ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 10007–10009

www.rsc.org/chemcomm

COMMUNICATION

A combinatorial approach toward smart libraries of discontinuous epitopes of HIV gp120 on a TAC synthetic scaffold†

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Received 23rd July 2012, Accepted 10th August 2012
DOI: 10.1039/c2cc35310e

We describe rapid and convenient access to smart libraries of protein surface discontinuous epitope mimics. Up to three different cyclic peptides, representing discontinuous epitopes in HIV-gp120, were conjugated to a triazacyclophane scaffold molecule *via* CuAAC. In this way protein mimics for use as synthetic vaccines and beyond will become available.

One of the greatest challenges in the construction of protein mimics is mimicry of the discontinuous epitope nature of the large surfaces of proteins. This mimicry is difficult to achieve, since linear peptides usually do not adopt the correct 3D-folded structure of these epitopes. For mimicry of discontinuous epitopes, the challenge is not only the proper folding of individual peptide segments but also the combination of several different peptides in a single molecule.

Among the many protein–protein interactions involving discontinuous epitopes, the interaction of HIV gp120 with the CD4 receptor is very interesting, since this interaction is a potential target for HIV-vaccine design.[1,2] Therefore, mimicry of the gp120 discontinuous epitopes in a (much) smaller protein mimic may possibly lead to a synthetic vaccine.

Based on the X-ray crystal structure of protein complexes, in this case the gp120-CD4 complex,[3] (Fig. 1) we[4] and others[5] have determined which interacting peptide segments may be most effective for inclusion in a synthetic discontinuous epitope mimic. For this purpose we have devised a conveniently accessible (TAC, triazacyclophane) scaffold, to which three different peptide sequences can be attached.[6] In order to obtain the best possible mimicry of epitope loops, previously we have developed a general approach for the non-stop solid phase synthesis of subsequently three different cyclic peptides onto a TAC scaffold.[4]

This earlier synthesized protein mimic was evaluated for its ability to prevent HIV-infection, however no inhibition of infection was observed in tissue culture. Although such an inhibition is difficult to realize, this result points at the potential problem of whether the ideal mimic has been synthesized. With respect to this, crucial issues related to designing and synthesizing the optimal discontinuous epitope mimics are: (i) do the cyclic peptides mimicking the epitope loops have the right size? (ii) is the arrangement of the cyclic peptides in the mimic an adequate representation of the situation in the protein complex? (iii) does the scaffold provide the proper flexibility or rigidity? (iv) does the scaffold provide a good platform for the relative involvement of the different loops of the discontinuous epitope?

In this communication, we provide an approach for solutions of several of these issues. Usually many combinations of peptides often from large libraries – obtained for example from phage display – have to be screened before finding a hit. Recent approaches[7–9] point at the importance of the introduction of cyclic peptides or loops in peptide libraries, and the use of these to find active hits.

We now present a reproducible combinatorial approach for the introduction of up to three cyclic peptides onto a scaffold, leading to clean collections or libraries of scaffolded loops, which can be unambiguously characterized by MS, conveniently separated by HLPC and screened as mixtures or as single compounds. This convergent approach as compared to our earlier non-stop solid phase approach allows a much faster accessibility of several protein mimics for screening purposes.

For the assembly of multiple peptides on a core molecule, a wide variety of bioorthogonal ligation methods are available.[10–12] However, when libraries are screened for actives, it is crucial that no unknown contaminations or impurities are present. These could give rise to false positives that cannot be re-synthesized, and are therefore not accessible. Our approach utilizes the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC)[13] reaction and no by-products were observed and reaction mixtures

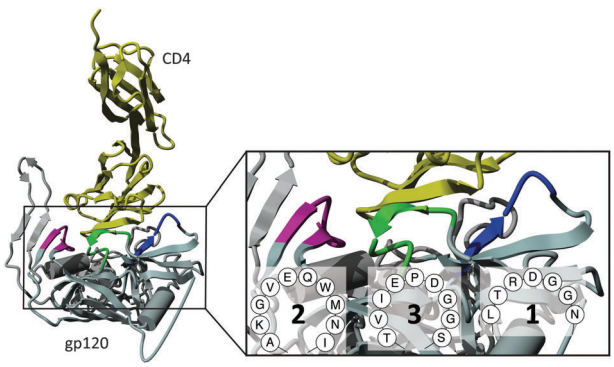

Fig. 1 Conserved epitopes in the CD4 binding site on gp120.

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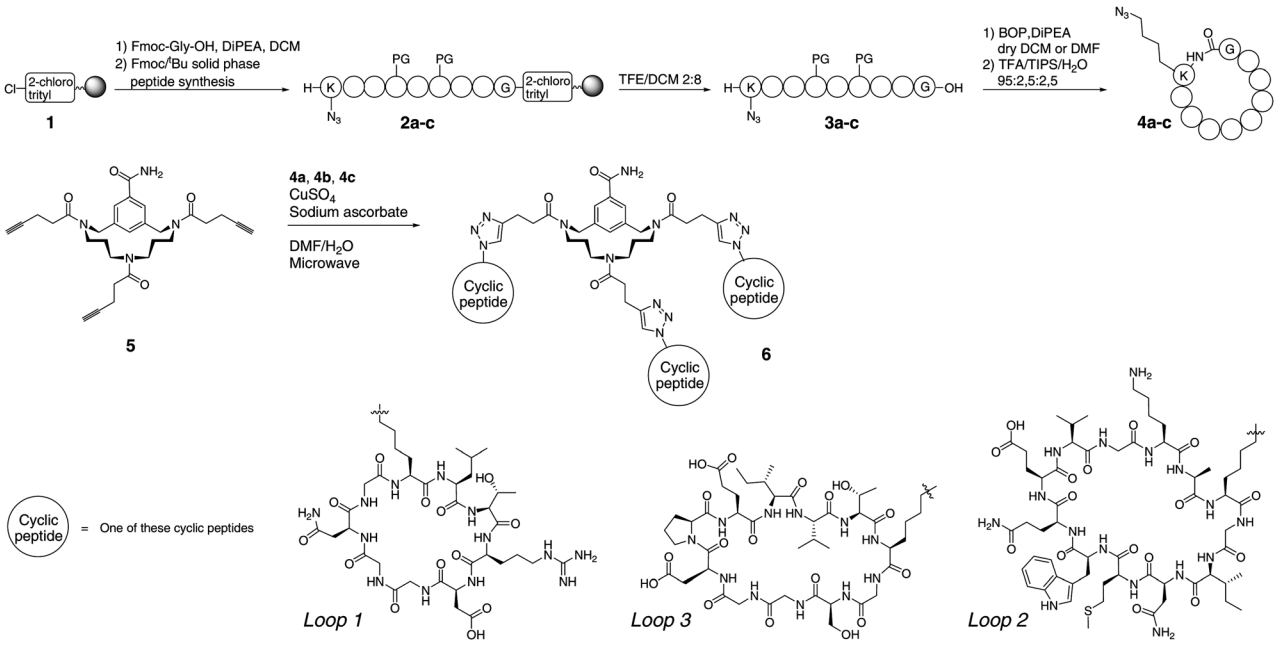
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2cc35310e



Scheme 1 Synthesis of azido-functionalized cyclic peptides followed by CuAAC onto the TAC-scaffold affording a discontinuous epitope library.

solely containing the desired discontinuous epitope mimics were obtained. Thus, this approach provided access to "clean" collections or libraries.

The triazacyclophane (TAC) scaffold was used as a template for pre-organization[4,14] and outfitted for CuAAC with three pentynoic acid residues leading to **5** (Scheme 1). As was mentioned above, the best mimicry of epitope loops can probably be achieved using cyclic peptides. Although widely used in every conceivable branch of chemistry and biology, synthesis and purification of cyclic peptides, irrespective of their size and amino acid composition, turned out to be a major hurdle.[15–17] The method shown in Scheme 1 proved to be most efficient and for this, first the linear peptide containing an azidolysine residue for CuAAC was synthesized on 2-chlorotrityl chloride resin. Next, the protected peptide was cleaved from the resin, followed by head-to-tail cyclization in solution. Because of the presence of a number of (lipophilic) protecting groups, normal (pressure) column chromatography could be carried out on silica, leading to pure protected cyclic peptides in large quantities.

Whereas "clicking" of protected cyclic peptides resulted in incomplete couplings and intractable mixtures, clicking of deprotected cyclic peptides appeared to be an ideal approach for obtaining separable libraries of discontinuous epitope mimics by the well-established HLPC-procedures for (unprotected) peptide separation (*vide infra*).

An equimolar mixture of the three gp120 cyclic peptides, representing three conserved loops of the CD4-binding site, was conjugated to the TAC scaffold by CuAAC (Fig. 1). The most efficient reaction conditions involved the use of $CuSO_4$, in the presence of TBTA.[18,19] The resulting very clean reaction mixtures were subjected to LC-MS and practically all combinations of cyclic peptide loops on the TAC-scaffold **5** could be identified from their mass value (Fig. 2). After separation, up to mg (!) quantities of the individual pure epitope mimics were obtained. Moreover, the library preparations were highly reproducible and virtually identical libraries were easily prepared when repeating the procedure. The prepared library

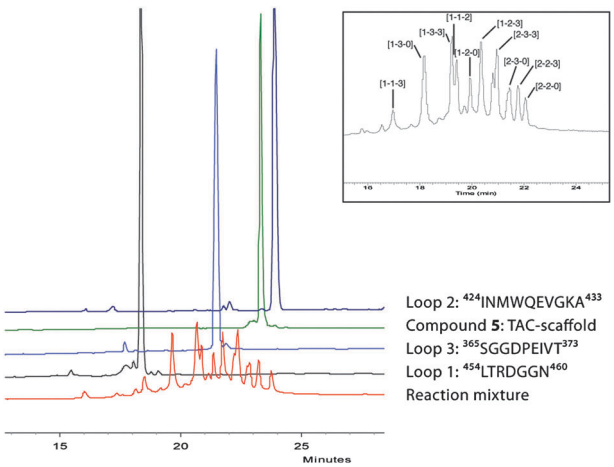


Fig. 2 HPLC-chromatogram of cyclic azidopeptides containing the conserved epitopes, TAC-scaffold **5** and reaction products of the CuAAC reaction depicted in Scheme 1.

containing practically[22] all combinations of epitope mimics enabled assessment of the relative importance of each epitope as well as the biological activity of mimics containing just one or two out of three different loop-mimicking cyclic peptides. Binding of our discontinuous epitope mimics to CD4 was examined in a gp120-capture ELISA experiment.[20] The "clean" mixture of all combinations of epitope mimics was able to compete with recombinant monomeric gp120(IIIB) for binding to CD4, whereas the individual loops did not show any activity. This clearly indicates a synergistic effect of combining multiple epitopes in one molecule, and demonstrates the importance of the TAC scaffold.

After this first indication of activity, the individual discontinuous epitope mimics were separated, purified and tested in an identical competition experiment. After screening at a single concentration (Fig. 3) it was found that in contrast to the individual cyclic

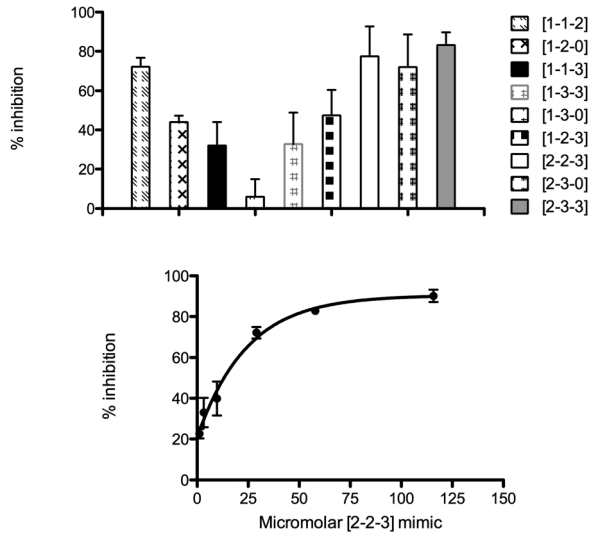


Fig. 3 Top: results of the competitive gp120(IIIB)-CD4 ELISA for all pure epitope mimics. Bottom: competition of the most potent mimic with gp120(IIIB) for CD4-binding (IC_{50} 16.8 μM).

peptides, the combination of just two cyclic peptides onto a scaffold already led to a molecule that can compete with gp120 for CD4-binding. Moreover, all discontinuous epitope mimics carrying a combination of the cyclic peptides mimicking loops 2 and 3 were stronger competitors than the other combinations.[21]

Also, the combination of these cyclic peptides exhibited a slightly higher affinity than an epitope mimic carrying the linear peptide fragments.[5] The importance of these two epitopes is in agreement with the crystal structure of the gp120-CD4 complexes.[3,22] In particular, amino acid residues in CD4 showed multiple contacts with amino acid residues in gp120 epitope ^{365}SGGDPEI^{371} – corresponding to loop 3 – and in epitope ^{425}NMWQEV^{430}, corresponding to loop 2.[3] Moreover, it has been shown that changes in gp120 epitope ^{454}LTRDGGN^{460}, corresponding to loop 1, did not affect the binding to CD4 significantly, since these residues are located in a relatively flexible portion of gp120.[23] The lack of increased affinity of a molecular construct containing all three loops may be explained by a non-optimal cyclic peptide ring size or positioning of the individual cyclic peptides, since for example a [1−2−3] and a [2−1−3] construct are not identical. In the delineated approach the best hits (*i.e.* [2−3−3] and [2−2−3]) will be starting points for libraries with varied cyclic peptide ring size and variation of the scaffold. Ultimately, the best library member can be re-synthesized using our earlier developed non-stop solid phase synthesis allowing evaluation of the relative positioning of the cyclic peptide on the scaffold.[4]

In summary, CuAAc of a mixture of cyclic peptides to a scaffold molecule provides rapid access to a diversity of peptide biomolecular constructs as a clean smart library for mimicry of the discontinuous protein surface. Using this method, for the first time up to three different cyclic peptides were incorporated into a single molecule in a convergent synthetic manner employing a TAC-scaffold. In addition, the individual epitopes and scaffold molecules required for assembly of these molecular constructs were conveniently accessible by chemical synthesis. Appreciable binding to CD4 was found for certain library members and these can be used as starting points for the preparation of focused libraries. Ultimately, these compounds might be applied as synthetic vaccines for the generation of antibodies capable of binding to HIV. Moreover, the approach described here is very promising for the generation of mimics of other proteins containing discontinuous epitopes, possibly ultimately leading to synthetic antibodies.

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