TITLE:

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CITATION:

Arafiles, Jan Vincent V. ...[et al]. Stimulating Macropinocytosis for Intracellular Nucleic Acid and Protein Delivery: A Combined Strategy with Membrane-Lytic Peptides to Facilitate Endosomal Escape. *Bioconjugate Chemistry* 2020, 31(3): 547-553

ISSUE DATE:

2020-03-18

URL:

<http://hdl.handle.net/2433/261214>

RIGHT:

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Stimulating Macropinocytosis for Intracellular Nucleic Acid and Protein Delivery: A Combined Strategy with Membrane-Lytic Peptides to Facilitate Endosomal Escape

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KEYWORDS: macropinocytosis, membrane-lytic peptide, siRNA, bioactive protein, drug
delivery

Abstract

Delivery of biomacromolecules via endocytic pathways requires the efficient accumulation of cargo molecules into endosomes, followed by their release to the cytosol. We propose a unique intracellular delivery strategy for bioactive molecules using a new potent macropinocytosis-inducing peptide derived from stromal-derived factor (SDF)-1 α (SN21). This peptide allowed extracellular materials to enter cells through the activation of macropinocytosis. To provide the ability to release internalized cargoes from endosomes, we conjugated SN21 with membrane-lytic peptides. The combination of a macropinocytosis-inducing peptide and a membrane lytic peptide successfully delivered functional siRNA and proteins, which include antibodies, Cre recombinase, and an artificial transcription regulator protein having a transcription activator-like effector (TALE) motif. This study shows the feasibility of combining physiological stimulation of macropinocytosis with physicochemical disruption of endosomes as a strategy for intracellular delivery.

Main Text

Intracellular delivery of biofunctional molecules contributes significantly to molecular cell biology, chemical biology, and drug discovery.¹ With recent developments in protein- and peptide-based therapeutics, the demand to deliver therapeutic materials into cells has also increased.^{2,3} Endocytosis has been employed as a practical pathway for intracellular delivery but is limited by endosome trapping. To address this problem, numerous methodologies were developed to facilitate endosomal escape⁴⁻⁶; however, initial recruitment of bioactive molecules (cargoes) into endosomes is crucial to exploit the full potential of these endosome-release strategies (Figure 1A). Thus, this study aims to establish the approach of combining a robust physiological activator of macropinocytosis with a physicochemical disruptor of endosomes to facilitate efficient cargo delivery.

Macropinocytosis is an actin-driven endocytosis, commonly induced by external stimuli, that leads to bulk uptake of extracellular fluid and materials into large endosomes, called macropinosomes.⁷ The diameter of which is reported to be up to 5 μm , considerably larger than those of endosomes formed in clathrin and caveolae endocytosis (said to be ~ 120 and ~ 80 nm, respectively).⁸ Earlier studies suggested the involvement of macropinocytosis in the cellular entry of specific viruses⁹ and cell-penetrating peptides (CPPs).^{10,11} Recent works have demonstrated its significance in the uptake of various molecules and materials, including stapled peptides,¹² liposomes,¹³ and exosomes.¹⁴ Macropinocytosis could thus be regarded as a general and a ubiquitous cellular uptake pathway than previously considered. The massive uptake and non-specific character of macropinocytosis may also provide flexibility in allowing entry of materials having different physicochemical properties.

We propose a strategy to incorporate bioactive materials into macropinosomes using a potent macropinocytosis-inducing peptide SN21 (Figure. 1A and B). This peptide, derived from positions 1-21 of stromal cell-derived factor 1 α (SDF-1 α), stimulates macropinocytic uptake of cargoes comparable with SDF-1 α and other known inducers. To complement its strong endosome uptake, we conjugated SN21 with the membrane-lytic peptide LK15.¹⁵ This addition facilitated endosomal disruption, which allowed endocytosed cargoes to be released into the cytosol, achieving effective intracellular delivery of siRNA, antibodies (IgGs), Cre recombinase, and an artificial transcription regulator protein having a transcription activator-like effector (TALE) motif.

Our previous study has demonstrated that stimulation of CXCR4 with dodeca-arginine (R12) induces its macropinocytic uptake. The same study found that the CXCR4 ligand, SDF-1 α , has a marked ability to stimulate macropinocytosis.¹⁶ The importance of the N-terminal 17 residues of SDF-1 α , particularly positions 1-8 (KPVLSYR) and 12-17 (RFFESH), was also suggested by other groups for receptor binding and for antagonizing HIV-1 entry via CXCR4.^{17,18} We, therefore, speculated that N-terminus peptides of SDF-1 α might have the ability to stimulate macropinocytosis. Increase in 70 kDa dextran uptake into cells served as a criterion to evaluate macropinocytosis induction.¹⁹ HeLa cells were treated for 30 min with fluorescein-labeled 70 kDa dextran (Dex70-FL) in the presence of the peptides (5 μ M each) comprised of n residues of SDF-1 α N-terminus (SNn peptides; n=8, 17, and 21, 23, and 25) (Figure 1B; Table S1), followed by flow cytometric analysis. Increased Dex70-FL uptake was dependent on chain length, and SN21 yielded a comparable Dex70-FL uptake to that obtained from 100 nM SDF-1 α

Figure 1. (A) Combining a macropinocytosis-inducing peptide (physiological stimulant of cellular uptake) and a membrane-lytic peptide (physicochemical means to disrupt the barriers to cytosolic translocation) for cytosolic delivery of bioactive cargoes. (B) Sequences of SDF-1 α -derived peptides. (C) Induction of Dex70-FL uptake in HeLa cells treated with 5 μ M peptide and 1 mg/mL Dex70-FL in α -MEM(-) for 30 min. Comparing Dex70-FL uptake induced by SN21 with (D) SDF-1 α and (E) R8 or TAT. Data presented as mean $\pm$ standard error (SE) of three biological replicates. One-way ANOVA followed by (C) Dunnett's post-hoc test, and (D, E) Tukey's post-hoc test. **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant

treatment, resulting in ~75% higher dextran uptake than no peptide treatment controls (Figure 1C and D). Since no significant increase in Dex70-FL uptake was observed in SN23 or SN25 treatments compared with SN21 (Figure S1), and for practical purposes, we used SN21 for further studies. Compared with CPPs inducing macropinocytosis,²⁰ SN21 stimulated ~30% higher Dex70-FL uptake than HIV-1 TAT and octaarginine (R8) at the same peptide concentration (Figure 1E). Likewise, SN21 was more efficient than known macropinocytosis inducers, 10 μ M 12-O-tetradecanoylphorbol-13-acetate (TPA)²¹, and 500 ng/mL platelet-derived growth factor (PDGF),²² yielding only ~10 and ~30% higher than no peptide treatment controls respectively (Figure S2).

Performing the Dex70-FL uptake assay in the presence of inhibitors further confirmed macropinocytosis induction by SN21. Treatment with macropinocytosis inhibitors [100 μ M 5-(*N*-ethyl-isopropyl)amiloride (EIPA),²³ and 500 nM wortmannin²⁴] decreased SN21-stimulated Dex70-FL uptake (Figure S3A and S3B). EIPA (Na^+/H^+ exchanger inhibitor) and wortmannin (phosphatidylinositol-3-kinase inhibitor) respectively suppress membrane ruffling and fusion needed to accomplish macropinocytosis.^{23,24} The significant effect of each inhibitor on SN21-induced Dex70-FL uptake is a strong indication of macropinocytosis-stimulated uptake. Other macropinocytosis inducers (such as R8) are delivered into cells not only by macropinocytosis but also by clathrin-mediated endocytosis depending on the condition.²⁵ A recent report also found that clathrin-mediated endocytic uptake could precede macropinocytosis induction through signaling from endolysosomes²⁶; nevertheless, both are not the case for SN21 since its activity was unaffected by the presence of clathrin endocytosis inhibitor (30 μ M pitstop 2²⁷; Figure S3C).

Dynamic actin reorganization, seen as veil-like actin accumulation and protrusions at the cell periphery (*i.e.*, lamellipodia), accompanies macropinocytosis.²⁸ Observing the formation of the said structure should further indicate the ability of SN21 to activate macropinocytosis. We performed time-lapse confocal laser scanning microscopy (CLSM) on HeLa cells expressing Lifeact-mCherry [visualizing filamentous actin (F-actin) structures] treated with SN21. In agreement with the previous data, the addition of SN21 resulted in dynamic lamellipodia and membrane ruffle formation (yellow arrows, Figure S4). Cells treated without the peptide showed no such change. This result along with the inhibitor experiments, firmly suggests the ability of SN21 to activate macropinocytosis and drive the uptake of the model macromolecule, 70kDa dextran. Co-localization of signals from SN21 labeled with fluorescein isothiocyanate (FITC) and tetramethylrhodamine-labeled 70kDa dextran (Dex70-TMR) suggests co-internalization of SN21 with macropinocytosed materials (Figure S5), indicating that SN21 could be potentially applicable for intracellular delivery.

SN21 induces macropinocytosis to allow entry of external cargoes into endosomes, seen as dot-like Dex70-TMR signals (Figure S5). However, liberation from endosomes is required to achieve cellular function. To enable macropinocytosed materials to escape from endosomes, a membrane-lytic peptide LK15 (KLLKLLLKLLLKLLK)¹⁵ was conjugated to SN21 via a glycylglycine spacer (Figure 2A). LK15 is highly cytotoxic due to its strong interaction with membranes.²⁹ Before evaluating the applicability of SN21-LK15 for cytosolic delivery, we first analyzed the cytotoxicity of SN21-LK15 and LK15 using the mitochondrial dehydrogenase activity-based WST-8 assay.³⁰ The result showed the reduction of LK15 cytotoxicity when conjugated to SN21 segment [Figure S6; EC50 (concentration of peptide yielding 50% cell

death) of SN21-LK15 and LK15: $8.5 \pm 1.8 \mu\text{M}$ and $3.7 \pm 0.2 \mu\text{M}$, respectively (mean $\pm$ standard error (SE)). Such a lytic peptide is usually composed of cationic and hydrophobic amino acids. The hydrophobic interactions of the peptide with the cell membranes results in the rupture of membrane structures.³¹ Conjugation with SN21 (SN21-LK15) could have allowed LK15 to enter and accumulate within endosomes, decreasing its concentration outside the cell and its cell membrane-lytic activity (Figures 1A and S6). Additionally, incubating cells with SN21-LK15 at concentrations below the EC₅₀ for 1 h or 6 h suggests no apparent long-term cytotoxicity (Figure S7).

Figure 2. (A) SN21-LK15 sequence. (B) Enhanced siRNA effect obtained by SN21-LK15. Luciferase activities of HeLa-GL3 cells following transfection using 1 μL Lipofectamine 2000 (LF2000), 10 μM SN21, LK15, and SN21-LK15 at specified charge ratios with 50 nM siLuc. Data presented as mean $\pm$ SE of three biological replicates. One-way ANOVA followed by Tukey's post-hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant

Previous works reported the use of LK15 for nucleic acid delivery.³² The benefit of SN21 conjugation was thus confirmed through the delivery of the small-interfering RNA (siRNA) against luciferase (siLuc) into HeLa cells, stably expressing GL3 firefly luciferase (HeLa-GL3).³³ The cells were treated with varying charge ratios of peptides/siRNA mixture (represented by the positive charges in peptides/negative charges in siRNA). SN21-LK15 (charge ratio ≥ 2.5) delivered siLuc into HeLa-GL3 cells and significantly diminished luciferase activity (Figure 2B). The decrease in luciferase activity at charge ratios 5 and 10 was comparable to that obtained using a known powerful transfection agent, Lipofectamine 2000 (LF2000). Similar results were obtained using the conjugate of SN21 with melittin as an alternative lytic peptide (Figures S8 - S10).^{34,35}

The accumulation of SN21-LK15 within endosomes following its uptake led to the disruption of endosomal membranes, confirmed by treating cells expressing galectin-3 fused with an enhanced green fluorescent protein (EGFP).³⁶ Galectin-3 is a protein that binds to β -galactosides.^{36,37} β -galactosides are commonly located on the extracellular side of the cell membrane and within endosomes. When endosomes are ruptured, EGFP-galectin-3 binds and accumulates on exposed β -galactosides within the broken endosomes, resulting in distinct fluorescent puncta (Figure 3A and S11, yellow arrows). Only cells treated with SN21-LK15 and the L-leucyl-L-leucyl methyl ester (LLOMe), a dipeptide known to destabilize endosomes,³⁸ formed these puncta. The lack of observed puncta in LK15-treated cells means that LK15 does not actively enter cells. Thus, SN21 is required to perform endosomal uptake and disruption. Also, endosomal membranes contain high amounts of negatively charged lipids, including bis(monoacylglycero)phosphate (BMP),^{39,40} to which cationic amphiphilic peptides are more lytic over cell membranes (mostly

Figure 3. (A) EGFP-galectin 3 accumulation assay. HeLa cells transiently expressing EGFP-galectin 3 were incubated with 5 μ M SN21, 2 μ M LK15, and 2 μ M SN21-LK15 for 30 mins. (B) Delivery of IgG-AF488 in the presence of SN21-LK15. (C) Nuclear pore complex targeting by anti-NPC IgG-AF594 achieved using SN21-LK15. Yellow arrows indicate the boundary of the Hoechst 33342-stained nucleus.

composed of neutral, zwitterionic lipids). This concept may also apply to SN21-LK15.

The synergy between the strong induction of macropinocytosis and endosome disruption enabled SN21-LK15 to deliver proteins into cells. We first demonstrated this through its delivery of Alexa Fluor 488-labeled immunoglobulin G (IgG-AF488). After confirming no substantive cytotoxicity from 2 μ M LK15 and SN21-LK15 treatments (Figure S12), we treated HeLa cells with 100 μ g/mL IgG-AF488 in the presence of the above peptides. CLSM observation of cells treated with 2 μ M SN21-LK15-treated showed dot-like, punctate signals (endosomes), together with fluorescent signals uniformly distributed throughout the cytosol (Figure 3B). These are indications of uptake facilitated by macropinocytosis, and the release of internalized cargoes to the cytosol, respectively. Neither the treatment with 5 μ M SN21 nor 2 μ M LK15, showed such cytosolic signals. Treating cells at 4°C (i.e., temperature at which endocytosis is suppressed),¹¹ as well as EIPA treatment, prevented SN21-LK15-mediated IgG-AF488 uptake. This result suggests the requirement of endocytosis (particularly macropinocytosis) for efficient intracellular delivery (Figure S13), and ruling out physicochemical and mechanical rupture of the cell membrane in SN21-LK15-mediated delivery. The importance of endosomal accumulation and maturation was also suggested by the time-dependent appearance of diffuse cytosolic IgG-AF488 signals and subsequent observation of punctate signals (cells having diffuse cytosolic signals: 35% and 61% in 30 and 60 min, respectively, Figure S14A) and by the decreased number of cells with diffuse cytosolic signals (~27%) in the presence of the early-to-late endosome maturation inhibitor bafilomycin A1 (Figure S14B).⁴¹ Performing IgG-AF488 delivery in the presence of serum revealed that a higher SN21-LK15 concentration (~20 μ M) is required to observe cells having a similar extent of diffused cytosolic signals compared with serum-free

conditions (2 μ M) (Figure S15). We are currently exploring further modifications that would enable SN21-LK15 to work in serum-containing environment.

Delivered IgGs retained their ability to target intracellular proteins, confirmed by incubating cells with Alexa Fluor 594 (AF594)-labeled anti-nucleopore complex IgG [anti-NPC IgG-AF594] (50 μ g/mL) in the presence of 2 μ M SN21-LK15. This experiment yielded significant labeling of the nuclear periphery (yellow arrows in Figure 3C, upper panels; nucleus stained with Hoechst33342). On the other hand, such accumulation was not observed in cells treated with AF594-labeled isotype control IgG (control IgG-AF594) (Figure 3C, lower panels). This result confirms that, although the degradation of antibodies could happen before and after their endosomal escape, a significant fraction of these are delivered to the cytosol while retaining their functions. Note that the administered amount of IgG in this experiment was lower than previous reports.⁴² The effectiveness of the SN21-LK15 strategy allowed the use of 6- to 10-fold lower IgG concentration (until 50 μ g/mL), without compromising the antibody function.

Applications of SN21-LK15 for intracellular protein delivery were expanded using the two genome-editing proteins with different physicochemical properties, namely, Cre recombinase and an artificial transcription activator protein (dHax3-VP64). A Cre-loxP recombination assay system was employed to assess the delivery of Cre recombinase (Figure 4A).^{25,43} This system uses HeLa cells transiently transfected with the *loxP-DsRed-loxP-EGFP-N1* reporter plasmid. Cells initially express the red-fluorescent protein, DsRed. When Cre is successfully introduced, the coding region of DsRed flanked by two loxP sites will be removed to allow the expression of EGFP (Figure 4A). Reporter cells were treated with Cre (5 μ M) in the presence of 3 μ M

Figure 4. (A) Cre-loxP recombination assay. (B) CLSM images of *loxP-DsRed-loxP* reporter cells treated with Cre recombinase in the presence of SN21, LK15, or SN21-LK15 for 1 h. (C) Recombination efficiencies expressed as percent of EGFP-expressing cells over the total number of fluorescence-emitting cells (DsRed + EGFP cells). (D) dHax3-VP64 delivery assay. Luciferase expressions of HeLa-TBS-Luci cells treated with dHax3-VP64 in the presence of (E) L17E, SN21, LK15, and SN21-LK15 or (F) SN21-LK15 and TAT-LK15 for 1 h, respectively. Data presented as mean $\pm$ SE of three biological replicates. One-way ANOVA followed by Tukey's post-hoc test. **, $P < 0.01$; ***, $P < 0.001$.

SN21-LK15 for 1 h, followed by protein washout and incubation for another 24 h. This treatment resulted in 59% of EGFP-positive cells over total fluorescence-emitting cells, representing the efficacy of gene recombination attained by successful Cre delivery (Figure 4B and C). On the contrary, treatment with the same concentration of Cre in the presence of 5 μ M SN21 or 2.5 μ M LK15 only yielded 10% and 21% of gene recombination, respectively.

SN21-LK15 delivery of dHax3-VP64 further validates the ability of this peptide. Transcription activator-like effectors (TAL effectors or TALEs) are designable DNA binding proteins that bind to specific DNA segments (Figure 4D).⁴⁴ When combined with nucleases or transcription activators, TALEs can be employed as useful tools for genome manipulation.^{45–47} A model transcription activator dHax3-VP64 is comprised of a *de novo* designed artificial TALE (dHax3), recognizing the specific DNA segment of 12 base pairs^{46,48} and VP64 transcription activation domains.⁴⁹ We established a stable HeLa cell line carrying the luciferase reporter gene under the control of a dHax3 TALE binding sequence (TBS) upstream of a minimal promoter⁴⁸ (HeLa-TBS-Luci). Cytosolic translocation and binding of dHax3-VP64 to the TBS leads to luciferase expression, measured as a function of luminescence. A ~90-fold luminescence was obtained by treating HeLa-TBS-Luci with 1 μ M dHax3-VP64 in the presence of 2 μ M SN21-LK15, compared with no peptide and SN21 treatments (Figure 4E). We compared this with the previously identified endosomolytic peptide L17E.^{42,50} To attain efficient intracellular delivery using L17E, 5 μ M of bioactive proteins should typically be used. However, in this condition (1 μ M dHax3-VP64), delivery of dHax3-VP64 using L17E resulted in only a marginal increase in luminescence as compared to SN21-LK15. A representative CPP, TAT, also induces macropinocytosis,¹⁰ although weaker than SN21 (Figure 1E). The LK15-conjugate of this peptide

(TAT-LK15) may have structural/functional similarity with SN21-LK15. Despite only being reported for nucleic acid delivery³², TAT-LK15 also promoted dHax3-VP64 internalization, possibly due to its macropinocytosis induction. However, luminescence obtained using SN21-LK15 is higher than that obtained by using TAT-LK15 (Figure 4F). Consistency between results in Figures 4F and 1E supports the idea that high macropinocytosis induction by SN21 leads to more efficient cytosolic translocation of the bioactive molecules. Note that Cre recombinase and dHax3-VP64 represents cargoes of different physicochemical properties. Cre (38 kDa) has a theoretical net charge of +11 neutral pH, while TALE-VP64 (94 kDa) is -5.6 .⁵¹ Being able to deliver macromolecules of different sizes and charges, adds to the advantages of stimulating non-specific uptake via macropinocytosis.

This study illustrates our concept of combining a macropinocytosis-inducing peptide (physiological means to stimulate cellular uptake) with a membrane-lytic peptide (physicochemical method to disrupt barriers to cytosolic translocation) for intracellular delivery. SN21, a peptide derived from the N-terminal of SDF-1 α , was identified as a potent inducer of macropinocytosis. Its conjugates with the membrane-lytic peptides LK15 or melittin led to the successful delivery of siRNA and bioactive proteins into cells to exert their activities. When compared to a peptide with a similar ability, the stronger induction of macropinocytosis by SN21 resulted in better efficacy for delivery. The high-efficiency intracellular delivery by SN21-LK15 can, therefore, be attributed to the synergy of the abilities of both peptides. Simple administration of this peptide, without the need for cross-link formation, should be beneficial for the delivery of native nucleic acids and proteins, while the high efficacy cytosolic delivery should also

contribute to reducing the amount of cargoes required. This factor is essential for excellent delivery strategies, especially for precious samples including antibodies.

Acknowledgment

This work was supported by JST CREST (Grant Number JPMJCR18H5) and in part by JSPS KAKENHI (Grant Numbers 18H04403 and 18H04017). M.A. is grateful for the JSPS Research Fellowship for Young Scientists. The authors thank Professors T. Itoh (Kobe University), T. Yoshimori (Osaka University) for their gifts of *Lifeact-mCherry* and *EGFP-Gal3* plasmids, respectively.

Supporting Information.

The following files are available free of charge.

Supplementary Information

Experimental Section, Table S1 and Figures S1 - S15 (PDF)

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Author contribution

J.V.A. and H.H. designed the study and executed the experiments. M.A., M.I., and S.T. were involved in experiments of siRNA and genome-editing proteins. J.V.A. wrote the initial draft of the manuscript. S.F. directed the project and wrote the paper with J.V.A. and H.H. All authors read and commented on the manuscript.

References

- (1) Stewart, M. P.; Sharei, A.; Ding, X.; Sahay, G.; Langer, R.; Jensen, K. F. (2016) In vitro and ex vivo strategies for intracellular delivery. *Nature* **538**, 183–192.
- (2) Guillard, S.; Minter, R. R.; Jackson, R. H. (2015) Engineering therapeutic proteins for cell entry: the natural approach. *Trends Biotechnol.* **33**, 163–171.
- (3) Craik, D. J.; Fairlie, D. P.; Liras, S.; Price, D. (2013) The future of peptide-based drugs. *Chem. Biol. Drug Des.* **81**, 136–147.
- (4) Pei, D.; Buyanova, M. (2019) Overcoming endosomal entrapment in drug delivery. *Bioconjug. Chem.* **30**, 273–283.
- (5) Stewart, M. P.; Langer, R.; Jensen, K. F. (2018) Intracellular Delivery by Membrane Disruption: Mechanisms, Strategies, and Concepts. *Chem. Rev.* **118**, 7409–7531.
- (6) Nakase, I.; Kobayashi, S.; Futaki, S. (2010) Endosome-disruptive peptides for improving cytosolic delivery of bioactive macromolecules. *Biopolymers*, **94**, 763–770.
- (7) Jones, A. T. (2007) Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. *J. Cell. Mol. Med.* **11**, 670–684.
- (8) Conner, S. D.; Schmid, S. L. (2003) Regulated portals of entry into the cell. *Nature* **422**, 37–44.
- (9) Meier, O.; Greber, U. F. (2004) Adenovirus endocytosis. *J. Gene Med.* **6**, S152–S163.
- (10) Wadia, J. S.; Stan, R. V.; Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **10**, 310–315.

- (11) Nakase, I.; Niwa, M.; Takeuchi, T.; Sonomura, K.; Kawabata, N.; Koike, Y.; Takehashi, M.; Tanaka, S.; Ueda, K.; Simpson, J. C.; et al. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol. Ther.* *10*, 1011–1022.
- (12) Sakagami, K.; Masuda, T.; Kawano, K.; Futaki, S. (2018) Importance of net hydrophobicity in the cellular uptake of all-hydrocarbon stapled peptides. *Mol. Pharm.*, *15*, 1332–1340.
- (13) Cardarelli, F.; Pozzi, D.; Bifone, A.; Marchini, C.; Caracciolo, G. (2012) Cholesterol-dependent macropinocytosis and endosomal escape control the transfection efficiency of lipoplexes in CHO living cells. *Mol. Pharm.* *9*, 334–340.
- (14) Costa Verdera, H.; Gitz-Francois, J. J.; Schiffelers, R. M.; Vader, P. (2017) Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. *J. Control. Release* *266*, 100–108.
- (15) Perry, H. A.; Alhaj Saleh, A. F.; Aojula, H.; Pluen, A. (2008) YOYO as a dye to track penetration of LK15 DNA complexes in spheroids: use and limits. *J. Fluoresc.* *18*, 155–161.
- (16) Tanaka, G.; Nakase, I.; Fukuda, Y.; Masuda, R.; Oishi, S.; Shimura, K.; Kawaguchi, Y.; Takatani-Nakase, T.; Langel, Ü.; Gräslund, A.; et al. (2012) CXCR4 stimulates macropinocytosis: implications for cellular uptake of arginine-rich cell-penetrating peptides and HIV. *Chem. Biol.* *19*, 1437–1446.
- (17) Crump, M. P.; Gong, J. H.; Loetscher, P.; Rajarathnam, K.; Amara, A.; Arenzana-Seisdedos, F.; Virelizier, J. L.; Baggiolini, M.; Sykes, B. D.; Clark-Lewis, I. (1997) Solution

structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J.* 16, 6996–7007.

(18) Loetscher, P.; Gong, J. H.; Dewald, B.; Baggiolini, M.; Clark-Lewis, I. (1998) N-terminal peptides of stromal cell-derived factor-1 with CXC chemokine receptor 4 agonist and antagonist activities. *J. Biol. Chem.* 273, 22279–22283.

(19) Li, L.; Wan, T.; Wan, M.; Liu, B.; Cheng, R.; Zhang, R. (2015) The effect of the size of fluorescent dextran on its endocytic pathway. *Cell Biol. Int.* 39, 531–539.

(20) Futaki, S.; Nakase, I.; Tadokoro, A.; Takeuchi, T.; Jones, A. T. (2007) Arginine-rich peptides and their internalization mechanisms. *Biochem. Soc. Trans.* 35, 784–787.

(21) Yamamoto, K.; Seki, T.; Yamamoto, H.; Adachi, N.; Tanaka, S.; Hide, I.; Saito, N.; Sakai, N. (2014) Deregulation of the actin cytoskeleton and macropinocytosis in response to phorbol ester by the mutant protein kinase C gamma that causes spinocerebellar ataxia type 14. *Front. Physiol.* 5, 126.

(22) Davies, P. F.; Ross, R. (1978) mediation of pinocytosis in cultured arterial smooth muscle and endothelial cells by platelet-derived growth factor. *J. Cell Biol.* 79, 663–671.

(23) Meier, O.; Boucke, K.; Hammer, S. V.; Keller, S.; Stidwill, R. P.; Hemmi, S.; Greber, U. F. (2002) Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J. Cell Biol.* 158, 1119–1131.

(24) Araki, N.; Johnson, M. T.; Swanson, J. A. (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol.* 135, 1249–1260.

- (25) Kawaguchi, Y.; Takeuchi, T.; Kuwata, K.; Chiba, J.; Hatanaka, Y.; Nakase, I.; Futaki, S. (2016) Syndecan-4 is a receptor for clathrin-mediated endocytosis of arginine-rich cell-penetrating peptides. *Bioconjug. Chem.* 27, 1119–1130.
- (26) Tejeda-Muñoz, N.; Albrecht, L. V; Bui, M. H.; De Robertis, E. M. (2019) Wnt canonical pathway activates macropinocytosis and lysosomal degradation of extracellular proteins. *Proc. Natl. Acad. Sci. U. S. A.* 116, 10402–10411.
- (27) von Kleist, L.; Stahlschmidt, W.; Bulut, H.; Gromova, K.; Puchkov, D.; Robertson, M. J.; MacGregor, K. A.; Tomilin, N.; Pechstein, A.; Chau, N.; et al. (2011) Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* 146, 471–484.
- (28) Yoshida, S.; Pacitto, R.; Sesi, C.; Kotula, L.; Swanson, J. A. (2018) Dorsal ruffles enhance activation of Akt by growth factors. *J. Cell Sci.* 131, 1–11.
- (29) Cornut, I.; Büttner, K.; Dasseux, J. L.; Dufourcq, J. (1994) The amphipathic α -helix concept: Application to the de novo design of ideally amphipathic leu, lys peptides with hemolytic activity higher than that of melittin. *FEBS Lett.* 349, 29–33.
- (30) Matsuoka, M.; Wispriyono, B.; Igisu, H. (2000) Increased cytotoxicity of cadmium in fibroblasts lacking C-Fos. *Biochem. Pharmacol.* 59, 1573–1576.
- (31) Matsuzaki, K. (2019) Membrane Permeabilization Mechanisms. In *Advances in Experimental Medicine and Biology*. Vol. 1117, pp 9–16.

- (32) Arthanari, Y.; Pluen, A.; Rajendran, R.; Aojula, H.; Demonacos, C. (2010) delivery of therapeutic shRNA and siRNA by Tat fusion peptide targeting Bcr–Abl fusion gene in chronic myeloid leukemia cells. *J. Control. Release* **145**, 272–280.
- (33) Akita, H.; Kogure, K.; Moriguchi, R.; Nakamura, Y.; Higashi, T.; Nakamura, T.; Serada, S.; Fujimoto, M.; Naka, T.; Futaki, S.; et al. (2010) Nanoparticles for ex vivo siRNA delivery to dendritic cells for cancer vaccines: programmed endosomal escape and dissociation. *J. Control. Release* **143**, 311–317.
- (34) Sessa, G.; Freer, J. H.; Colacicco, G.; Weissmann, G. (1969) Interaction of a lytic polypeptide, melittin, with lipid membrane systems. *J. Biol. Chem.*, **224**, 3575–3582.
- (35) Meyer, M.; Philipp, A.; Oskuee, R.; Schmidt, C.; Wagner, E. (2008) Breathing life into polycations: functionalization with PH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery. *J. Am. Chem. Soc.* **130**, 3272–3273.
- (36) Maejima, I.; Takahashi, A.; Omori, H.; Kimura, T.; Takabatake, Y.; Saitoh, T.; Yamamoto, A.; Hamasaki, M.; Noda, T.; Isaka, Y.; et al. (2013) Autophagy sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury. *EMBO J.* **32**, 2336–2347.
- (37) Paz, I.; Sachse, M.; Dupont, N.; Mounier, J.; Cederfur, C.; Enninga, J.; Leffler, H.; Poirier, F.; Prevost, M.-C.; Lafont, F.; et al. (2010) Galectin-3, a marker for vacuole lysis by invasive pathogens. *Cell. Microbiol.* **12**, 530–544.
- (38) Jacobson, L. S.; Lima, H.; Goldberg, M. F.; Gocheva, V.; Tshiperson, V.; Sutterwala, F. S.; Joyce, J. A.; Gapp, B. V.; Blomen, V. A.; Chandran, K.; et al. (2013) Cathepsin-mediated

necrosis controls the adaptive immune response by Th2 (T Helper Type 2)-associated adjuvants.

J. Biol. Chem. 288, 7481–7491.

(39) Van Meer, G.; Voelker, D. R.; Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell Biol.* 9, 112–124.

(40) Kobayashi, T.; Beuchat, M. H.; Lindsay, M.; Frias, S.; Palmiter, R. D.; Sakuraba, H.; Parton, R. G.; Gruenberg, J. (1999) Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat. Cell Biol.* 1, 113–118.

(41) Bayer, N.; Schober, D.; Prchla, E.; Murphy, R. F.; Blaas, D.; Fuchs, R. (1998) Effect of bafilomycin a1 and nocodazole on endocytic transport in HeLa Cells: implications for viral uncoating and infection. *J. Virol.* 72, 9645–9655.

(42) Akishiba, M.; Takeuchi, T.; Kawaguchi, Y.; Sakamoto, K.; Yu, H. H.; Nakase, I.; Takatani-Nakase, T.; Madani, F.; Gräslund, A.; Futaki, S. (2017) Cytosolic antibody delivery by lipid-sensitive endosomolytic peptide. *Nat. Chem.*, 9, 751–761.

(43) Méndez-Ardoy, A.; Lostalé-Seijo, I.; Montenegro, J. (2019) Where in the Cell Is Our Cargo? Methods Currently Used To Study Intracellular Cytosolic Localisation. *Chembiochem* 20, 488–498.

(44) Bogdanove, A. J.; Voytas, D. F. (2011) TAL Effectors: customizable proteins for DNA targeting. *Science.* 333, 1843–1846.

(45) Joung, J. K.; Sander, J. D. (2013) TALENs: a widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Biol.* 14, 49–55.

- (46) Mahfouz, M. M.; Li, L.; Shamimuzzaman, M.; Wibowo, A.; Fang, X.; Zhu, J.-K. (2011) De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. *Proc. Natl. Acad. Sci. U. S. A.* 108, 2623–2628.
- (47) Tsuji, S.; Futaki, S.; Imanishi, M. (2016) Sequence-specific recognition of methylated DNA by an engineered transcription activator-like effector protein. *Chem. Commun.* 52, 14238–14241.
- (48) Tsuji, S.; Futaki, S.; Imanishi, M. (2013) Creating a TALE protein with unbiased 5′-T binding. *Biochem. Biophys. Res. Commun.* 441, 262–265.
- (49) Beerli, R. R.; Segal, D. J.; Dreier, B.; Barbas, C. F. (1998) Toward controlling gene expression at will: specific regulation of the ErbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc. Natl. Acad. Sci.* 95, 14628–14633.
- (50) Akishiba, M.; Futaki, S. (2019) Inducible membrane permeabilization by attenuated lytic peptides: a new concept for accessing cell interiors through ruffled membranes. *Mol. Pharm.* 16, 2540–2548.
- (51) Zuris, J. A.; Thompson, D. B.; Shu, Y.; Guilinger, J. P.; Bessen, J. L.; Hu, J. H.; Maeder, M. L.; Joung, J. K.; Chen, Z.-Y.; Liu, D. R. (2015) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat. Biotechnol.* 33, 73–80.

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