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Stable Expression of Soluble Therapeutic Peptides in Eukaryotic Cells by Multimerisation: Application to the HIV-1 Fusion Inhibitory Peptide C46

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A major drawback of therapeutic peptides is their short half-life, which results in the need for multiple applications and high synthesis costs. To overcome this, we established a eukaryotic expression system that allows the stable expression of small therapeutic peptides by multimerisation. By inserting the sequence encoding the therapeutic peptide between a signal peptide and the multimerising domain of the α-chain from the human C4bp plasma protein, therapeutic peptides as small as 5 kDa are secreted as multimers from transfected cells; this allows easy purification. As proof of principle, we show that the T20-derived HIV-1 fusion inhibitory peptide C46 in its multimeric form: i) was efficiently secreted, ii) was more stable than the current antiviral drug T20 in vitro and in vivo, and iii) inihibited HIV-1 entry with similar efficiency in vitro. Besides the gain in stability, multimerisation also leads to increased valency and allows the combination of several therapeutic functions. Furthermore, by expressing the multimers from cells, post-translational modifications could easily be introduced.

Introduction

Multivalency is a general principle found in many biological systems that allows increased molecular interactions. Molecular systems can be either structurally multivalent, such as antibodies, C1q,[1] C4bp[2] and CR1,[3] or functionally multivalent, such as MHC-cl II[4] and the complex TCR/CD3/CD4-8, or both. The first depends on the intrinsic oligomeric structure of a given molecule, the latter brings together several types of molecules into complex structural association, in order to create an orchestrated functional effect.[5,6]

Protein oligomerisation leads to functional advantages, such as i) higher binding strength, ii) increased structure stabilisation, iii) combination of more than one function in the same molecule and iv) modification of the in vivo pharmacokinetic properties.[7] These features, seen in naturally occurring proteins, can be engineered by combining oligomerisation domains with functional domains through molecular cloning.

We have previously described the use of a multimerising system as a biological tool to express proteins in a soluble, multimeric form from eukaryotic cells.[8–11] The multimerising domain was derived from the C-terminal end of the α-chain of the human C4b-binding plasma protein ($C4bp_\alpha$).[2,12,13] Fusion of the genetic sequence of this domain to a given gene allows the spontaneous multimerisation of the corresponding protein after transfection into eukaryotic cells through intermolecular disulfide-bridge formation.

In this study, we developed the $C4bp_\alpha$ multimerising system further to achieve stable expression of soluble peptides with therapeutic interest that are too small in their monomeric form to be routed correctly through the endoplasmic reticulum (ER) and the Golgi for proper secretion from eukaryotic cells. This is the reason why naturally expressed peptides, like hormones, are often synthesised as larger prehormones that are later cleaved to produce the active hormones.[14,16] Also, abundant antimicrobial peptides, α-defensins, which are expressed from polymorphic leukocytes and play an important role in innate immunity, are expressed as larger proteins (prepropeptides) containing about 45 additional amino acids between the signal peptide and the actual peptide.[17,18] This allows the effective transfer of the peptide precursor into the ER; proteolyt-

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ic cleavage occurs later during the peptide maturation process.[14]

Therapeutic peptides are generally produced synthetically. However, due to their small size, many peptides have short half-lives in vivo and require multiple applications, which are not favourable in a therapeutic setting. Increasing their size by multimerisation should lead to increased stability and would allow the expression of multimers from eukaryotic cells. This offers additional advantages like the introduction of post-translational modifications, the amplification of functional domains and the possibility of combining different functional moieties within the same molecule. Further, the addition of a signal peptide leads to secretion of the multimers, which can then be easily purified from cell culture supernatants.

For proof of principle, we multimerised the C46 peptide, a T20-derived peptide inhibitor of HIV-1 entry,[19,20] by combining it with the C4bp C-terminal α-chain multimerising domain ($C4bp_{\alpha}$, MD).

T20 (*Enfuvirtide*) is the first entry inhibitor approved by the FDA for antiviral treatment of HIV-1 infection.[21] The peptide is derived from the transmembrane (TM) protein gp41 of HIV-1, and its potent antiviral activity is due to inhibition of the six-helix bundle formation required for fusion of viral and cellular membranes.[22] However, the short in vivo half-life of T20 requires subcutaneous injections of 100 mg of peptide twice a day, often resulting in inflammation at the sites of injection.[23] A related peptide, T1249, which has a similar length to T20 but is shifted 10 amino acids towards the N terminus of gp41, interacts with a highly conserved hydrophobic pocket of the N-heptad repeat of gp41.[24] Therefore, T1249 is also active against most T20-resistant isolates in vitro and in vivo. [25–27] C46 combines all amino acids of T20 with the N-terminal extension of T1249 to result in a peptide of 46 amino acids.[20] This peptide has very potent antiviral activity in vitro when expressed in a membrane-anchored form after retroviral transduction into CD4-positive cells.[19,20]

In this study, we expressed the HIV-1 fusion inhibitory peptide C46 in a soluble multimeric form from eukaryotic cells. Different constructs were generated in order to optimise the expression of the peptide. The purified multimeric peptides were characterised biochemically and analysed for their stability and antiviral activity in HIV-1 entry assays in vitro. The biodistribution of multimeric and monomeric peptides was analysed in mice.

Results

Biochemical characterisation of multimeric, dimeric and monomeric C46 peptides

A number of different constructs of C46 were generated (Figure 1) in order to compare the expression of the peptides in supernatants and cell lysates as well as their antiviral activity. The basic construct consists of the signal peptide from the low-affinity nerve growth factor (LNGFR) followed by the C46 peptide and the MD (Figure 1b, group I, construct 363). Linkers of different lengths were inserted between C46 and the MD; a C-terminal His-tag was also added (Figure 1b, group I, construct 364H-(X)L). Furthermore, one or both cysteins of the MD were exchanged for glycines in order to generate dimeric and monomeric C46 peptides, respectively (Figure 1b, groups III and II). The minimal construct consists of the signal peptide

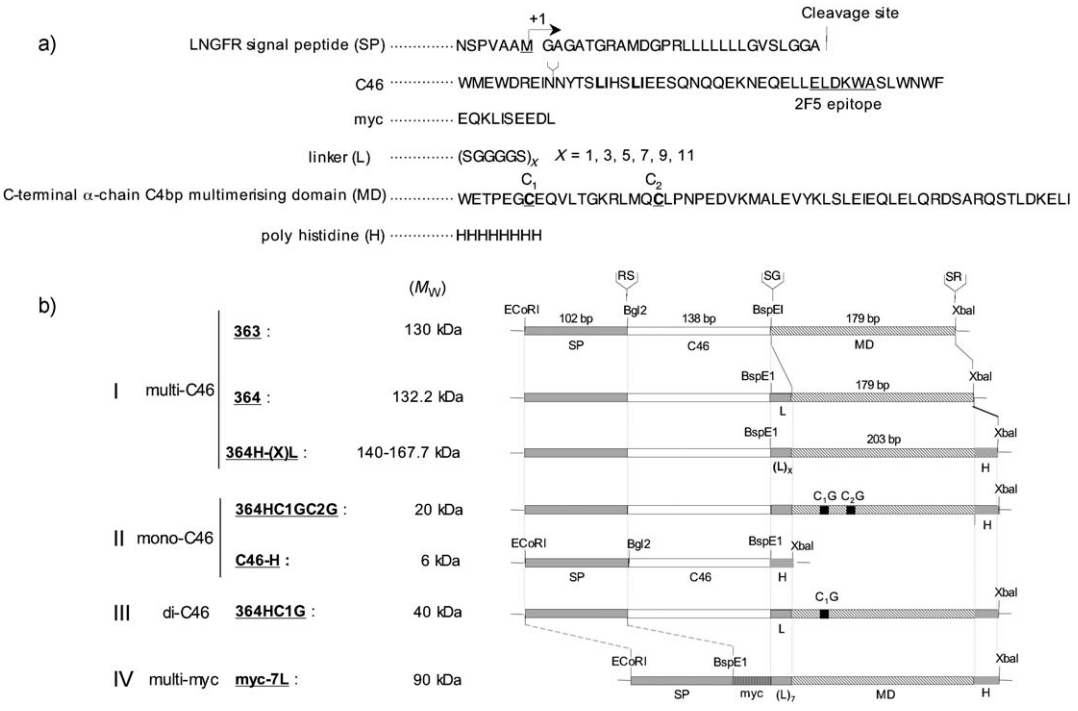

Figure 1. Multimeric constructs analysed in this study. a) Amino acid sequences of the different functional domains. The *N*-glycosylation site and the 2F5 epitope are marked in the C46 sequence. The two cysteins responsible for multimerisation are underlined in the MD sequence. b) Molecular architecture of I: multimeric, II: monomeric, and III: dimeric C46 constructs as well as the multimeric myc (IV). Mutated cysteins are indicated with black boxes.

and C46 without the MD (Figure 1b, group II, construct C46-H). As a control peptide, the C46 gene (in the construct 364H-7L) was replaced by the myc epitope to express the multimeric myc (Figure 1b, group IV, construct myc-7L).

To compare the expression of the different multimeric, dimeric and monomeric versions of C46 in transiently transfected 293T cells, Western blotting was performed after immunoprecipitation of the peptides from crude supernatants by using a mouse antibody bound to magnetic beads (Figure 2). Secretion of the peptides into culture supernatants was clearly correlated with the size of the constructs. Whereas the multimeric versions of C46 (364H and mut364H) were found mostly in the supernatants of the cells (Figure 2a), dimeric and monomeric versions ($364HC_1G$, $364HC_2G$ and $364HC_1GC_2G$) predominated in the cell lysates (Figure 2b and c, upper panel). The minimal construct (C46-H) lacking the MD, was present only intracellularly (Figure 2c, lower panel). Thus, the data suggest that peptides with a molecular weight of 5 kDa (C46-H) were too small to be secreted from the cells. Peptides of 20 to 40 kDa can be secreted, but were retained predominantly in the cell, whereas dimeric and multimeric peptides with respective molecular weights of 40 and 130 kDa were efficiently secreted.

Interestingly, the secreted peptides were mostly uniform in size under nonreducing conditions for both multimers (Figure 2a, right panel) and dimers (Figure 2b, right panel), whereas two species were found in the cell lysates. Under reducing conditions, the multimers were reduced to their respective monomeric molecular species (Figure 2a and b, left panels) with apparent molecular weights of about 20–22 kDa. The size of the monomers in the supernatants was about 4–5 kDa higher than those from the cell lysates. This difference was multiplied in the multimers under nonreducing conditions.

In order to analyze the composition of multimeric C46 in the culture supernatants, purified protein expressed from the basic construct (363, Figure 1b) was treated with increasing concentrations of β-2-mercaptoethanol (0 to 638 mM) to get partially or completely reduced molecules. Western blot analysis on polyacrylamide gels (7% and 11.5%) revealed diverse molecular intermediates starting from the unreduced multimeric form to the completely reduced monomeric form of C46 (Figure 3). Like the major native form of C4bp, which consists of 7 α-chains, unreduced multimeric C46 appeared to be heptameric.[2]

Similar analyses were performed with all the constructs shown in Figure 1 after stable transfection into 293T cells. For all constructs, the unique multimeric form in the culture supernatants was heptameric, irrespective of the linker length or the addition of a His- or a myc-tag (data not shown). Thus, the intrinsic structure of the C4bp α-chain MD itself allows the formation of unique heptameric forms of soluble peptides by establishing covalent bonds through disulfide-bridge formation between the different monomers.

Glycosylation of C46 peptide

The C46 N-terminal amino acid sequence: WDREIN**NYT**SLIH contains a potential *N*-glycosylation site (NXT, underlined). Indeed, multimeric C46 secreted from transfected 293T cells was glycosylated, as treatment with PNGase F shifted the apparent molecular weight of the multimers from 140 kDa to 100 kDa (data not shown).

Analysis of the antiviral activity of multimeric C46 peptides in vitro by single-round infection assays

To analyze the effect of multimerisation on the antiviral activity of the fusion inhibitory peptide C46, single-round infection assays were performed with pseudotyped murine leukemia virus (MLV) GagPol/HIV-1 Env replication-incompetent virus particles carrying a luciferase marker gene.[28] The results of in-

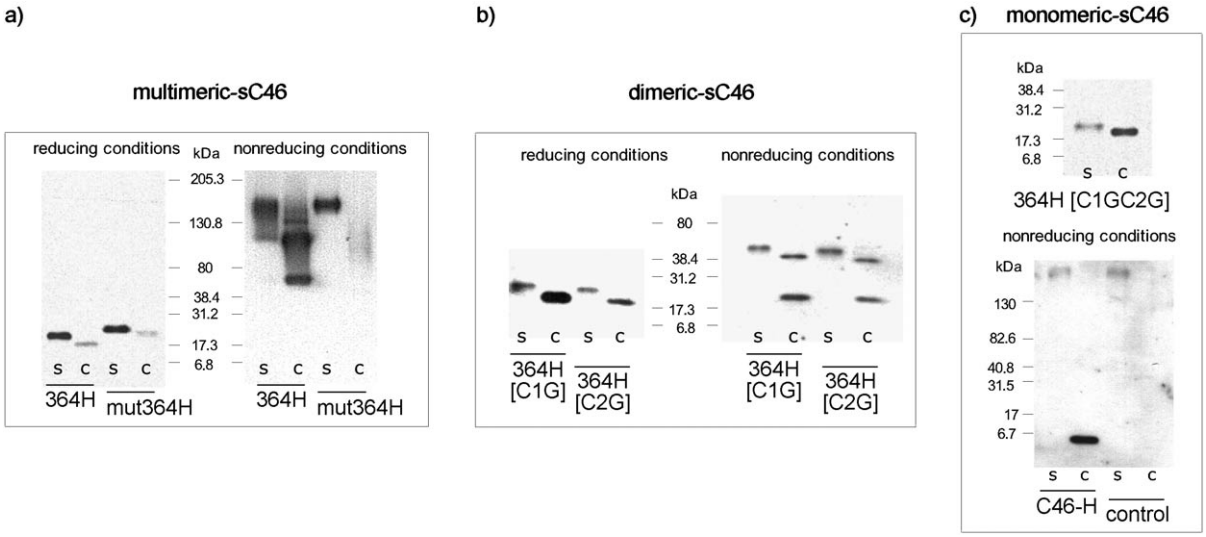

Figure 2. Analysis of the a) multimeric, b) dimeric and c) monomeric variants of C46 by SDS-PAGE and Western blotting. Supernatants (S) or cell lysates (C) from 293T cells transiently transfected with the diverse constructs were immunoprecipitated with magnetic beads coated with an anti-His monoclonal antibody. Proteins were detected after SDS-PAGE and Western blotting by using the human 2F5 monoclonal antibody under reducing or nonreducing conditions. Mut364H carries a C46 peptide with multiple single-point mutations that render the peptide inactive.

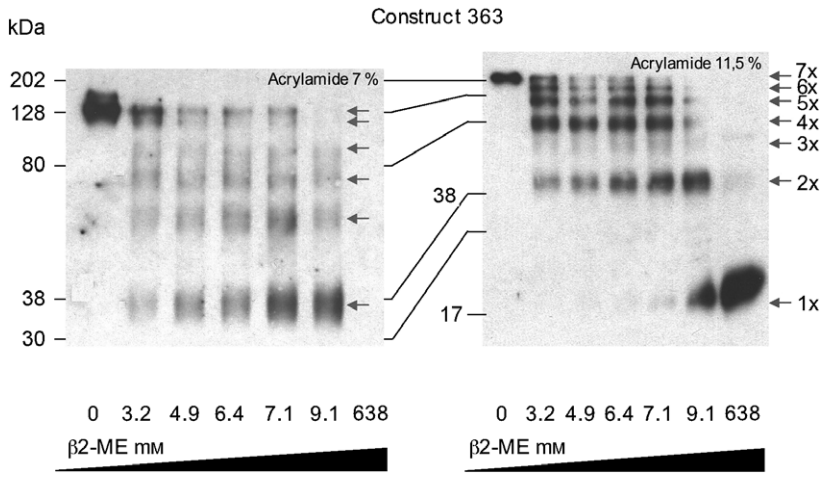

Figure 3. Composition of multimeric soluble C46 (construct 363) as deduced by partial reduction after SDS-PAGE and Western blot analysis. 293T cells were transfected with 30 μg DNA of the construct 363. Two days after transfection, cell culture supernatant was immunoprecipitated overnight with magnetic beads coated with the murine 2F12 anti-C4bp MD monoclonal antibody. Samples were reduced by using increasing concentrations of β-2-mercaptoethanol (β-2-ME). After SDS-PAGE and Western blotting, proteins were detected by the human 2F5 anti-C46 monoclonal antibody.

hibition of HIV-1 entry in vitro by the different constructs and different Env pseudotypes are summarized in Figure 4.

The multimeric constructs differed in their inhibitory potential depending on the HIV-1 strain and the length of the linker between C46 and MD. The X4-tropic HxB2 pseudovirus was inhibited at a lower IC_{50} than the R5-tropic D117III, Bal or JR-FL by all constructs including T20. Generally, inhibition of entry of the different HIV-1 pseudotypes by the multimeric versions with one linker or three linkers (364H, 364H-3L) varied depending on the Env pseudotype, as did inhibiton by T20. The IC_{50} values were between 13 and 92 nM for T20,

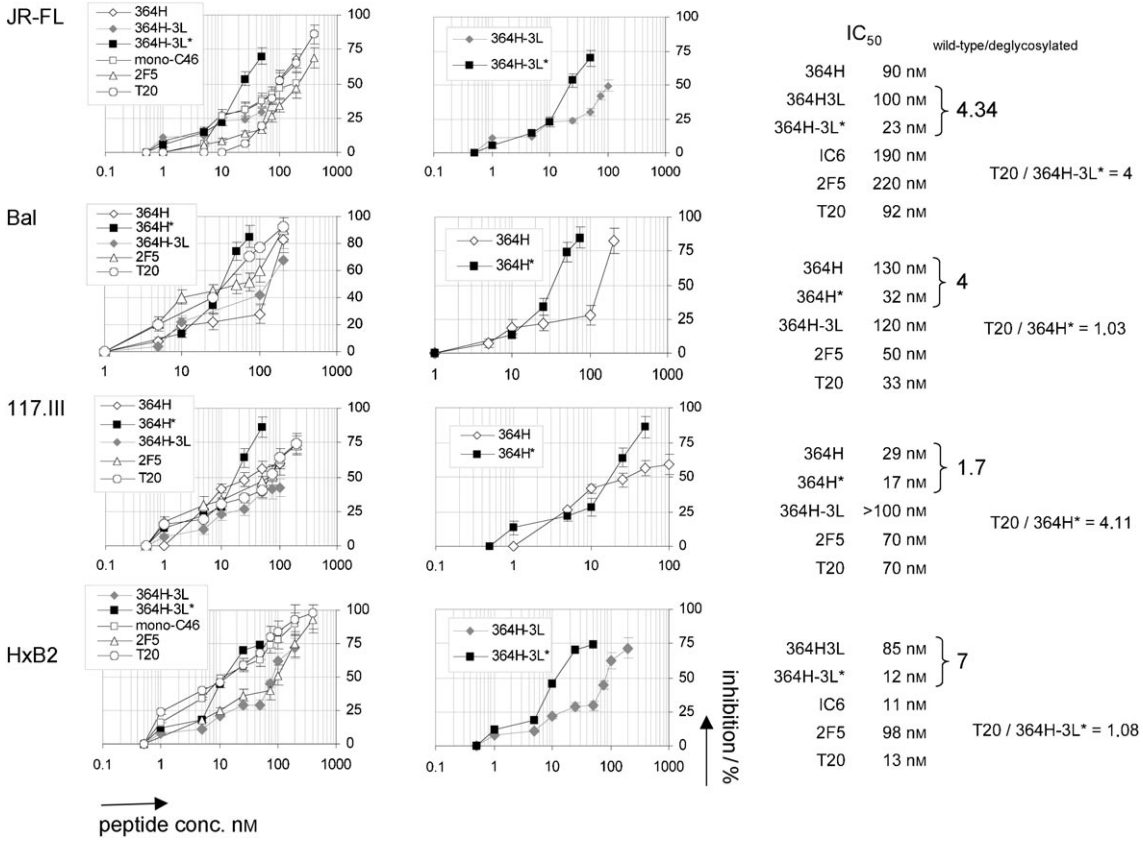

Figure 4. Inhibition of HIV-1 entry into U87.CD4.CCR5/CXCR4 cells by the different C46 constructs in single-round infection assays. Target cells (10000 cells per well) were infected with MLV Gag-Pol/HIV-1 Env pseudoviruses (R5-tropic 117III, Bal and JR-FL, X4-tropic HxB2) carrying a luciferase reporter gene. Cells were incubated with the virus particles in the presence of increasing amounts of the different purified C46 peptides for 48 h. T20, 2F5 and monomeric C46 were used as control. Infection was quantified by measuring the luciferase activity in the cell lysates. Percent inhibition (*y*-axis) was plotted against peptide concentration (*x*-axis). The right panel compares inhibition of HIV-1 entry by the glycosylated and nonglycosylated versions of the same C46 multimers. The IC_{50} values for the different isolates and constructs are indicated on the right as well as the quotient of IC_{50} values of the deglycosylated versus the glycosylated versions of the molecules.

29–130 nM for 364H, and 85–120 nM for 364H-3L, and thus fall in a similar range. Increasing the linker length by more than 3 L resulted in impaired HIV-1 entry inhibition (data not shown).

We then compared the inhibitory capacity of the deglycosylated and the glycosylated versions of the constructs 364H-1L and 364H-3L with respect to different HIV-1 Env pseudotypes (Figure 4, right panels). Again, the X4-tropic HxB2 pseudovirus was inhibited at lower IC_{50} values than the R5-tropic 117III, Bal or JR-FL by the deglycosylated multimers. Also, independently of the 1L or 3L constructs, the deglycosylated versions of multimeric C46 always inhibited better than their glycosylated counterparts by a factor of 1.7 to 7.

In vitro evaluation of serum stability of multimeric, dimeric and monomeric versions of C46

To compare the effect of multimerisation on the stability of the peptides in vitro, multimers (364H and 364H-3L), dimers (364HC1G) and monomers (364HC1GC2G) were incubated in the presence of mouse plasma, rat plasma or PBS/1% BSA for a period of 10 days (Figure 5). The amount of peptide was evaluated every 24 h by ELISA. Clearly, multimeric C46 peptides were much more stable than dimeric and monomeric peptides. Whereas multimers were stable over the incubation period of 10 days with about 80% of the initial peptide concentration still present at day 10, dimeric and monomeric peptides decayed rapidly. The half-life of monomeric C46 was 4.75 days in mouse plasma (Figure 5a), 2 days in rat plasma (Figure 5b) and 0.75 days in PBS/1% BSA (Figure 5c). The half-life of the dimeric C46 was 1.75 days in mouse plasma, 1 day in rat plasma and 4.25 days in PBS/1% BSA. The data could be reproduced in three independent experiments (data not shown). Thus, multimerisation clearly stabilised the peptides in the presence of plasma.

Blood clearance and biodistribution study of multimeric and monomeric C46 in mice

To compare the biodistribution of multimeric and monomeric C46 peptides, we radiolabeled the molecules with ^{125}I and ^{131}I, respectively and injected the same molar concentration in 200 μL of PBS into the tail vein of mice. Different organs were collected at various time points from diverse animal groups over 24 h, and the radioactivity was measured and standardised by defining the percentage of the injected dose per gram of tissue for each group of organs analysed (Figure 6a and c). Although there was no obvious advantage in the amount of multimeric C46 present after 24 h as compared to monomeric C46, the biodistribution of the molecules differed considerably. Whereas multimeric C46 accumulated in the liver (about 30% of the injected dose (i.d.) was found there after 30 min), monomeric C46 rapidly accumulated in the kidney (35% i.d. after 30 min).

The uptake of the multimeric C46 in the liver might be due to its hydrophobic character. In order to show that this is not due to the C4bp MD, we also analysed the biodistribution of a less hydrophobic multimeric peptide, the myc-epitope (Fig-

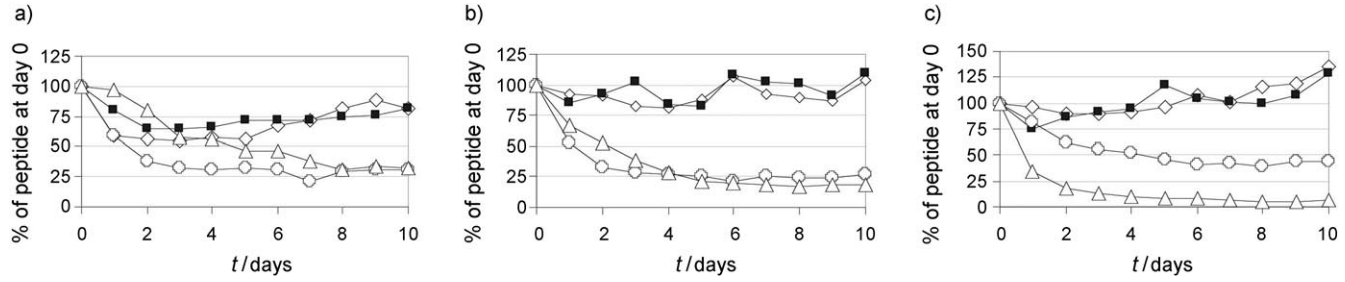

Figure 5. In vitro stability assay of the multimeric (364H-1L: ◇, 364H-3L: ■), dimeric (○) and monomeric (△) soluble recombinant C46 peptides in the presence of a) mouse plasma, b) rat plasma or c) PBS–1% BSA. 40 ng peptide/200 μL plasma or buffer were incubated at 37 °C. 20 μL were collected each day for 10 days and kept at −20 °C. After 10 days all samples were analyzed by quantitative ELISA.

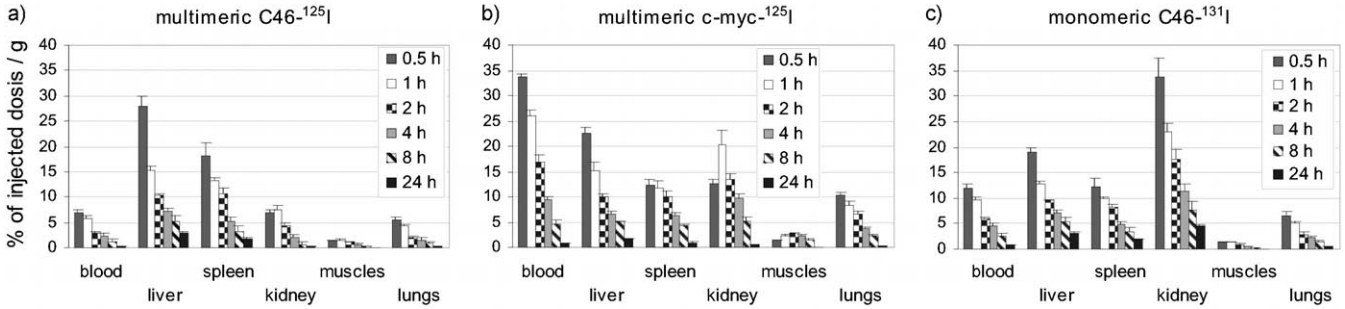

Figure 6. Biodistribution of a) multimeric C46, b) multimeric myc , and c) monomeric C46 peptides in mice. Multimeric molecules were radiolabeled with ^{125}I, monomers with ^{131}I and 0.55 MBq each were injected into the tail veins of a group of mice. Organs were taken at the indicated time points. The radioactivity was counted and normalized to the percentage of the injected dose per gram tissue.

ure 6b). This multimer did not accumulate to this extent in the liver; it was found predominantly in the bloodstream (35% of the i.d. after 30 min). Its accumulation in the kidney was delayed, with 15% i.d. after 30 min and 20–22% after 1 hour.

Discussion

To overcome the short half-life and the high synthetic costs of therapeutic peptides, such as the HIV-1 entry-inhibitor peptide T20, we describe here a biotechnological tool that allows the expression of such peptides in a soluble multimeric form from eukaryotic cells. Several advantages are associated with this multimerising system: i) the efficiency of export of the peptides through the ER and Golgi is improved; this results in better secretion, ii) their functional activity potentially increases due to the multivalency based on the presence of multiple copies of the biologically active domain within the same molecule, iii) if the molecular weight is above the renal-filtration threshold, the serum stability and bioavailibility are increased.

As proof of principle, we analysed the expression of the gp41-derived peptide C46.[20] We compared the expression of different monomeric, dimeric and multimeric C46 constructs after transfection into 293T cells, both in the cytoplasm and the culture supernatants (Figures 1 and 2). The minimal form of C46 without the MD (C46-H: 6 kDa) was efficiently translated, as it was detected in the cytoplasm of the transfected cells, however it was not secreted. Expression of C46 as an N-terminal fusion with the 60 amino acid C4bp C-terminal α-chain MD with two mutated cysteines (C4bpC1GC2G), resulted in a monomeric peptide of increased size (20 kDa). Although this peptide was still found predominantly in the cytoplasm, it also was detectable in the culture supernatant. However, for the larger (ca. 40 kDa) dimeric C46 molecule, resulting from mutation of one of the two cysteins in the MD, more than 50% of the peptide was not secreted. In contrast, the multimeric form (with both cysteins and a size of about 140 kDa) was predominantly secreted. Thus, the minimal size for efficient routing of a peptide through the secretory pathway of the cell seemed to be around 20 kDa.

We have shown that multimeric C46 was expressed in 293T cells as a unique heptameric species like the native form of C4bp, from which the MD originated.[2] Thus, the instrinsic structure of the MD with the two cysteins was sufficient to drive heptamerisation of linked peptides in the ER. In the cell-culture supernatants, we only detected the unique heptameric form.

Irrespective of the single glycosylation site in the C46 domain, all the multimeric, dimeric, and monomeric versions of C46 had slightly different apparent molecular weights, depending on whether they originated from cell lysates or from cell culture supernatants, both under reducing as well as non-reducing conditions. This could be due to additional post-translational modifications, which were not further analysed in this study.

We further showed, that multimeric C46 was functionally active in terms of HIV-1 entry inhibition in vitro using single-round infection assays with different HIV-1 Env pseudotypes. Overall, C46 constructs inhibited fusion in a similar range to T20 in terms of potency. Taking into consideration that one molecule of multimeric C46 carries seven functional valencies, this was not reflected in better inhibition, which could be due to steric hindrance when accessing the N-terminal heptads of gp41. A similar effect was described by Katinger et al. for the HIV-1-neutralizing IgG 2F5, which also inhibits HIV-1 entry at the level of fusion.[29] They constructed a pentameric 2F5 IgM antibody by class switching, which did not show better inhibition than the original 2F5 IgG. In contrast, the neutralizing activity of the 2G12 neutralizing antibody, which acts prior to fusion was improved by pentamerisation.

In line with this, the smaller deglycosylated versions of the multimeric C46 (heptameric, 100 kDa) in our study were more potent than their glycosylated counterparts and also more potent than the dimeric 2F5 antibody (150 kDa). A similar effect was recently described by Wang and co-workers for deglycosylated versions of the fusion inhibitory peptide C34.[30] Also, by fusing peptides derived from the C-heptads to different cargo proteins, Hamburger and colleagues clearly showed that inhibition of fusion strongly depended on the size of the inhibitors.[31] Interestingly, increasing the size of the linker between peptide and cargo also restored the inhibitory potency of the molecules, thus showing that the flexibility of the inhibitor is also important for efficient inhibiton. In our case, the three-linker constructs generally inhibited better than the one-linker constructs. However, further increase in the size of the linker resulted in impaired inhibition.

The comparative study of in vitro stability between the multi-C46 versus its dimeric and monomeric versions clearly showed much higher stability of the multimeric versions of C46 (Figure 5). However, in the in vivo study of the biodistribution of labelled multimeric and monomeric proteins in mice, this prolonged stability was not reflected in a longer persistence of multimeric C46 in different organs. Nevertheless, the biodistribution between the multimeric and monomeric C46 molecules differed: whereas monomeric C46 was found predominantly in the kidney, from where it is expected to be eliminated from the body, multimeric C46 accumulated in the liver and to a lesser extent, the spleen. The accumulation in the liver is probably due to the very hydrophobic character of the multimeric C46. As the hydrophobic amino acids are essential for the inhibitory function of the molecule, mutation to reduce hydrophobicity would not make sense. Therefore, we expressed the unrelated, less hydrophobic multimeric-myc peptide, and analyzed its biodistribution in the same way (^{125}I). The persistence in the blood was higher for the multimeric myc construct than for multimeric C46, and it had good correlation in terms of its size/biodistribution ratio compared to other studies.[7] Thus, better bioavailability of peptides can be achieved by C4bp-based multimerisation, however success will depend on the physical properties of the peptides. In vivo, the critical molecular mass for a therapeutic peptide is 20–40 kDa, which corresponds to the renal-filtration threshold. A slight increase in size can result in major effects concerning the persistence of a peptide in the blood as opposed to elimination via the kidney. In this respect, even if the inhibitory potency by

multimeric C46 was not improved in vitro, inhibition in vivo could still be more efficient than by T20 due to a more favourable biodistribution.

Experimental Section

Cloning of the multimeric constructs: The basic construct was first generated in a retroviral vector (M363) derived from M235 that expressed the C46 peptide in a membrane-bound form.[20] The combination of the C46 peptide preceded by the signal peptide (SP) from the low affinity nerve growth factor receptor (LNGFR) and the MD of the C4bp$_\alpha$ was generated by three-step PCR. The first PCR was performed to amplify the SP and C46 from M235 by using the primers M235-F-4391 (CTCGACAAAGTTAACTAATAG) and BspE1-PCR1-C46-r (TCCGGAGAACCAGTTCCACAGGCTGG). The second PCR amplified the MD from the plasmid pCI/sCD4-C4bpα[10] with the primers BspE1-PCR2-C4bp-F (TCCGGATGGGAGACCCCCGAAGG) and SalI-PCR2-C4bp-R (TATTTGTCGACCTAGATTAGTTCTTTATCCAAAG). Both fragments were combined in a third PCR by using the primers M235-F-4391 and SalI-PCR2-C4bp-R to produce a fragment of 508 bp, which was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen). After digestion with AfeI and SalI, the fragment was reintroduced into the M235 retroviral vector and digested with the same enzymes to replace the membrane-bound C46 by the new construct with the MD to produce construct M363. The SP-C46-MD fragment was amplified by using the primers EcoRI-F (ACCGGAATTCACCGGTCGCCGCCATGGG) and XbaI-R (CTAGTCTAGACTAGATTAGTTCTTTATC), digested with EcoRI and XbaI and cloned into the eukaryotic expression plasmid pEF-IRESp[32] to generate the basic construct 363.

The linker SG$_4$S was introduced by ligating a double-stranded oligonucleotide linker encoding this sequence into the BspEI site of the 363 vector. The individual oligonucleotides BspEI-SG$_4$S-F (CCGGAGGCGGTGGCTCGA) and BspEI-SG$_4$S-R (CCGG*T*CGAGCCACCGCCT) were annealed, phosphorylated by T4 polynucleotide kinase (New England Biolabs) and ligated into the 363 vector after digestion with BspEI and dephosphorylation by T4 alkaline phosphatase (New England Biolabs). In case of the correct orientation of the linker, the BspEI site was only regenerated at the N terminus of the linker; this gave the construct 364. All pEF-IRESp constructs were checked by sequence analysis with the primer pEF-5′seq (ACTCCCAGTTCAATTACAG) on an ABI-Prism 3100 Avant genetic analyser.

The 8x polyhistidine tail was introduced into the 364 construct by substituting the EcoRI-XbaI fragment with a PCR fragment generated by using the primers EcoRI-F and XD-Htag-R (CTAGTCTAGATCAGTGATGGTGATGGTGATGGTGGTGGATTAGTTCTTTATCCAAAGT) and the 364 construct as template. The PCR fragment was digested with EcoRI and XbaI to substitute the previous fragment, to afford construct 364H.

The minimal form of soluble recombinant C46 was generated by PCR, by using the EcoRI-F 5′ direct primer previously described, and the 3′R monoC46His8x primer (CTAGTCTAGATCAGTGATGGTGATGGTGATGGTGGTGTCCGGAGAACCAGTTCCACAG), containing *Xba*I and *Bsp*E1 restriction sites. Construct 364 was used as a template. After digestion with EcoRI and XbaI, the PCR product was ligated into the pEF-Iresp vector and digested with the same enzymes to generate sC46His8x.

Monomeric and dimeric versions of C46-C4bpα were generated by site-directed mutagenesis of both or one of each of the cysteins responsible for multimerisation (C498 and C510). The first C498G exchange was performed by PCR with the direct primer 5C4bp$_\alpha$C$_1$>G (5′-TGGGAGACCCCCGAAGGCGGTGAACAAGTGCTCACAG-3′), the reverse primer 3C4bp$_\alpha$C$_1$>G (CTGTGAGCACTTGTTCACCGCCTTCGGGGGTCTCCCA) and the template 364H. Similarly, the second C510G exchange was performed by using the direct primer 5C4bp$_\alpha$C$_2$>G (GGCAAAAGACTCATGCAGGGTCTCCCAAACCCAGAGG) and the reverse primer 3C4bp$_\alpha$C$_2$>G (CCTCTGGGTTTGGGAGACCCTGCATGAGTCTTTTGCC) and either 364H or the product from the first mutagenesis (364HC$_1$G) as template to give rise to 364HC$_2$G and 364HC$_1$GC$_2$G, respectively.

Constructs with optimized linkers between the soluble recombinant C46 and its C-terminal C4bpα multimerising domain were generated by sequential addition of oligonucleotide linkers containing the SG$_4$S linker. The 36 bp codon optimised oligonucleotides (SG$_4$S)$_2$-linker-d (CCGGAGGCGGTGGAAGCTCCGGTGGAGGAGGGAGCG) and its 36 bp reverse complement (SG$_4$S)$_2$-linker-r (CCGGCGCTCCCTCCTCCACCGGAGCTTCCACCGCCT) were annealed, purified by 4% Metaphore agarose gel, phosphorylated and ligated into the construct 364H, previously cut with *Bsp*E1, and dephosphorylated. The double-stranded DNA fragments had been designed such that only the inserts incorporated in the correct orientation contained the *Bsp*E1 restriction site at their N terminus, in order to allow easy substitution of the C46 peptide by other peptides. We selected and characterised several bacterial clones containing three (SG$_4$S)$_3$, five (SG$_4$S)$_5$, seven (SG$_4$S)$_7$, nine (SG$_4$S)$_9$ and 11 linkers (SG$_4$S)$_{11}$, respectively (constructs 364H-3L, 364H-5L, 364H-7L, 364H-9L and 364H-11L).

An additional construct was generated from 364H-11L containing a myc tag (recognized by the mouse anti-human c-myc monoclonal antibody, BD Pharmingen, Biosciences, Erembodegem, Belgium) between C46 and the linker (SG$_4$S)$_{11}$, by insertion of an oligonucleotide linker into the BspE1 restriction site. The sequences of the oligonucleotides myc-*Bsp*E1-d (CCGGAGAACAAAAACTCATCTCAGAAGAGGATCTAG) and myc-*Bsp*E1-r (CCGGCTAGATCCTCTTCTGAGATGAGTTTTTGTTCT) were annealed, phosphorylated and ligated into the 364H-11L construct, previously linearised with *Bsp*E1 and dephosphorylated. The final construct was termed 364H-myc-11L.

The multimeric myc-peptide was generated based on the 364H-7L construct. After digestion with *Bgl*2 and *Bsp*E1, the C46 peptide was replaced by an oligonucleotide linker encoding the myc peptide. The sequences of the oligonucleotides were myc-*Bgl*2-*Bsp*E1-d (GATCTGAACAAAAACTCATCTCAGAAGAGGATCTAT) and myc-*Bgl*2-*Bsp*E1-r (CCGGATAGATCCTCTTCTGAGATGAGTTTTTGTTCA). After annealing and phosphorylation, the 36 bp DNA fragment was ligated into the digested 364H-7L construct to give myc-7L-C4bp$_\alpha$His8x.

Expression and biochemical analyses of multimeric, dimeric and monomeric C46: 293T cells were transiently transfected in 10 cm diameter culture dishes (3.5×10^6 293T cells per dish at day −1, + 30 μg DNA per transfection) by using the calcium phosphate precipitation method,[33] and left overnight at 37 °C under 5% CO_2. Supernatants were collected 48 h after transfection, cleaned by centrifugation at 3000 rpm for 5 min at 4 °C and analysed for protein expression, as described below. Cells were washed once with phosphate buffered saline (PBS) and lysed in lysis buffer (40 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA, 5 mM EDTA, 1% Triton X100, 0.5% NP40) containing protease inhibitors (0.4 μM AEBSF, 3.6 μM EDTA-Na$_2$, 0.5 nM Leupeptine, 0.5 nM Pepstatine and 5 mM PMSF, all from ICN, Cleveland, OH). Cells were energically vortexed, left in ice-cold lysis buffer for 30 min, vortexing every 5 min, and centrifugated for 30 min at 4 °C at 24000 *g*. Lysate supernatants were kept and analysed for protein expression, as described below.

Supernatants were immunoprecipitated overnight at 4 °C with anti-mouse IgG coated-magnetic beads (Dynal, Hamburg, Germany), bearing an IgG1 mouse anti-poly-His monoclonal antibody (Dianova, Hamburg, Germany). For constructs without a His-tag (363, 364) the peptides were precipitated with the mouse monoclonal antibody 2F12 against C4bpα (kindly provided by Prof. B. Bouma, Academic Medical Center, Amsterdam, The Netherlands). Beads were collected, washed with PBS-BSA 0.1% and analysed by SDS-PAGE followed by Western blotting with 0.2 μm polyvinylidene difluoride (PVDF) blotting membranes (Millipore). After blocking with PBS–5% milk powder/0.1% Tween 20, the membranes were incubated with the anti-gp41 2F5 human monoclonal antibody (Dr. H. Katinger, Polymun Scientific Inc, Vienna, Austria), an anti-human HRP-conjugated antibody (Jackson ImmunoResearch, Cambridge, UK) and revealed using a chemoluminescent detection ECL kit (Amersham, Braunschweig, Germany).

Reduction of the precipitated multimers was perfomed with β-2-mercaptoethanol by using concentrations between 3.2 and 638 mM.

For *stable transfections*, cells transfected as described above were cultured for 48 h in complete medium DMEM (Gibco), supplemented with 1% penicillin/streptomycin antibiotics (Cambrex Biosciences, Verviers, Belgium), 10% FCS (Pan Biotech GmbH, Karlsruhe, Germany), 2% glutamine (PAA Laboratories GmbH, Pasching, Austria) and puromycin (20 $\mu g\,mL^{-1}$, ICN). The medium was replaced daily for 5 days after transfection in order to achieve full selection pressure. After 10 to 14 days, single cell colonies were subcloned and expanded separately, first in 96-well, then in 24-well cell culture plates (Costar, San Diego, CA), always in the presence of puromycin (20 $\mu g\,mL^{-1}$). The supernatants of each single clone were analysed by Western blot, as described above, and quantified by ELISA, as described below. The clones expressing the highest amounts of peptides (ranging from 5 to 10 $\mu g\,mL^{-1}$) were kept and expanded.

Affinity chromatography: Supernatants from stable tranfected cells were collected and filtered through 0.22 μm molecular weight cut off (MWCO) filter devices (Millex Millipore) before use. HiTrap NHS-activated affinity columns (Amersham Biosciences) were prepared according to the instructions from Amersham by using either the human 2F5 or the mouse 9E10 anti-myc monoclonal antibodies (Serotec, Oxford, UK). Supernatants were passed through the column overnight at 4 °C by using a peristaltic pump with a flow rate between 1 and 1.5 mL per minute, as per the instructions. The column was washed with sterile Dubelcco's PBS (50 mL, DPBS, Gibco). Peptides were eluted with a low-pH elution buffer (500 mM NaCl, 100 mM glycin–HCl pH 2.7). Three cycles of elution were performed with elution buffer (7 mL each), The column was washed with DPBS between cycles. The eluates were neutralised with NaOH. Purified peptides were concentrated by using centrifugal filter devices (Amicon Ultra - Millipore) of either 100 kDa MWCO for the multimers, 10 kDa for the monomers and dimers C46 (20 kDa and 40 kDa, respectively) or 5 kDa for the smallest C46 peptides (sC46-His8x, 5 kDa). Peptides were finally washed with phosphate buffer (100 mM, pH 7.4).

For *deglycosylation*, 2F5-affinity chromatography purified multi-C46 (construct 364H, 364H-3L, ca. 200 μg) was deglycosylated over night at 37 °C by addition of *N*-glycosydase F in PBS (500 units PNGase, New England Biolabs). The deglycosylated multi-C46 was purified by passing the solution through the 2F5-affinity column at 4 °C, washed with sterile DPBS (3×15 mL) and concentrated after elution as described above through a 50000 MWCO centrifugal filter device at 4000 rpm. Molecules were analysed by SDS-PAGE followed by Western blotting, as described above. Quantification was done by ELISA, as described below.

Quantification of the soluble recombinant C46 peptides: The multimeric C46 peptides (363, 364, 364H), as well as dimeric (364HC1G) and monomeric (364HC1GC2G) versions of C46 were separated by SDS-PAGE under reducing conditions followed by Coomassie brilliant blue staining. A standard band series was also loaded onto the gel, which was generated by serial dilutions of bovine serum albumin (BSA, Serva, Heidelberg, Germany), with concentrations ranging from 0.1 to 3.0 μg per lane. Gels were dried without heating to avoid damaging them. Bands were quantified by using the calibration curve by imaging with the Quantify One software package version 4.1.1. from Bio-Rad. The molar concentrations were then calculated for each peptide, according to their respective molecular weights.

The quantified peptides were then used as standards for the establishment of a specific *quantitative ELISA*. The 2F5 mAb was coated overnight in DPBS (100 ng per well per 100 μL DPBS) at 4 °C on a 96-well microtiter immunoassay plate (Dynatech Laboratories, Inc., Chantilly, VA, USA). After being washed with PBS–1% BSA (4×), plates were blocked with PBS–5% BSA for 1 h at 37 °C. After two additional washings, dilutions of samples to quantify as well as standards, previously diluted in a final volume of PBS–1% BSA (100 μL), were incubated for 1 h at 37 °C. After four washings, the plate was incubated with a rabbit polyclonal anti-His antibody (ABR, Affinity BioReagents) in PBS–1% BSA per well (100 μL) for 1 h at 37 °C. After four further washings, the plate was incubated with an anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch Laboratories) in 100 μL PBS–1% BSA per well for 1 h at 37 °C. The plate was washed five times and incubated in a 100 mM phosphate-citrate buffer pH 5.0 (Sigma) with the *o*-phenylenediamine (OPD, Sigma)/H_2O_2 chromogenic substrate combination for peroxydase for 5 min (100 μL per well). The reaction was stopped with H_2SO_4 (0.5 N, 100 μL per well), and the plate was read with a Spectra-MAX 340 reader and the software Softmax Pro version 1.1.1 at 492 nm.

The sC46-myc-$(SG_4S)_{11}$-C4bp$_\alpha$-His8x peptide quantified as described above was further used to establish a quantitative myc-ELISA for the multimeric myc-peptide. The ELISA plate was coated with the mouse 9E10 anti-myc monoclonal antibody (20 ng per well) in a final volume of 100 μL PBS. The following steps were performed as described above, by using the polyclonal rabbit anti-His antibody (ABR, Affinity BioReagents) as secondary antibody.

Analysis of antiviral activity of the peptides: The antiviral activity of the peptides was analysed in single-round infection assays by using recombinant luciferase reporter viruses, pseudotyped with the R5-tropic HIV Env glycoproteins JR-FL, D117III, Bal and the X4-tropic HIV Env protein HxB2. Lentiviral vector supernatants were generated from a three-plasmid transfection system[34] by cotransfecting 293T cells with pRRLsinCMV LUC transfer vector[35] (7.5 μg), the packaging construct pCMVΔR9[33] (12.5 μg) containing Gag-Pro-Pol from HIV-1, Tat, Rev and RRE controlled by a CMV promoter and pSVIII (JR-FL, 117III, Bal or HxB2) env plasmids (2 μg, NIH) encoding the HIV-1 Env proteins by the calcium phosphate DNA-precipitation method.[32] Cell culture supernatants (DMEM, 2% Glu, 1% Pen/Strep, 5% FCS) were collected 24, 36, 48 and 60 h after transfection, cleared through 0.22 μm Millex HA filter units (Millipore) and concentrated up to six times by using Vivaspin100 filter tubes and centrifuging for 45 min at 2500 rpm at 4 °C. Virus particle containing supernatants were titered on U87MG cells (HTB 14, ATCC,

Rockville MD) expressing CD4 and CCR5 or CXCR4. The cells were infected for 48 h with serial dilutions of the particle stocks in DMEM. Infection was quantified by luciferase assay. Cells were washed with PBS, lysed with harvest buffer (0.5 M Mes-Tris, 1 M DTT, 10% Triton X-100 and glycerol), and light emission was measured on a Lumistar Galaxy Luminometer (BMG Labbiotechnologies, Offenburg). Viral stocks were stored at −80 °C.

The diverse C46-derived peptides were tested for their ability to prevent HIV-1 entry into in vitro neutralisation assays on U87CD4$^+$ CCR5$^+$/CXCR4$^+$ cells. Cells (10^4 per well) were plated and incubated for 24 h at 37 °C under 5% CO_2. Peptide dilutions were prepared in complete DMEM and were added to the cells at a volume of 20 μL per well. Viral pseudotyped particle dilutions (20 μL per well) were immediately added to the cells. Cells were incubated for 48 h at 37 °C under 5% CO_2 in DMEM (40 μL final volume) containing the peptide inhibitors and the pseudotyped viral particles. Cells were then lysed to determine luciferase activity with beetle luciferin (Promega) in luciferase buffer (0.5 M Mes-Tris, 0.5 M $MgCl_2$, ATP). Inhibition was expressed as the percentage of the positive control (containing virus but no peptide) after substraction of the negative control without virus.

In vitro stability test of the multimeric, dimeric as well as monomeric C46: The multimeric (364H-1L and 364H-3L), dimeric (364HC1G) and monomeric (364HC1GC2G) versions of C46 were compared for stability in vitro at 37 °C over a period of 10 days. The peptides (40 ng μL^{-1}, as determined by quantitative ELISA see above) were incubated in either mouse or rat plasma or PBS–1% BSA at 37 °C (200 μL final volume). Peptide-containing sera (10 μL) were collected daily and kept at −20 °C. After 10 days, a quantitative C46 ELISA was performed as described above. The samples corresponding to day 0 were used as references for the calibration of the ELISA and the establishment of the optimal dilutions to perform the analysis in the linear range of the standard calibration curve.

In vivo biodistribution of multimeric versus monomeric C46 peptides: The 364H-1L multimeric C46-construct was radioactively labelled with ^{125}I, whereas the 364H-1LC_1GC_2G monomeric C46-construct was labelled with ^{131}I. In vivo studies were performed in BALB/C mice of about 8–10 weeks with an average body weight of 20 g. Five mice were used for each measurement. PBS injection solution (200 μL) was used for each mouse. This solution contained ^{125}I-multi-sC46 (6.22 μg, corresponding to 0.55 MBq ^{125}I), ^{131}I-mono-sC46 (1 μg, corresponding to 0.57 MBq ^{131}I) and HSA (100 μg) as carrier protein. The peptide solutions were injected intraveneously into the tail vein, or subcutaneously into the flank of the thorax area. Organs were taken after 0.5, 1, 2, 4, 8 and 24 h after injection. Intraveneous and subcutaneous injections were compared after 4 h. The organs analysed were: liver (~0.95 g), spleen (~0.08 g), kidney (~0.25 g), lungs (~0.12 g), whole blood (~8% of the whole body weight, ~1.6 g) and muscles (~45% of the whole body weight, ~9 g). At each time point, a batch of five mice was sacrificed, and the counting of ^{125}I-multi-sC46 and ^{131}I-mono-sC46 was performed. All data were normalized to percentage of the injected dose per gram tissue.

Multimeric myc-peptides were labelled with ^{125}I and analyzed in vivo as described above.

Animal experiments were performed at the DKFZ according to the ethical guidelines.

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