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## Modular assembly of dimeric HIV fusion inhibitor peptides with enhanced antiviral potency

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### ABSTRACT

The HIV-1 envelope gp120/gp41 glycoprotein complex plays a critical role in virus-host cell membrane fusion and has been a focus for the development of HIV fusion inhibitors. In this Letter, we present the synthesis of dimers of HIV fusion inhibitor peptides C37H6 and CP32M, which target the trimeric gp41 in the pre-hairpin intermediate state to inhibit membrane fusion. Reactive peptide modules were synthesized using native chemical ligation and then assembled into dimers with varying linker lengths using Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) 'click' chemistry. Cell-cell fusion inhibition assays demonstrated that dimers with a (PEG)<sub>7</sub> linker showed enhanced antiviral potency over the corresponding monomers. Moreover, the bio-orthogonal nature of the CuAAC 'click' reaction provides a practical way to assemble heterodimers of HIV fusion inhibitors. Heterodimers consisting of the T20-sensitive strain inhibitor C37H6 and the T20-resistant strain inhibitor CP32M were produced that may have broader spectrum activities against both T20-sensitive and T20-resistant strains.

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The human immunodeficiency virus type 1 (HIV-1) is an enveloped virus, and its envelope glycoprotein complex gp120/gp41 plays a critical role in HIV entry into host cells. The current model of virus entry (Fig. 1A) involves binding of the trimeric gp120 to the host cell CD4 receptor and chemokine co-receptors CCR5 or CXCR4. Upon binding, gp120 undergoes conformational changes and dissociates from gp41, which results in insertion of the N-terminal fusion peptide of gp41 into the host cell membrane and formation of a pre-hairpin intermediate. Later, the N-terminal heptad repeat 1 region (HR1) and the C-terminal heptad repeat 2 region (HR2) of gp41 fold back to form a six-helix bundle. In this fusion active hairpin structure, the virus membrane and the host membrane are brought close enough for fusion.<sup>1,2</sup> HIV fusion inhibitor peptides derived from the HR2 region of gp41 (referred to as C-peptides) were discovered to prevent hairpin structure formation by binding to the HR1 region of gp41 in the pre-hairpin intermediate. This stabilizes the pre-hairpin structure and consequently inhibits membrane fusion and viral entry.<sup>3,4</sup> Among these peptides, T20 (also known as DP-178, Enfuvirtide, and Fuzeon)<sup>5,6</sup> and C34<sup>7</sup> are well-studied inhibitors with IC<sub>50</sub> values in the nM range (Fig. 1B). T20 is also the only member of this class of peptide fusion inhibitors approved by FDA as an antiretroviral drug. Although T20 has been successfully used in the treatment of HIV infection, a

variety of T20-resistant HIV mutation strains have been isolated from patients.<sup>8</sup> Therefore, new potent HIV fusion inhibitor peptides against T20-resistant HIV strains have been developed.<sup>9–15</sup>

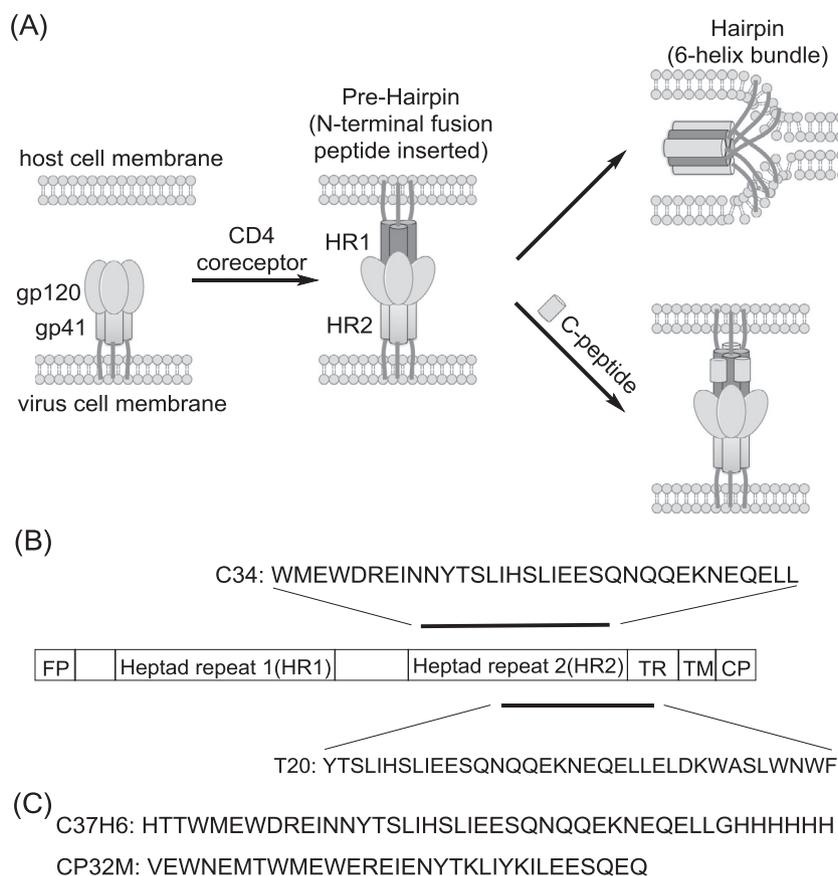
Both structural and biochemical studies have demonstrated that gp120 and gp41 are active as trimers.<sup>16,17</sup> Because of this, both gp120 and gp41 have three binding sites for their targets and for potential inhibitors. The trimeric states of gp120 and gp41 have been the focus for development of more potent inhibitors and vaccine candidates utilizing simultaneous multivalent interactions. Wang and co-workers have synthesized dimeric and trimeric CD4-mimetic miniproteins that target the CD4-binding sites of trimeric gp120 and block gp120 binding to CD4. These multivalent miniproteins showed enhanced antiviral activity over the monovalent miniprotein.<sup>18,19</sup> Trimeric N- and C-peptides of gp41, designed by Tam and co-workers for mimicking the pre-hairpin intermediate of gp41, were more potent than the corresponding monomers and may be useful as vaccine candidates for blocking HIV infection.<sup>20,21</sup> Recently, We and other groups also developed dimeric and trimeric C-peptide HIV fusion inhibitors with improved potency.<sup>22–26</sup> These studies demonstrate that multivalent approaches to targeting gp120 and gp41 can be utilized to increase inhibitor potency.

The Cu(I)-catalyzed azide-alkyne cycloaddition, termed CuAAC 'click' chemistry, is a highly chemoselective reaction that has been widely used to form selective conjugates of small molecules, peptides, and proteins for drug discovery and investigations of biological function.<sup>27–33</sup> Recently, we have developed a novel strategy to synthesize homodimers and heterodimers of expressed proteins and peptides via a combination of native chemical ligation

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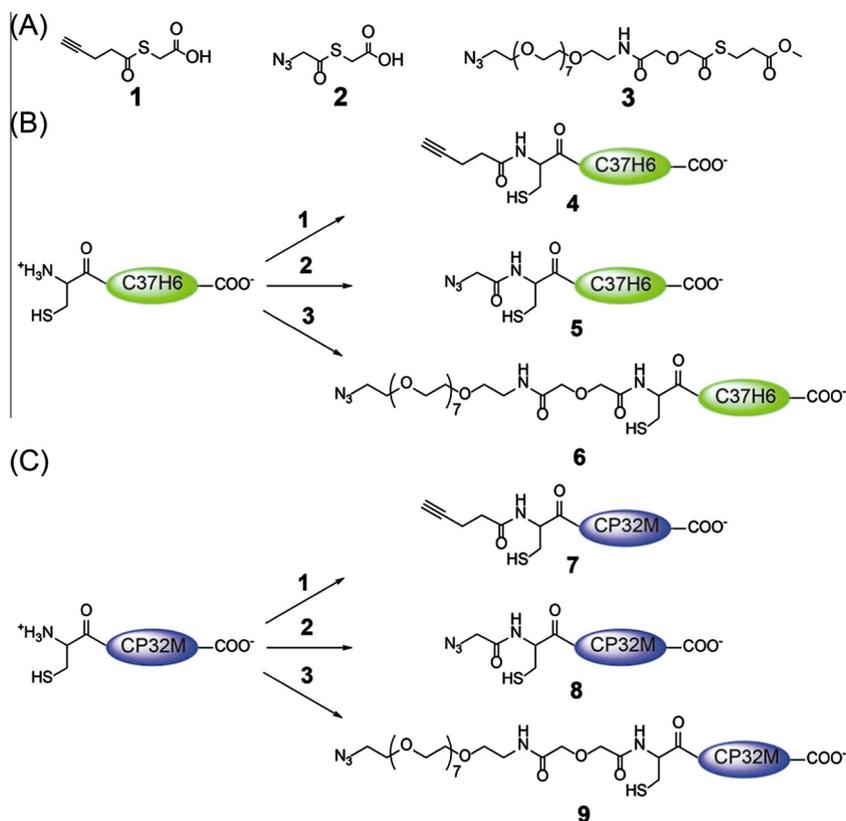
**Figure 1.** HIV fusion and HIV fusion inhibitors. (A) Schematic representation of the mechanism for HIV membrane fusion and fusion inhibition by C-peptides.<sup>1,2</sup> (B) Schematic representation of structure of gp41, and the locations and sequences of HIV fusion inhibitor C34 and T20. FP, fusion peptide; TR, tryptophan-rich domain; TM, transmembrane domain; CP, cytoplasmic domain. (C) The sequences of C37H6 and CP32M.

(NCL)<sup>34</sup> and CuAAC.<sup>35</sup> In this strategy, the N-terminus of proteins and peptides are first functionalized with azide and alkyne groups by NCL. The azide and alkyne groups are then coupled together to form protein homodimers or heterodimers through CuAAC. To develop more potent fusion inhibitor peptides, we present the application of this strategy to assemble homodimers of HIV fusion inhibitor peptides C37H6,<sup>36,37</sup> an analog of the HIV fusion inhibitor C34, and CP32M,<sup>9</sup> a newly developed HIV fusion inhibitor against both T20-sensitive and T20-resistant strains targeting the trimeric gp41 in the pre-hairpin intermediate (peptide inhibitor sequences shown in Fig. 1C). Moreover, the bio-orthogonal nature of the CuAAC reaction also provides us a practical way to assemble heterodimers of C37H6 and CP32M that are functionalized with alkyne and azide moieties respectively. The heterodimers assembled in this way could have broader spectrum activities against both T20-sensitive and T20-resistant strains due to the presence of both the T20-sensitive strain inhibitor C37H6 and the T20-resistant strain inhibitor CP32M. Treatment of HIV infection with heterodimers made up of monomeric HIV fusion inhibitors active against different drug resistant strains could also potentially slow or prevent the development of drug-resistance. Functionalization of peptides with alkyne and azide groups by NCL requires alkyne and azide thioesters and N-terminal cysteine containing peptides (Scheme 1), which were generated by tobacco etch virus N1a (TEV) protease cleavage.<sup>38</sup> A fusion protein, GPRT-C37H6, that contains a TEV protease cleavage sequence between the carrier protein GPRT and C37H6 was expressed and purified from *Escherichia coli* as described previously.<sup>37</sup> Treatment of this fusion protein with TEV protease released the C37H6 peptide with an N-terminal cysteine (Fig. S1A). Due to the hydrophobic nature of

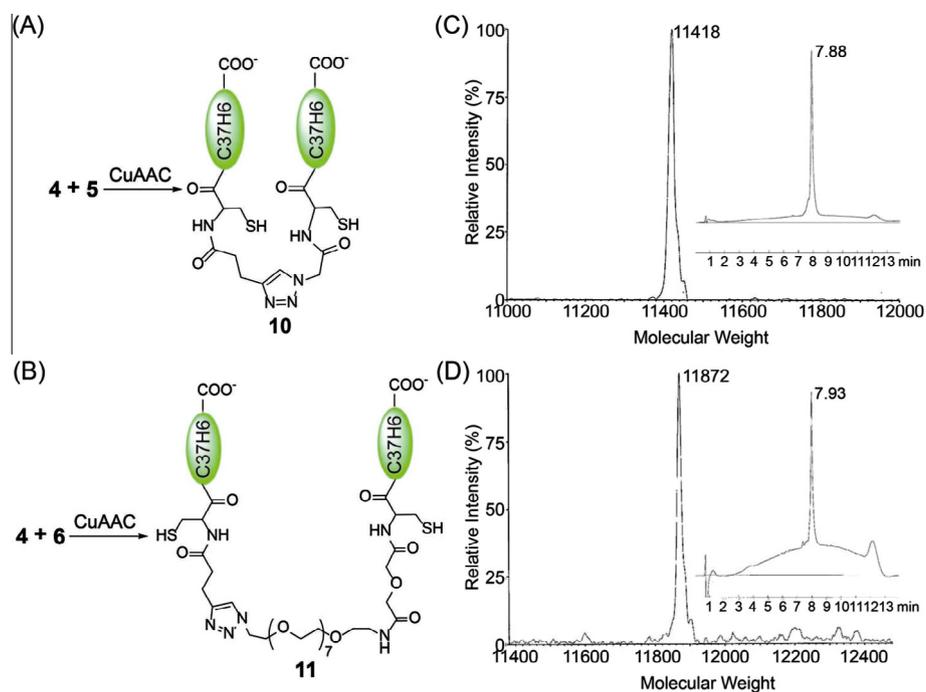
the CP32M peptide, the carrier protein SUMO was used as the fusion partner because of its ability to enhance protein expression and solubility.<sup>39,40</sup> Similarly, the fusion protein SUMO-CP32M that contains the TEV protease cleavage sequence was expressed, purified and treated with TEV protease to produce N-terminal cysteine containing CP32M (Fig. S2A). The cleaved C37H6 and CP32M peptides were separated from their fusion partners by Ni<sup>2+</sup>-NTA affinity chromatography, and then further purified by reverse phase HPLC and lyophilized. The alkyne thioester **1** and the azide thioester **2** were synthesized as described previously.<sup>35</sup> An azide thioester **3** with a polyethylene glycol (PEG)<sub>7</sub> linker was also synthesized to allow the production of dimers with an extended linker between peptide monomers (Scheme S1).

The alkyne functionalized C37H6 (alkyne-C37H6 **4**) and azide functionalized C37H6 (azide-C37H6 **5** and azide-PEG-C37H6 **6**) were obtained by incubating 2 mg/mL of C37H6 with 4 mM alkyne thioester **1**, and azide thioesters **2** and **3**, respectively, in the presence of 30 mM sodium 2-mercaptoethanesulfonate in denaturing buffer (7 M guanidine hydrochloride, 100 mM sodium phosphate pH 7.5) (Scheme 1B). After 24 h the reaction mixtures were purified by reverse phase HPLC and lyophilized to give **4**, **5** and **6** (Fig. S1B, C and D). The same reaction conditions were also used to produce alkyne-CP32M **7**, azide-CP32M **8** and azide-PEG-CP32M **9** (Scheme 1C, Fig. S2B, C and D).

To assemble homodimers of HIV fusion inhibitors, alkyne functionalized peptide monomers and azide functionalized peptide monomers were coupled together through CuAAC. Incubation of alkyne-C37H6 **4** with azide-C37H6 **5** or azide-PEG-C37H6 **6** in the presence of 1 mM CuSO<sub>4</sub> and 2 mM L-ascorbic acid in denaturing buffer gave the C37H6-C37H6 homodimer **10** or C37H6-PEG-C37H6



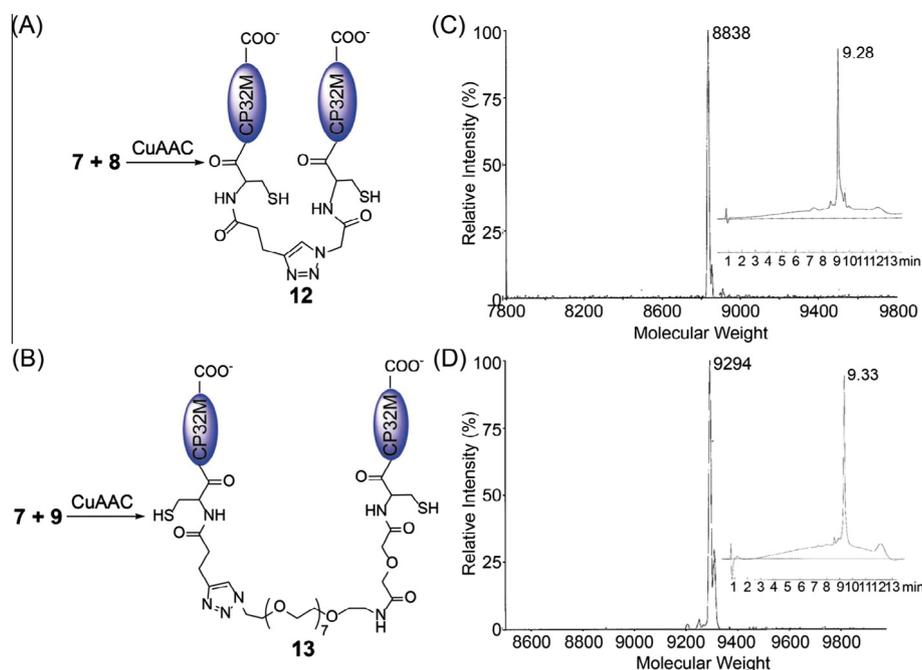
**Scheme 1.** Functionalization of C37H6 and CP32M with alkyne and azide groups by NCL. (A) Chemical structure of alkyne and azide thioesters **1–3**. (B) Synthesis of alkyne and azide functionalized C37H6 **4–6**. (C) Synthesis of alkyne and azide functionalized CP32M **7–9**.



**Figure 2.** Synthesis of C37H6 homodimers. (A) Synthesis of C37H6-C37H6 homodimer **10**. (B) Synthesis of C37H6-PEG-C37H6 homodimer **11**. (C) Analytical HPLC profile and ESI-MS of **10**, calcd 11419, found 11418. (D) Analytical HPLC profile and ESI-MS of **11**, calcd 11873, found 11872.

homodimer **11** with a short and long linker between two monomers, respectively (Fig. 2A and B). The dimer products were separated from the unreacted monomers by size-exclusion chromatography and further purified by reverse phase HPLC and lyophilized (74% yield for **10**

and 63% yield for **11**). The analytical HPLC and ESI-MS profiles of purified homodimers **10** and **11** are shown in Figure 2C and D, which indicates the desired products were obtained with high purity. CP32M-CP32M homodimer **12** with a short linker and CP32M-PEG-



**Figure 3.** Synthesis of CP32M homodimers. (A) Synthesis of CP32M-CP32M homodimer **12**. (B) Synthesis of CP32M-PEG-CP32M homodimer **13**. (C) Analytical HPLC profile and ESI-MS of **12**, calcd 8839, found 8838. (D) Analytical HPLC profile and ESI-MS of **13**, calcd 9294, found 9294.

CP32M homodimer **13** with a long (PEG)<sub>7</sub> linker were also produced, purified and characterized using the same procedures (57% yield for **12** and 55% yield for **13**) (Fig. 3).

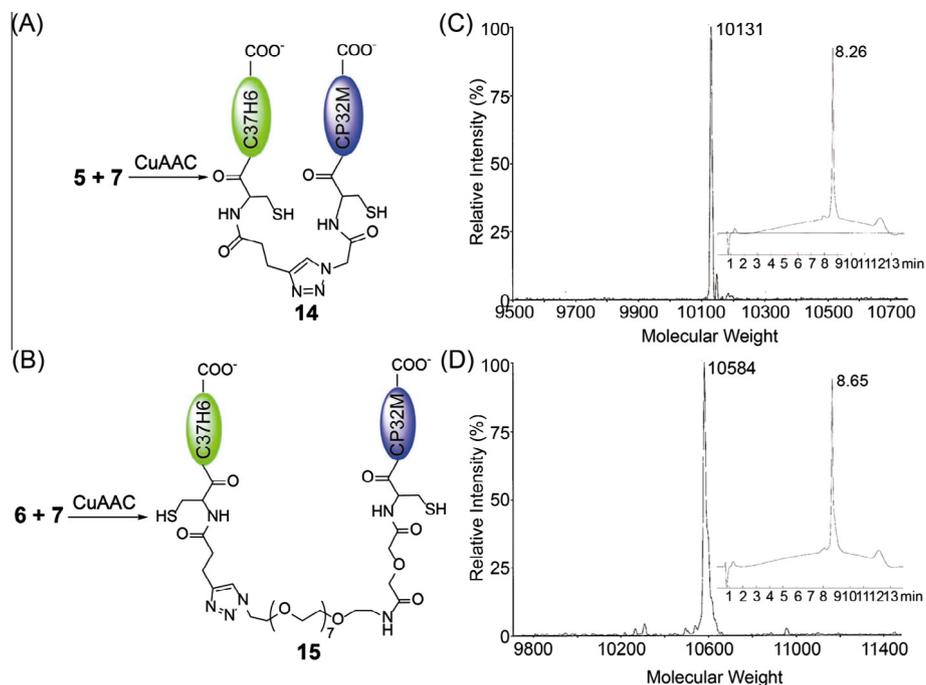
To examine whether the divalent HIV fusion inhibitors have increased antiviral activities compared to monovalent inhibitors, the antiviral activities were examined using a luciferase-based cell–cell fusion inhibition assay.<sup>41</sup> Briefly, two cell lines, HL2/3<sup>42</sup> and TZM-bl<sup>43</sup>, were used in this assay. The HL2/3 cell line stably expresses HIV Gag, Env, Tat, Rev and Nef proteins. The TZM-bl indicator cell line stably expresses large amounts of CD4 and CCR5 and contains integrated copies of the luciferase gene under control of the HIV-1 promoter. Fusion between HL2/3 cells and TZM-bl cells can be monitored by luciferase activity induced by cell fusion, and can also be inhibited by the addition of HIV fusion inhibitor peptides into the cell culture media. Results from three independent assays are summarized in Table 1 (also see Fig. S3).

As we expected, the bacterial expressed fusion inhibitors C37H6 and CP32M are active to block cell–cell fusion with an IC<sub>50</sub> of 4.4 nM and 3.3 nM, respectively. Although the homodimers with a short linker, C37H6-C37H6 **10** and CP32M-CP32M **12**, showed very little or even no improvement of potency over the corresponding monomers (1.6-fold and 0.8-fold, respectively), the homodimers with a long (PEG)<sub>7</sub> linker, C37H6-PEG-C37H6 **11** and CP32M-PEG-CP32M **13**, showed approximately fourfold enhanced antiviral potency over the corresponding monomers. The difference in activities among the dimers with different linker length indicates that the space between the two monomer units may have some influence on the activities of the dimers. The spacing in **10** and **12** might be too short to allow the second peptide monomer of the dimer to reach the second binding site of trimeric gp41 in the pre-hairpin state while the first monomer is simultaneously binding to the first binding site of gp41. Therefore, these two dimers may only act as monomeric inhibitors. In contrast, **11** and **13** have a longer (PEG)<sub>7</sub> linker, which apparently gives the two monomer units enough space to bind two different binding sites on trimeric gp41 simultaneously to achieve higher affinity binding and consequently obtain enhanced antiviral potency.

**Table 1**  
Antiviral activities of the HIV fusion inhibitors

Compound	IC <sub>50</sub> (nM)	Relative activity (C37H6)	Relative activity (CP32M)
C37H6	4.4 ± 0.1	1.0	–
CP32M	3.3 ± 0.3	–	1.0
C37H6-C37H6 <b>10</b>	2.7 ± 0.3	1.6	–
C37H6-PEG-C37H6 <b>11</b>	1.1 ± 0.1	4.0	–
CP32M-CP32M <b>12</b>	4.2 ± 0.2	–	0.8
CP32M-PEG-CP32M <b>13</b>	0.8 ± 0.1	–	4.1
C37H6-CP32M <b>14</b>	4.9 ± 0.4	0.9	0.7
C37H6-PEG-CP32M <b>15</b>	1.2 ± 0.1	3.7	2.8

In addition to homodimers, the bio-orthogonal nature of the CuAAC reaction also provides us a more practical way to assemble heterodimers of HIV fusion inhibitors with broader spectrum activities by joining a T20-sensitive strain inhibitor with a T20-resistant strain inhibitor that are functionalized with alkyne and azide moieties respectively. As an example, two heterodimers C37H6-CP32M **14** and C37H6-PEG-CP32M **15** were synthesized by coupling azide-C37H6 **5** and azide-PEG-C37H6 **6** with alkyne-CP32M **7**, respectively (Fig. 4A and B). After purification and lyophilization (68% yield for **14** and 56% yield for **15**), the products were characterized by analytical HPLC and ESI-MS (Fig. 4C and D), which indicated the desired heterodimers were produced. The antiviral activities of **14** and **15** were determined by cell–cell fusion inhibition assays as shown in Table 1. Similar to the homodimers **10** and **12**, the heterodimer C37H6-CP32M **14** showed no improvement of antiviral activity, presumably due to the short linker between the monomers. However, the heterodimer C37H6-PEG-CP32M **15** inhibited the cell–cell fusion with an IC<sub>50</sub> of 1.2 nM, which was 3.7-fold more potent than the C37H6 monomer and 2.8-fold more potent than the CP32M monomer. In addition to improved potency, the heterodimers of C37H6 and CP32M should have broader activity against both T20-sensitive and T20-resistant virus strains. Moreover, treatment of HIV infection with heterodimers of HIV fusion inhibitors could potentially slow or prevent the devel-



**Figure 4.** Synthesis of C37H6-CP32M heterodimers. (A) Synthesis of C37H6-CP32M heterodimer **14**. (B) Synthesis of C37H6-PEG-CP32M heterodimer **15**. (C) Analytical HPLC profile and ESI-MS of **14**, calcd 10130, found 10131. (D) Analytical HPLC profile and ESI-MS of **15**, calcd 10584, found 10584.

opment of drug-resistance. This is because the two peptides in the heterodimer have different sequences and different binding sites on gp41, and it will likely require more mutations to create resistance to the heterodimer than to either monomeric inhibitor alone.

In conclusion, a series of homodimers and heterodimers of HIV fusion inhibitor peptides C37H6 and CP32M have been assembled using a combination of NCL with CuAAC. Peptide inhibitors were produced by recombinant protein expression and then ligated to alkyne and azide linkers using NCL. Alkyne and azide adapted HIV fusion inhibitor modules could then be assembled to produce homodimeric and heterodimeric inhibitors with varied linker lengths by utilizing CuAAC. Cell–cell fusion inhibition assays demonstrated that although the dimers with a short linker did not improve antiviral activities compared to monomers, the dimers with a longer (PEG)<sub>7</sub> linker did show about fourfold enhanced antiviral potency over the corresponding monomers. Furthermore, by synthesizing a heterodimer consisting of T20-sensitive strain inhibitor C37H6 and T20-resistant strain inhibitor CP32M, it is likely that the resulting heterodimeric inhibitor will have a broader spectrum activity against both T20-sensitive and T20-resistant virus strains. Treatment of HIV infection with heterodimers of HIV fusion inhibitors could also potentially slow or prevent the development of drug-resistance. This strategy could be applied to assemble homodimers and heterodimers, or even higher order multimers of different types of HIV inhibitors to create a library of HIV inhibitors with potentially greater potency, broader specificity and less chance for the development of drug-resistance.

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#### Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2013.09.034>.

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