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Multivalent presentation of the cell-penetrating peptide nona-arginine on a linear scaffold strongly increases its membrane-perturbing capacity☆

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ABSTRACT

Arginine-rich cell-penetrating peptides (CPP) are widely employed as delivery vehicles for a large variety of macromolecular cargos. As a mechanism-of-action for induction of uptake cross-linking of heparan sulfates and interaction with lipid head groups have been proposed. Here, we employed a multivalent display of the CPP nona-arginine (R9) on a linear dextran scaffold to assess the impact of heparan sulfate and lipid interactions on uptake and membrane perturbation. Increased avidity through multivalency should potentiate molecular phenomena that may only play a minor role if only individual peptides are used. To this point, the impact of multivalency has only been explored for dendrimers, CPP-decorated proteins and nanoparticles. We reasoned that multivalency on a linear scaffold would more faithfully mimic the arrangement of peptides at the membrane at high local peptide concentrations. On average, five R9 were coupled to a linear dextran backbone. The conjugate displayed a direct cytoplasmic uptake similar to free R9 at concentrations higher than 10 μM. However, this uptake was accompanied by an increased membrane disturbance and cellular toxicity that was independent of the presence of heparan sulfates. In contrast, for erythrocytes, the multivalent conjugate induced aggregation, however, showed only limited membrane perturbation. Overall, the results demonstrate that multivalency of R9 on a linear scaffold strongly increases the capacity to interact with the plasma membrane. However, the induction of membrane perturbation is a function of the cellular response to peptide binding.

1. Introduction

Conjugation to cell-penetrating peptides (CPPs) is considered a highly promising strategy to mediate cellular delivery of molecules that otherwise poorly enter cells [1–3]. Natural and synthetic CPPs are continuously being (re-)designed to improve delivery [4,5]. In contrast to the original concept of the CPP acting as a Trojan horse that passages passively through the plasma membrane by virtue of its structural characteristics it has become clear that with the exception of direct membrane permeation at low concentrations [6,7] CPPs actively induce cellular uptake [8]. In particular for large molecular weight cargos, endocytosis is the route of uptake [9]. Furthermore, concentrations above about 10 μM, arginine-rich CPPs induce activation of acid sphingomyelinase, which leads to rapid cytoplasmic import via ceramide-rich membrane microdomains [10,11].

While the molecular mechanism underlying the activation of acid sphingomyelinase has not been resolved, for induction of endocytosis, it has been proposed that binding to negatively charged oligosaccharides of the glycocalyx leads to a clustering of syndecans [12], which activates Rac-dependent actin remodeling [13]. However, uptake is also observed for cells that are poor in glycosaminoglycans such as Jurkat T cell leukemia cells which calls for the involvement of additional processes [11].

Strikingly, in spite of its activity as a CPP, for nona-arginine (R9) little to no enrichment at cellular membranes is observed. Only when

Abbreviations: CPP, cell-penetrating peptide; R9, nona-arginine; Dex-$(R9)_5$, pentavalent display of R9 on a linear dextran backbone; PI, propidium iodide; N.A., numerical aperture; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin

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internalization is compromised, a staining of the plasma membrane is visible which can be attributed to the interaction with glycosaminoglycans [14]. This indicates that in spite of a documented ability to interact with the head groups of the lipid bilayer and the capacity to partition into a hydrophobic environment in the presence of negatively charged chelating groups [15–17], on cells there is little enrichment at the level of the plasma membrane. Also, among a panel of nine tested CPPs R9 had the least membrane-disruptive potential [18], overall indicating that enrichment at the plasma membrane plays only a small role in the uptake of this CPP.

The geometry in which positive charge is presented greatly influences uptake efficiency. On one hand, it has been demonstrated that import can be enhanced through rigidifying the peptide backbone through cyclization [19]. On the other hand, multivalency is a well-established principle to potentiate molecular interactions through introduction of avidity [20–22]. Multivalent interactions in nature include binding of DNA at several sites by transcription factors, like the retinoid X receptor [20] or immunogenic recognition by multivalent antibodies such as IgM [23]. In the context of CPP, oligoarginines are an example for a multivalent display of the guanidino-group and up to a certain length, activity of oligoarginines increases with the number of residues [24,25]. With respect to a multivalent display of CPP, multivalency has been investigated for TAT, oligolysine, HSV-1 VP22, oligoarginine, Antp, (reviewed in Ref. [26]), zinc coordinated oligotyrosine peptides [27] and recently for TP10, pVEC and polyproline helix SAP [28]. A tetravalent presentation of deca-arginine fused to the p53 protein was shown to improve delivery efficiency even at low concentrations without increasing toxicity [29]. In the latter study, this increased activity was associated with enhanced interactions with cell surface heparan sulfates. However, these multivalent arrangements were either based on globular scaffolds or branched dendrimers, geometries that confine the area of interaction with the membrane components of the cell. To be effective for promoting uptake, a stretch of arginines has to be present. A dispersed presentation of individual arginines on a polymer results in considerably reduced uptake also at higher charge density [30]. Overall, the multivalent geometries that have been investigated so far for CPPs are more restricted than those addressed for antimicrobial peptides for which presentation along linear scaffolds has also been investigated, already [31].

In all these applications, the multivalent CPPs were well tolerated, showing little signs of toxicity. Also, individual oligoarginine peptides are generally well tolerated to concentrations in the mean micromolar range [11,24].

Beyond yielding a benefit in efficiency, so far, the multivalent display of CPPs has yielded little insights into the molecular mechanisms triggering import. Also, no functional characteristics were reported that differed greatly for those of the individual CPPs. As for individual CPPs, it has been proposed that the multivalent systems crosslink glycosaminoglycans followed by endocytosis [32]. This may be due to the globular nature of these structures that renders them very similar to CPPs linked to macromolecules.

Therefore, in the present study we compared a multivalent configuration of nona-arginine (R9) in which on average five copies of the CPP were coupled to a linear and flexible dextran backbone (Dex-$(R9)_5$) to the monovalent R9 counterpart. We hypothesized that in comparison to multivalent displays on globular structures, this configuration would result in contact of the molecule with a larger surface area of the plasma membrane or in a structurally adaptive binding due to the flexibility of the dextran backbone. In particular, we hoped that this configuration would shed further light into the structural requirements for triggering the rapid sphingomyelinase-dependent uptake mechanism. All data presented so far, indicated that this import route is restricted to free CPPs or CPPs conjugated to small molecular weight cargo [9,10].

Next to addressing uptake and toxicity for HeLa and Jurkat cells we also included human erythrocytes in our studies. These cells lack endocytosis and should therefore reveal to which degree a membrane-disruptive activity is a function of the conjugate–membrane interaction or the triggering of a cellular response. Since multivalency can generate strong interactions for low affinity binders, we also addressed whether inside the cell the multivalent display of R9 leads to the disruption of protein–protein interactions. Our results demonstrate that the multivalent display strongly enhances interactions with the plasma membrane. The restriction of toxicity to cells that show uptake demonstrates that toxicity is a consequence of the reaction of the cells to these conjugates. Interactions of the conjugates with the plasma membrane alone are insufficient to evoke strong toxicity.

2. Materials and methods

2.1. Reagents

Nona-arginine (R9) with an amidated C-terminus and an N-terminal carboxyfluorescein was purchased from EMC microcollections (Tübingen, Germany). Dex-$(R9)_5$ was synthesized using a 10 kDa dextran (Dex) backbone comprising on average of 62 glucose monomers. Carboxyethyl groups were introduced using acrylamide followed by amide hydrolysis to form 2-carboxyethyl dextran (CED). *N*-(2-aminoethyl)maleimide was synthesized and coupled to the modified polymer. Using maleimide-thiol coupling, in a single step *NCPI*cysteinyl-lysinyl-5(6)-carboxyfluorescein and *N*-cysteinylnona-arginine amide were coupled to the modified dextran achieving an average loading of five nona-arginine peptides and one fluorophore per polymer as determined by quantitative amino acid analysis and NMR [22]. Dex-$(R9)_5$ had a final molecular weight of 22 kDa (for details see SI, Figure S1.1 and S1.2). Dex and CED were used as negative controls. Imipramine, resazurin and Tween-20 were from Sigma-Aldrich (Zwijndrecht, the Netherlands). Propidium iodide and Alexa-647-labeled Annexin-V were from Invitrogen (Eugene, USA) and Ficoll-Paque from GE Healthcare (Uppsala, Sweden). Complete Ringer (pH 7.4) solution was prepared using 32 mM HEPES, 125 mM NaCl, 5 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$.

2.2. Tissue culture

Jurkat T cell leukemia cells (ACC-282, DSMZ, Braunschweig, Germany) and HeLa cells (CCL-2, ATCC, LGC Standards, Wessel, Germany) were cultured in RPMI1640 with stable glutamine and 2.0 g/L $NaHCO_3$ (PAN Biotech, Aidenbach, Germany) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAN Biotech). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. Jurkat cells were passaged every 2–3 days when the cells had grown to a density of approximately $4 * 10^5$ cells/mL while HeLa cells were passaged every 2 days at around 80%–90% confluency.

2.3. Confocal microscopy

Confocal laser scanning microscopy was performed on a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an HCX PL APO 63x 1.2 N.A. water immersion lens. Cells were maintained at 37 °C on a temperature-controlled microscope stage. For multichannel recordings with propidium iodide (PI) or Annexin-V in addition to the labeled R9 or Dex-$(R9)_5$, images were recorded using the 488 nm line of an argon-ion laser and a HeNe 561 or 633 nm laser. To avoid crosstalk, fluorescence was recorded sequentially where necessary.

2.4. Dex-$(R9)_5$ uptake

HeLa cells ($4 * 10^4$/well) were seeded in 8-well microscopy chambers (Nalge Nunc International, New York, USA), 24 hours prior to peptide addition. Cells were incubated with 4 μM Dex-$(R9)_5$ or 20 μM R9 in RPMI 1640 supplemented with 10% FBS (heat inactivated) for

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