to this test system. All the values show that only very low amounts of infectious HIV (less than 0·5% of the input virus; see Table 1) are detectable.

To analyse further the effects that the peptides may have on HIV-infected cells and the production of infectious HIV-particles, and which step during the viral propagation cycle is affected, we subjected peptide-treated, HIV-1-infected Jurkat and H9 cells to thin section transmission electron microscopy (TEM). After 3 days of treatment with the respective peptides, virus-infected Jurkat cells and chronically infected H9 cells, as well as non-treated infected and uninfected control cells were fixed with 2·5% glutaraldehyde and processed for TEM using standard techniques (Gelderblom *et al.*, 1987). After treatment with OsO_4 the cells were enclosed in agarose blocks, treated with 1% tannic acid and, after dehydration, embedded in Epon. Ultrathin sections, approximately 50 nm, were stained with lead citrate and evaluated using a Zeiss EM 10 A TEM. These experiments revealed that after treatment with the respective peptides particulate viral structures were formed at the surface of the cells. However the majority appeared to be immature or malformed and showed a tendency to remain associated with the cell surface. HIV-1-infected H9 cells treated with peptide 29 (40 μg/ml medium) are shown in Fig. 2 as an example. No mature virus particles with conical capsid core structures budding from the surface of the cell could be found. Chronically infected H9 cultures or freshly infected Jurkat cells treated with the other reactive peptides showed similar effects in electron microscopy.

We have described Gag-derived peptides that inhibit the release of infectious HIV particles from infected Jurkat and H9 cells. The mechanism by which the peptides may exhibit their inhibitory effect remains unclear at present. A similar peptide effect has been reported only by Collier *et al.* (1991) using compounds derived from influenza virus haemagglutinin protein sequences which reduce the formation of influenza virus particles. In our system, two Gag-derived peptide domains could be defined that inhibit the correct assembly of the viral components in the cell and the maturation process. One region could be aligned with 13 residues of the matrix protein p17 (amino acids 47 to 59), the second with 11 residues of the capsid protein p24 (amino acids 339 to 349) (Table 1, Fig. 1). The peptide effect results in a drastic loss of infectivity of up to 100%, and only immature particulate structures are released from the cell surface (Fig. 2). The observations that viral Gag proteins are synthesized as shown by electron microscopy and that viral envelope polypeptides are produced (as the treated cells are able to induce syncytium formation) exclude the possibility that early or regulatory steps are affected by the peptides. The release of morphologically distinct viral particles points to a step during virus assembly as the target.

One of the late processes known to be affected by peptides is the maturation cleavage of the Gag precursor protein by the viral protease. Inhibition of the HIV-specific protease shows a similar immature virus to be released from the cell surface (Schätzl *et al.*, 1991). Preliminary experiments did not show that the peptides found to be active in our test systems affect the activity of the viral protease. However, the peptides could alter the protein conformation in such a way that correct interactions or proteolytic cleavage into the mature proteins cannot be performed. There are indications by other groups (K. von der Helm, personal communication) that conformational changes have to occur in Gag precursor proteins to allow successive recognition of the individual proteolytic cleavage sites.

The peptides may display their activity in a different way. They may lower the concentration of Gag precursor proteins in the submembrane compartment, leading to incorrect budding. The electron microscopical observations indicate that the observed effect occurs late in the viral replicative cycle. Incorrect budding could involve a mechanism similar to that described for the inhibition of herpesvirus-encoded ribonucleotide reductase by sequence-derived peptides (Dutia *et al.*, 1986; Cohen *et al.*, 1986; Gaudreau *et al.*, 1990; Telford *et al.*, 1990). These peptides represent protein interaction domains which inhibit the dimerization of the monomers necessary for enzyme activity. As shown by Trono *et al.* (1989), HIV-1 Gag mutants can interfere with the replication of wild-type virus.

Recent results by our group using site-directed mutagenesis of residues TLEEM and CQG (von Poblotzki *et al.*, 1993) have shown that this region is functionally active in Gag particle formation. The system used by von Poblotzki *et al.* is different to that used in this paper since all Gag proteins synthesized in the cell are mutated, leading to complete blocking of the virus assembly process. In contrast, when peptides derived from that region are added to infected cultures the wild-

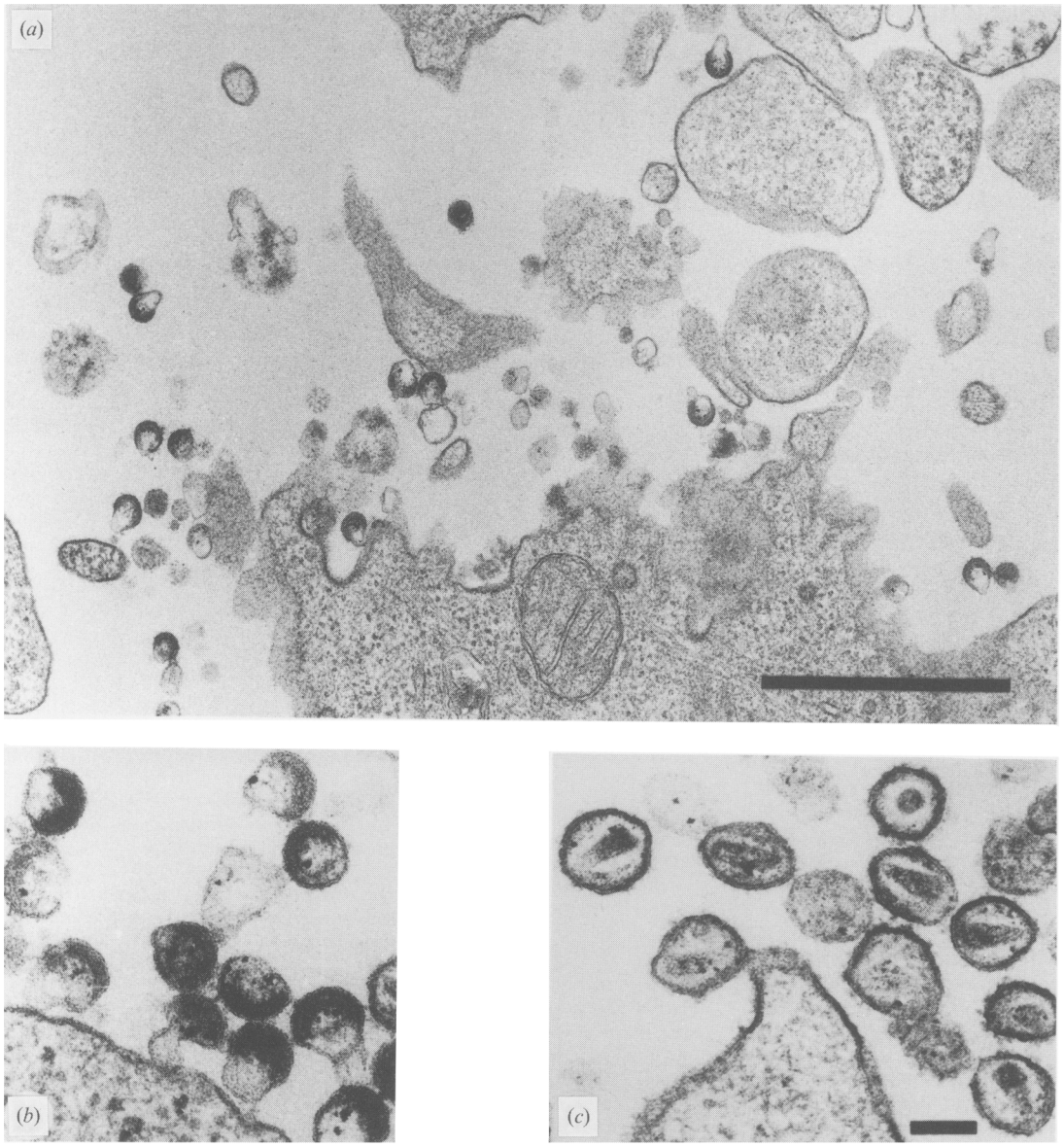

Fig. 2. TEM of HIV-1-infected H9 cells treated with peptide 29 (100 μg/ml). (*a*) HIV-1-infected H9 cell treated with peptide 29 for 3 days; particulate structures similar to immature HIV are shed from the cell surface. Bar marker represents 1 μm. (*b*) Magnification of the particulate structures budding from HIV-1-infected H9 cells treated with peptide 29 showing malformed, immature particles. (*c*) Magnification of particulate structures budding from HIV-1-infected H9 cells without peptide treatment. These particles show the conical capsid core structure characteristic for mature, infectious HIV particles. Bar marker represents 100 nm and applies also to (*b*).

type protein is hindered only in its normal function but is sufficiently active for induction of the budding process.

Both *gag* regions are conserved in most HIV strains, particularly for the domain defined in p24. In addition there is a high degree of similarity in sequence to simian and feline immunodeficiency viruses suggesting that this region is functionally very important in virus assembly. The activity of HIV-1-derived peptides in suppression also of HIV-2 further emphasizes the independence of this region of *gag*, conserved between different isolates (M. Niedrig, unpublished results). Yu *et al.* (1992) also found, for the region active in p17, that amino acid deletions affect the release of HIV particles from the cells.

The peptides used in our assays were not toxic for cells, unlike most protease inhibitors; this allows their potential use as therapeutically active compounds. In comparison to inhibitors of RT they should have the advantage that during their application immature virus particles are released from the cell and these could stimulate the immune system of the patient.

As a first step towards designing therapeutically active

stable compounds, the individual residues active in the mechanism were identified by synthesis of alanine-modified peptides. Further modifications including the replacement of natural amino acids by other sequences would be necessary for the design of compounds with a long half-life and active in concentrations that can be given to patients.

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