Bioorganic & Medicinal Chemistry Letters 28 (2018) 378–381

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Enhanced intracellular peptide delivery by multivalent cell-penetrating peptide with bioreducible linkage

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ARTICLE INFO

Article history:
Received 21 September 2017
Revised 6 December 2017
Accepted 14 December 2017
Available online 15 December 2017

Keywords:
Cell-penetrating peptide
Multivalency
Bioreducible linkage
Intracellular delivery
Apoptosis

ABSTRACT

Multivalent cell-penetrating peptides (CPPs) have been reported to show enhancement in cellular uptake and endosomolytic activity. However, its application was limited to *trans*-delivery of cargo which is lower in cellular uptake efficiency of cargo than *cis*-delivery. Here, we tried the *cis*-delivery of cargo with multivalent CPP by preparing bioreducible dimeric CPP–cargo with apoptotic activity using TatBim peptide, a fusion of Tat CPP and Bim peptide derived from Bim apoptosis-inducing protein. Dimeric TatBim was almost twice as highly internalized by cells and significantly induced apoptosis compared to monomeric TatBim. Contribution of bioreducible linkage of dimeric TatBim towards apoptotic activity was also confirmed.

Cell penetrating peptides (CPPs), which are 8–30 amino acids long with cationic or amphipathic sequences, can efficiently deliver membrane impermeable bioactive macromolecules such as peptides, proteins, and oligonucleotides into cells.[1,2] Generally, CPP-conjugated macromolecules (CPP–cargos) enter cells by various modes of endocytosis.[1] However, CPP–cargos often get trapped in the endosome and are unable to reach their intracellular targets. Thus, CPP–cargos should be able to escape from the endosomal compartment in order to improve the biological activity of the cargo.[3]

Recently, multivalent CPPs have been reported to show enhancement in the cellular uptake and escape from endosomes with high efficiency as compared to that of their parent monomers.[4–10] For instance, dimeric Tat peptide gets twice as highly internalized by cells as compared to monomeric Tat peptide.[4,7] Multivalent Tat peptide also works as an endosomolytic agent and can deliver proteins into cytoplasm by simple co-incubation procedure.[5,6] In another example, co-incubation of branched multivalent Tat peptide has highly improved the adenoviral transduction into human mesenchymal stem cells (MSCs) by three orders of magnitude compared to that of the monomer.[8]

Despite the enhanced cellular uptake and endosomolytic activity of multivalent CPP, its application has been restricted to *trans*-delivery (co-incubation) of cargo as described above. However, cellular uptake efficiency of cargo by *trans*-delivery is quite low as compared to that by *cis*-delivery (covalent conjugation) and is easily affected by the nature of cargo. Therefore, *cis*-delivery of cargo with multivalent CPP peptide would significantly increase the cellular uptake of cargo and augment its activity by improvement in endosomal escape. Thus, we prepared bioreducible dimeric CPP–cargo with apoptotic activity using TatBim peptide, which is a fusion of Tat CPP and Bim peptide derived from Bim apoptosis-inducing protein.[11,12] Dimeric TatBim was prepared by the formation of a disulfide bridge to cleave itself in the reductive intracellular environment, where the glutathione concentration is 100–1000 times higher than that in the extracellular milieu.[13,14]

Monomeric TatBim (mTatBim) with a cysteine residue at C- terminus was prepared by the solid-phase peptide synthesis using standard Fmoc protocols (Fig. 1a). Molecular weight of mTatBim was confirmed by MALDI-TOF mass spectrometry measurement (Calculated mass $[M+H]^+$; 4262.36 Da, Observed mass $[M+H]^+$; 4261.83 Da, Fig. S1). Dimeric TatBim (diTatBim) was prepared by intermolecular disulfide bond formation of mTatBim (Fig. 1b), and the reactant was subjected to reverse-phase HPLC.[15] The HPLC fraction with a retention time of ~24 min (fraction #3) corresponded to diTatBim (Fig. 1c, d). The purity of diTatBim was more than 95% (Fig. S2). diTatBim showed a molecular weight comparable with that of mTatBim under reduced conditions, which indicated that mTatBim was dimerized by disulfide bond formation (Fig. 1d).

To visualize the cellular uptake of diTatBim, the peptide was fluorescently labeled with ATTO 520 NHS-ester (AT520) (Fig. S3).[16]

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https://doi.org/10.1016/j.bmcl.2017.12.035
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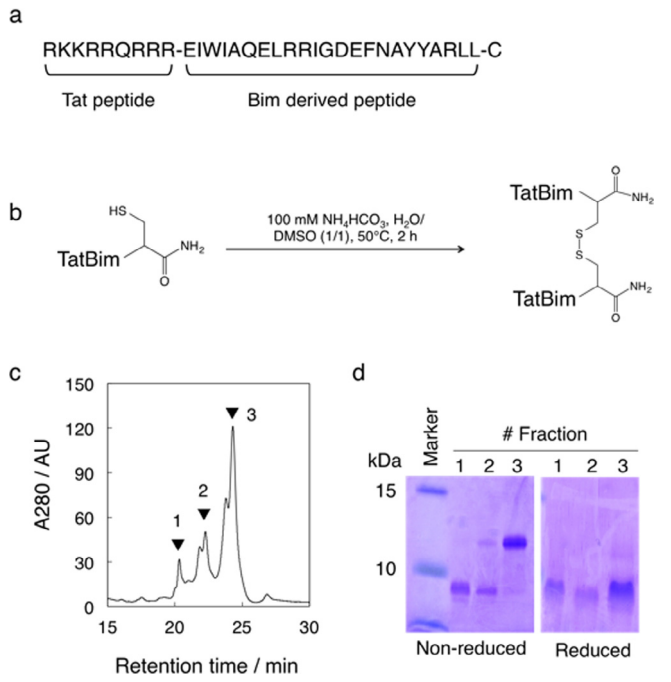

Fig. 1. TatBim peptide was efficiently dimerized by disulfide bond formation. (a) Amino acid sequence of TatBim peptide. (b) Dimerization of TatBim by intermolecular disulfide bond formation. (c) HPLC purification of the reaction solution to obtain the dimeric TatBim. Numbers on the peaks indicate the collected fractions. (d) 18% SDS-PAGE analysis of the collected fractions.

Then, to prepare AT520-labeled diTatBim, the purified AT520-labeled mTatBim was dimerized by intermolecular disulfide bond formation (Fig. S4). The molar ratios of AT520 attached to the TatBim unit in mTatBim and diTatBim were 0.15 and 0.13, respectively, and the former was adjusted to 0.13 with the unlabeled mTatBim. HeLa cells were treated for 30 min at 37 °C with 10 μM mTatBim or 5 μM diTatBim, so that the concentration of TatBim unit remains the same in both treatments. After the 30-min treatment, the medium was changed to complete medium supplemented with 10% FBS. Then the cellular uptake was evaluated using a fluorescence microscope (Olympus IX51). Because of the presence of free AT520 (1.2 μM) in 10 μM AT520-labeled mTatBim and 5 μM diTatBim samples (Figs. S3 and S4), cellular uptakes of free AT520 were also tested. Internalization of free AT520 was not observed under this condition (Fig. S5). As shown in Fig. 2a, diTatBim was significantly internalized by cells to a higher extent compared to mTatBim, although the same concentration of TatBim units was used during both the treatments. Mean fluorescence intensity was evaluated by calculating the average of red pixel intensities of three individual images using Image J software (NIH, Bethesda, MD). Mean fluorescence intensity of diTatBim was approximately twice as higher than that of mTatBim (Fig. 2b). More than twofold increase in cellular uptake was also reported by the dimerization of Tat peptide.[4,7] This enhancement might be derived from the increase in local concentration of the TatBim peptide on cell membrane, which would in turn increase membrane permeability of the peptide by intensified guanidinium–phospholipid interactions.[17]

Next, we evaluated the apoptotic activity of mTatBim (10 μM) and diTatBim (5 μM).[18] diTatBim significantly induced higher apoptosis compared to mTatBim (Fig. 3a), and cell proliferation was strongly inhibited by treatment with diTatBim (32%) compared to that with mTatBim (63%) (Fig. 3b). Dimerized Tat peptide (Fig. S6) did not show apoptotic activity, which demonstrated that the high cationic charge density of diTatBim is not the reason for the enhanced apoptotic activity. Consistent with the caspase-3 assay, the higher extent of apoptosis with diTatBim was also observed in the Annexin V assay, which evaluated exposed phosphatidylserine on the surface of apoptotic cells (Fig. S7).

To investigate the contribution of bio-reducible linkage (disulfide bridge) on the enhanced apoptotic activity of diTatBim, we prepared and purified non-reducible diTatBim (NR-diTatBim) linked by N,N′-1,4-phenylenedimaleimide (Fig. 4a–c). The peptide in fraction #3, with a retention time of ~20 min, was not cleaved even under reduced conditions (Fig. 4b and c). Therefore, we assigned the fraction #3 to NR-diTatBim and used it for the following cellular assay. Interestingly, apoptotic activity of diTatBim was higher than that of NR-diTatBim (Fig. 4d). Cell proliferation was inhibited to a higher extent by the treatment with diTatBim (34%) compared to that with NR-diTatBim (49%) (Fig. 4e), suggested that cleavage of diTatBim in a reductive intracellular environment accelerated the apoptotic activity of diTatBim. Compared to NR-diTatBim, a higher degree of freedom of the Bim domain in reduced diTatBim might have better accessibility to its binding domain for Bax or Bak, which mediate apoptosis through the release of cytochrome *c* from the mitochondria.[19]

In conclusion, diTatBim got twice as highly internalized by cells as compared to mTatBim, even at the same concentration of TatBim units. In relation to this enhanced cellular uptake of diTatBim, diTatBim significantly induced apoptosis compared to mTatBim. Then, we demonstrated that the bioreducible diTatBim with disulfide linkage was more potent to induce apoptosis than the non-reducible diTatBim. These results provide guidelines to design other multivalent CPP–cargos for the development of enhanced intracellular delivery of bioactive peptide cargos.

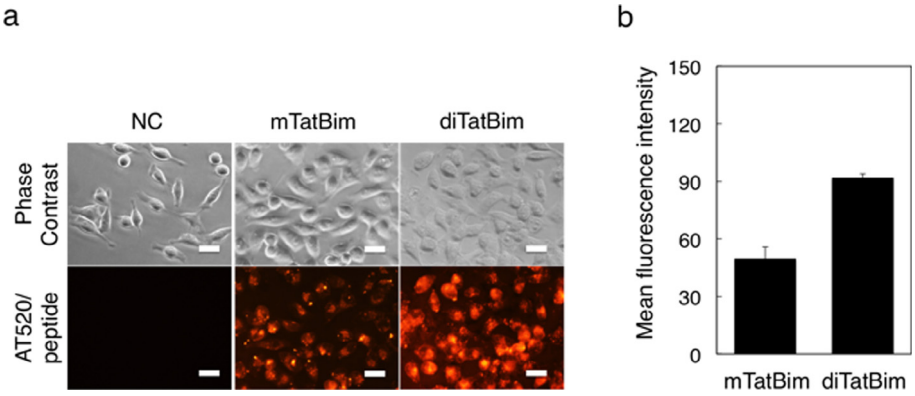

Fig. 2. diTatBim was highly internalized by HeLa cells compared to mTatBim. (a) mTatBim and diTatBim were fluorescently labeled with AT520. HeLa cells were treated with 10 μM mTatBim or 5 μM diTatBim for 30 min at 37 °C. *Scale bar*, 20 μm. (b) Mean fluorescence intensity was calculated by the red pixel intensity of the images.