Published in final edited form as:
Bioconjug Chem. 2010 December 15; 21(12): 2164–2167. doi:10.1021/bc100130r.

Generation of Endosomolytic Reagents by Branching of Cell-Penetrating Peptides: Tools for the Delivery of Bioactive Compounds to Live Cells in Cis or Trans

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Abstract

We describe the synthesis and cellular delivery properties of multivalent and branched delivery systems consisting of cell-penetrating peptides assembled onto a peptide scaffold using Native Chemical Ligation. A trimeric delivery system presenting three copies of the prototypical cell-penetrating peptide TAT shows an endosomolytic activity much higher than its monomeric and dimeric counterparts. This novel reagent promotes the endosomal release of macromolecules internalized into cells by endocytosis and, as a result, it can be used to achieve cytosolic delivery of bioactive but cell-impermeable macromolecules in either cis (covalent conjugation) or trans (simple co-incubation).

Cell-penetrating peptides (CPPs) can be conjugated to cell-impermeable macromolecules and carry their cargo into mammalian cells.(1-3) A prototypical CPP is the cationic TAT peptide (GRKKRRQRRR). TAT-macromolecule conjugates interact with cell-surface proteoglycans and are internalized into cells by TAT-induced macropinocytosis and other endocytosis mechanisms. TAT and its cargo then enter the cytosolic space of the cell by escaping from endosomes.(4) This endosomolytic activity is however only modest and a large fraction of macromolecules remain trapped inside endocytic organelles and unable to reach their intracellular targets.(4,5) In order to increase delivery of cargo to the cytoplasm, the challenge is to enhance the endosomolytic activity of TAT. This is particularly difficult given that the exact mechanisms involved in this critical step of the delivery process are unclear. A potential solution to this problem however is to create multivalent delivery agents displaying multiples copies of the peptide.(6) This is based on the principle that multivalency results in an increase of the CPP's local concentration at the sites where the peptide interacts with cellular components. It has been clearly established that such an approach can increase the endocytic uptake of cationic peptides, and that branched molecules can function and multitask in ways their linear homologues cannot. (7-9) Whether multivalency enhances the endosomolytic activity of CPPs remains however uncertain. In this report, we hypothesize that branched TAT derivatives would also have an increased endosomolytic activity as compared to TAT and that these reagents could induce the release of macromolecules trapped inside endocytic organelles into the cytosolic space of cells. To test this hypothesis, we report a simple and modular synthetic route to generate branched

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multivalent CPPs carrier agents. We show that dimerization of TAT does not improved its delivery properties while species with more than 3 branches lead to unproductive membrane binding. In contrast, a trimeric TAT translocates into live cells with much higher efficiency than its monomeric counterpart. Importantly the endosomolytic activity of the trimeric peptide is sufficiently increased that it is able to deliver a bioactive peptide *in trans*. Together, these results should be important for the future design of multivalent delivery agents and establish branched CPP species as non-cytotoxic endosomolytic agents that could complement numerous delivery methodologies.

Our synthetic approach consists of assembling multiple CPPs onto scaffold peptides using Native Chemical Ligation (NCL).(10) The scaffold peptides contain several lysines that have been modified at the e NH_2 with a cysteine residue (Figure 1).(11) Each cysteine is available to react with TAT containing a *C*-terminal thioester to generate branched species. The scaffold peptides were synthesized by SPPS using standard Fmoc protocols. Two to six lysine residues bearing Mtt protecting groups at the e NH_2 were incorporated on rink amide resin and separated by glycine residues to reduce steric hindrance during synthesis and NCL. The lysine side chains were deprotected orthogonally with 2% TFA, and Boc-Cys(S^tBu)-OH was coupled onto the exposed e NH_2. This was followed by *N*-terminal Fmoc deprotection and subsequent coupling of 5(6)-carboxyfluorescein (Fl). TFA cleavage and HPLC purification afforded the scaffold peptides containing 2 to 6 sites for NCL. TAT thioester was obtained by SPPS on a sulfamylbutyryl resin using reported protocols.(12) The multivalent TAT compounds were obtained by reacting the scaffold peptides containing n cysteines with 3 n equivalents of TAT-thioester at room temperature in aqueous buffer at pH 7.5. Formation of the products, Fl-TATn, was detected by HPLC as early as 1 hour, and the reactions were complete in less than 12 hours.

The cellular import of the multivalent compounds was first investigated by live-cell fluorescence microscopy. The fluorescein molecule installed at the *N*-terminus of the peptides was used as both a fluorescent label and a model of a small molecule cargo. A fluorescein labeled TAT (Fl-TAT) was evaluated as a “non-branched” control. Fl-TAT, Fl-TAT2, and Fl-TAT3 were incubated with HeLa cells for 30 min at 1 or 5 μM. These concentrations were chosen because Fl-TAT is known to penetrate cells poorly under a concentration threshold of 5-10 μM.(13) We therefore considered that it would be possible to observe any enhancement of activity by Fl-TAT2 and Fl-TAT3 as compared to Fl-TAT under conditions where the monomeric peptide shows low activity. After washing the cells with fresh media, cells were imaged by fluorescence confocal microscopy (Figure 1b). In the case of Fl-TAT and Fl-TAT2, a punctate distribution consistent with accumulation of the compounds inside endocytic organelles was observed at both concentrations. No accumulation of the peptides in the cytosol was detected. In contrast, Fl-TAT3 exhibited a clear diffuse and cytosolic distribution at 1 or 5 μM indicative of an increase in the translocation efficiency as compared to the monomeric and dimeric compounds. Importantly, this was not accompanied by an increase in cytotoxicity of Fl-TAT3 (see next paragraph and Figure 2). The transport of Fl-TAT3 into the cytosol was inhibited by amiloride, an inhibitor of macropinocytosis. These results indicate that Fl-TAT3 is predominantly internalized by macropinocytosis and that it reaches the cytosol of cells by escaping from the endocytic pathway. Fl-TATn with n= 4, 5, and 6 were also tested but could not be washed from the cell surface to a satisfactory level after incubation and analysis of their transport properties was therefore difficult. These results are consistent with the work of Futaki and coworkers who have reported the synthesis of peptides with four branched chains of the type $(R_n)_4$ (n = 1-6).(14) $(R_2)_4$ was shown to internalized into cells with an efficiency similar to that of its linear homologue R_8 while $(R_6)_4$ was found to only accumulate at the plasma membrane without being able to enter cells.(15) Likewise, Fl-TATn with n= 4, 5, and 6 might bind to cell-surface components to such an extent that, in

this case, it leads to a membrane-bound state that is not productive. Accordingly, these reagents were not considered further in our experiments.

To characterize the delivery activity of Fl-TAT3, we tested whether Fl-TAT3 might be capable of inducing the release of macromolecules from endosomes. This idea is based on the principle that, if Fl-TAT3 and a macromolecule are co-incubated and accumulate together within endocytic vesicles, Fl-TAT3 might cause leakage of the endosomal membrane and release of the macromolecule into the cell.(16) This was first suggested by the observation that 70 kDa Dextran-TMR, a marker of pinocytosis that typically accumulates inside endocytic vesicles when co-incubated with cells, was present into the cytosol of cells when co-incubated with Fl-TAT3 (Figure 1 C). To test this hypothesis further and evaluate the delivery of a bioactive macromolecule, the pro-apoptotic peptide $KLAKLAKKLAKLAKNH_2$ (PAD) was used. PAD is cell-impermeable and has a negligible cytotoxicity towards mammalian cells.(17) However, if delivered into the cytosol of cells, PAD permeabilizes the mitochondrial membrane and induces apoptosis.(18,19) Cell-death was therefore used as an indicator of the efficiency with which Fl-TAT3 might deliver PAD into cells. PAD labeled with carboxytetramethylrhodamine (TMR-PAD, 10 μM) was co-incubated with Fl-TAT (1 to 10 μM) or Fl-TAT3 (1 to 3 μM) for 30 min. The import of TMR-PAD, Fl-TAT and Fl-TAT3 into cells was monitored by confocal microscopy and cell viability was assessed by SYTOX® Blue staining. SYTOX® Blue stains the nucleus of apoptotic or necrotic cells with a compromised plasma membrane without staining live cells. When TMR-PAD was co-incubated with Fl-TAT, TMR-PAD was present inside endocytic organelles but did not appear to enter the cytosol of cells (as monitored by TMR fluorescence, not shown) regardless of the concentration of Fl-TAT used. In addition, the cells' viability was not affected by treatment with these peptides (Figure 2). These results are consistent with a model where Fl-TAT cannot promote endosomal release of TMR-PAD and, as a result, TMR-PAD is unable to induce cell-death. In contrast, cells incubated with Fl-TAT3 and TMR-PAD showed a diffuse and cytosolic distribution for both TMR-PAD and Fl-TAT3 (Figure 3). These cells were not stained by SYTOX® Blue 60 min post-incubation. However, 40 and 80% of the cells became SYTOX® Blue positive 240 min post-incubation when 1 or 3 μM Fl-TAT3 were used, respectively. Progression to classic morphological changes such as membrane blebbing and nuclear fragmentation further confirmed that cells were undergoing apoptosis. Treatment with amiloride inhibited delivery of Fl-TAT3 and TMR-PAD into cells as well as cell-death (Figure 2 and 3). These results therefore indicate that the cytotoxicity observed when Fluo-TAT3 and TMR-PAD are co-incubated arises from the delivery of TMR-PAD by Fl-TAT3 as opposed to cytotoxic effects that might arise when the peptides are combined and not related to the transport of TMR-PAD. Together, these results support a model where Fl-TAT3 and TMR-PAD are first internalized into cells by endocytic mechanisms and where Fl-TAT3 then releases TMR-PAD into the cytosol of cells.

The NCL synthesis scheme used in this report generates Fl-TATn compounds that contain n free thiols. One hand, these thiols could be used to attach molecular cargos to the multivalent delivery reagents. On the other hand, the thiols, when left unmodified, could promote the aggregation of Fl-TATn through the formation of intermolecular disulfide bridges. Such aggregates could participate in the cellular activities observed. The presence of aggregates could not be detected when the Fl-TATn compounds dissolved in PBS were oxidized by blubbing oxygen in the media (HPLC and mass spectrometry analysis), suggesting that intramolecular disulfide bonds might form preferentially over intermolecular bonds (Supporting Information). Nonetheless, in vitro assays might not reproduce what happens inside cells and it is possible that aggregates form in the lumen of endosomes. To address this issue, the thiols of Fl-TAT3 were blocked with iodoacetamide to form an acetamidated analogue of Fl-TAT3 (synthesis and characterization in Supporting

Information). This product, Fl-TAT3-(S-CH2-CO-NH2)3, can not form disulfide bonds because the thiols are replaced by thioethers. To compare the delivery activity of Fl-TAT3-(S-CH2-CO-NH2)3 with that of Fl-TAT3, Fl-TAT3-(S-CH2-CO-NH2)3 (3 μM) and TMR-PAD (10 μM) were co-incubated for 30 min with HeLa cells and cell-viability was assessed by microscopy. While incubation with Fl-TAT3-(S-CH2-CO-NH2)3 alone resulted in a cell viability of 90%, co-incubation with TMR-PAD resulted in a cell-viability of 25% 240 min post-incubation (Supporting Information). These results are similar to those obtained with Fl-TAT3. This suggests that the delivery activity of Fl-TAT3 is not a result of aggregation of the compound and that the presence of the thiols is not necessary to achieve delivery.

Overall, the trimeric TAT reported appears to have an endosomolytic activity much greater than its monomeric and dimeric counterparts without displaying the unproductive membrane-binding observed for constructs containing more branches. As a result, it can deliver the small molecule cargo fluorescein *in cis* at concentrations much lower than that required for Fl-TAT. In addition, it can also be used to achieve delivery of a bioactive but cell-impermeable peptide *in trans*. This is important because this demonstrates that a macromolecule does not need to be labeled with an extraneous delivery peptide to be delivered, thereby greatly simplifying delivery protocols for *in vitro* applications. We are now testing the applicability of this delivery method to other macromolecules and exploring the effect of the structure and spatial orientation of the scaffold peptide on the delivery activity of the TAT trimer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Award Number R01GM087227 from the National Institute of General Medical Sciences. A. E.-O. was supported by the Chemistry-Biology Interface training grant NIH 3 T32 GM008523.

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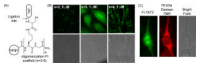

Figure 1.

(A) Structure of the multivalent delivery agents Fl-TATn. Multiple copies of the TAT peptide are ligated to a cysteine-containing oligomerization scaffold by NCL while a cell-impermeable cargo, fluorescein (Fl), is incorporated during SPPS. (B) Fluorescence images of live HeLa cells treated with Fl-TAT2 (n=2), Fl-TAT3 (n=3), and Fl-TAT4 (n=4). The fluorescent cargo is trapped inside endocytic organelles with Fl-TAT2 (punctate distribution, 5 μM, same results were obtained for Fl-TAT at 5 μM) but released into the cytosol with Fl-TAT3 (diffuse distribution in cytosol and nucleus) at only 1 μM. Fl-TAT4 is bound to the plasma membrane. (C) Fluorescence images of live HeLa cells treated with Fl-TAT3 (1 μM) and 70 kDa Dextran-TMR (10 μM), a marker of pinocytosis. The cells represented in B or C were not stained by SYTOX® Blue. This indicates that the membrane of these cells was not compromised.

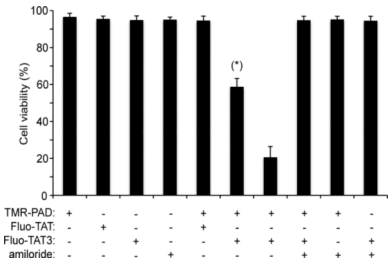

Figure 2.

Delivery of TMR-PAD into HeLa cells mediated by Fl-TAT (10 μM) and Fl-TAT3 (1 μM where highlighted with * or 3 μM otherwise) as measured by a reduction in cell viability. SYTOX® Blue staining was used to detect dead or dying cells 240 min after incubation with the peptides. Amiloride was used to inhibit macropinocytosis. Cell viability is expressed in % in comparison to an untreated sample. These results represent the average cell viability and corresponding standard deviation from three experiments.

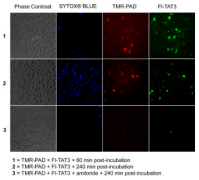

Figure 3.

Images of cells incubated with TMR-PAD (10 μM), Fl-TAT3 (3 μM), and with or without the macropinocytosis inhibitor amiloride. Cells were incubated with the peptides for 30 min, washed with fresh media, and SYTOX Blue was added to detect dead cells. Images were acquired 60 min or 240 min after addition of SYTOX® Blue. Blue nuclei in the SYTOX® Blue images represent dead or dying cells. Cells containing a cytosolic distribution of both TMR-PAD and Fl-TAT3 can be observed at 60 min.