Cholesterol-Lowering Effect of Octaarginine-Appended \( \beta \)-Cyclodextrin in \( \text{Npc1-Trap-CHO} \) Cells

Keiichi Motoyama,\(^a\) Rena Nishiyama,\(^a\) Yuki Maeda,\(^a,b,c\) Taishi Higashi,\(^a\) Yoshimasa Kawaguchi,\(^d\) Shiroh Futaki,\(^d\) Yoichi Ishitsuka,\(^a\) Yuki Kondo,\(^a\) Tetsumi Irie,\(^a,c\) Takumi Era,\(^c\) and Hidetoshi Arima\(^a,b,c\)

\(^a\)Graduate School of Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-honmachi, Chuou-ku, Kumamoto 862–0973, Japan; \(^b\)Research Fellow of Japan Society for the Promotion of Science; 5–3–1 Kojimachi, Chiyoda-ku, Tokyo 102–0083, Japan; \(^c\)Program for Leading Graduate Schools “HIGO (Health life science: Interdisciplinary and Glocal Oriented) Program,” Kumamoto University; 5–1 Oe-honmachi, Chuou-ku, Kumamoto 862–0973, Japan; \(^d\)Institute for Chemical Research, Kyoto University; Uji, Kyoto 611–0011, Japan; \(^e\)Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University; 2–2–1 Honjo, Chuou-ku, Kumamoto 860–0811, Japan.

Received May 2, 2016; accepted August 22, 2016; advance publication released online September 6, 2016

Niemann–Pick disease type C (NPC) is an autosomal recessive lysosomal storage disorder, which is an inherited disease characterized by the accumulation of unesterified cholesterol in endolysosomes. Recently, 2-hydroxypropyl-\( \beta \)-cyclodextrin (HP-\( \beta \)-CyD) has been used for the treatment of NPC, and ameliorated a hepatosplenomegaly in the patients. However, to obtain the treatment efficacy, a high dose of HP-\( \beta \)-CyD was necessary. Therefore, the decrease in dose by using active intracellular delivery system of \( \beta \)-CyD to NPC cells is expected. In this study, to efficiently deliver \( \beta \)-CyD to NPC-like cells, we newly synthesized octaarginine (R8)-appended \( \beta \)-CyD with a spacer of \( \gamma \)-aminobutyric acid (R8-\( \beta \)-CyD) and evaluated its cytotoxicity, intracellular distribution, endocytosis pathway and cholesterol-lowering effect in \( \text{Npc1-Trap-CHO} \) Chinese hamster ovary (CHO) cells, cholesterol-accumulated cells through the impairment of NPC1 function. R8-\( \beta \)-CyD did not show cytotoxicity in the cells. In addition, Alexa568-labeled R8-\( \beta \)-CyD was actively internalized into \( \text{Npc1-Trap-CHO} \) cells, possibly through micropinocytosis. Notably, R8-\( \beta \)-CyD significantly decreased intracellular cholesterol content compared with HP-\( \beta \)-CyD. These results suggest that R8-\( \beta \)-CyD may be a promising therapeutic agent for ameliorating cholesterol accumulation in NPC.

Key words cyclodextrin; octaarginine; Niemann–Pick disease type C; cholesterol; cell-penetrating peptide

Niemann–Pick disease type C (NPC) is an atypical lysosomal storage disorder, which is an inherited disease characterized by the accumulation of unesterified cholesterol in endolysosomes. NPC is elicited by the mutations in either \( \text{Npc1} \) or \( \text{Npc2} \) gene, and elicits hepatosplenomegaly, neurodegeneration and failure to thrive childhood.\(^1,3\) NPC1 protein in endolysosomes is dominantly associated with cholesterol trafficking in cells.\(^3,4\) Therefore, in NPC patients with loss of function of NPC1 protein, an excessive accumulation of unesterified cholesterol in endolysosomes and a shortage of esterified cholesterol in other cellular compartments are observed. Therefore, decreasing the cholesterol level in endolysosomes was found to be crucial approach for the treatment of NPC.

To evaluate the cholesterol-decreasing ability of drug candidates against NPC, \textit{in vitro} cell culture by utilizing NPC-like cells are necessary. Recently, Higaki et al. established the \( \text{Npc1-Deficient Chinese Hamster Ovary (CHO)} \) cell mutants (\( \text{Npc1-Trap-CHO} \) cells) by gene trap mutagenesis.\(^5\) \( \text{Npc1-Trap-CHO} \) cells exhibit the phenotype characteristics of \( \text{Npc1-Deficient CHO} \) cells; the accumulation of free cholesterol in endocytic vesicles and the upregulation of cholesterol synthesis through mevalonate pathway.\(^5,6\) Therefore, \( \text{Npc1-Trap-CHO} \) cells can be applicable for the model of NPC cells.

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of 6–8 glucose units and have been utilized for improvement of certain properties of drugs such as solubility, stability and bioavailability, \textit{etc}., through the formation of inclusion complexes.\(^7,8\) Recently, of various CyD derivatives, 2-hydroxypropyl-\( \beta \)-cyclodextrin (HP-\( \beta \)-CyD) has utilized for the treatment of NPC.\(^9–11\) Based on the several reports demonstrating the gain of longevity of \( \text{Npc1-Deficient mice} \) by a cholesterol decreasing ability of HP-\( \beta \)-CyD.\(^9,11\) Unfortunately, HP-\( \beta \)-CyD was found to produce an increasing in hearing threshold.\(^12\) Most recently, it is demonstrated that HP-\( \gamma \)-CyD, which is more biocompatible than HP-\( \beta \)-CyD, can also lower the accumulation of cholesterol in NPC-like cells.\(^13\) However, further dosing as well as dose escalations are needed to ascertain sufficient efficacy of HP-\( \beta \)-CyD or HP-\( \gamma \)-CyD.

To deliver the various bioactive molecules into cells, arginine-rich cell-penetrating peptides (CPPs) such as the oligoarginine peptides and human immunodeficiency virus (HIV)-1 Tat peptide are promising carriers. So far, various cellular uptake mechanisms of arginine-rich CPPs were proposed, \textit{i.e}., physiological cellular uptake (\textit{i.e}., endocytosis) and direct permeation through the plasma membranes,\(^14,15\) and recently macropinocytosis (accompanied by actin reorganization, plasma membrane ruffling, and the stimulated engulfment of large volumes of extracellular fluid) has been shown to be an important pathway.\(^16–20\) Therefore, CPP-mediated intracellular delivery is likely to be promising approach to deliver CyDs to NPC disease cells. In this study, to efficiently deliver \( \beta \)-CyD to \( \text{Npc1-Trap-CHO} \) cells, we newly fabricated octaarginine (R8)-appended \( \beta \)-CyD with a spacer of \( \gamma \)-aminobutyric acids (R8-\( \beta \)-CyD) and evaluated its cytotoxicity, cholesterol-lowering effect and intracellular distribution in \( \text{Npc1-Trap-CHO} \)
MATERIALS AND METHODS

**Materials** β-CyD was donated by Nihon Shokuhin Kako (Tokyo, Japan). LysoTracker® was purchased from Life Technologies Japan (Tokyo, Japan). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Nichirei (Tokyo, Japan) and Nissui Pharmaceuticals (Tokyo, Japan), respectively.

**Peptide Synthesis** A synthetic scheme of R8-β-CyD is shown in Fig. 1. The peptide chain GABA-[Arg(Pbf)]$_8$-amide (GABA: γ-aminobutyric acid; Pbf: 2,2,4,6,7-penta-methyldihydrobenzofuran-5-sulfonyl) was constructed on a TGS-RAM resin (Shimadzu, Kyoto, Japan) by the Fmoc solid-phase peptide synthesis with the standard coupling system using N-hydroxybenzotriazoles (HOBT)/O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU)/N,N-diisopropylethylamine (DIEA). The peptide was cleaved from the resin and deprotected by treatment with trifluoroacetic acid (TFA)–ethanedithiol (EDT) (95:5), and purified by a reverse phase high-performance liquid chromatography (RP-HPLC). Introduction of β-CyD to the N-terminus of GABA-R8-amide was conducted using 6-deoxy-6-(N-hydroxysuccinimide)-β-CyD (NHS-CyD)$^{21}$ (5 equiv.) and N-methylmorpholine (2 equiv.) in 200 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 6.8) at room temperature, followed by a RP-HPLC purification to yield CyD-GABA-R8 (R8-β-CyD). The product was confirmed by a matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOFMS).

**Effect of a Macropinocytosis Inhibitor on Cellular Uptake of R8-β-CyD** Npc1-trap-CHO cells (1×10$^5$ cells/35 mm dish with glass bottom) were incubated with 150 μL of DMEM containing 10 μM Alexam568-R8-β-CyD at 37 or 4°C for 1 h. After washing with PBS, the fluorescence derived from Alexam568 and LysoTracker® in Npc1-trap-CHO cells was detected by an IN Cell Analyzer 6000 (GE Healthcare Life Sciences, U.K.).

**Effect of Endocytosis Inhibition on Cellular Uptake of R8-β-CyD** Npc1-trap-CHO cells (1×10$^5$ cells in 35 mm dish with glass bottom) were incubated with 1 mL of culture medium containing 10 μM Alexam568-R8-β-CyD at 37 or 4°C for 1 h. After washing with PBS, the fluorescence derived from Alexam568 in Npc1-trap-CHO cells was detected by an IN Cell Analyzer 6000 (GE Healthcare Life Sciences). A BZ-II analyzer (Keyence, Osaka, Japan) was used for determination of the fluorescence intensities.

**Intracellular Distribution of R8-β-CyD** Npc1-trap-CHO cells (5×10$^4$ cells/35 mm dish with glass bottom) were pretreated with 1 mg/mL of amiloride, a macropinocytosis inhibitor, for 30 min. Then, the cells were incubated with 10 μM Alexam568-R8-β-CyD at 37°C for 1 h. After washing with PBS, the fluorescence derived from Alexam568 in Npc1-trap-CHO cells was detected by a fluorescence microscope (Keyence Biozero BZ-8000, Osaka, Japan).

**Intracellular Distribution of Cholesterol** Npc1-trap-CHO cells (5×10$^4$ cells in 35 mm dish with glass bottom) were incubated in the presence of 150 μL of R8-β-CyD (0.1, 1, 5 or 10 μM) in DMEM for 24 h. After washing with PBS, the intracellular cholesterol was visualized by the cholesterol cell-based detection assay kit (Ann Arbor, MI, U.S.A.). Then, the cells were fixed by cell-based assay fixative solution (4% formaldehyde) and added 150 μL of cholesterol detection assay buffer containing 50 μg/mL of Filipin III. After incubation for 1 h, the fluorescence derived from Filipin III in Npc1-trap-CHO cells was detected by a fluorescence microscope (Keyence Biozero BZ-8000). A BZ-II analyzer (Keyence) was used for determination of the fluorescence intensities.

**Data Analysis** Data are presented as the mean±standard error of the mean (S.E.M.) for each group. Statistical signifi-
cance of mean coefficients for the studies was performed by ANOVA followed by Scheffe’s test. $p$-Values for significance were set at 0.05.

RESULTS AND DISCUSSION

Cytotoxicity of R8-β-CyD To evaluate cytotoxicity of R8-β-CyD in Npc1-trap-CHO cells as NPC-like cells,5,6 we examined the WST-1 method. As shown in Fig. 2, no cytotoxicity of R8-β-CyD was observed in Npc1-trap-CHO cells up to 100 µM for 24 h. The following studies were performed under the present experimental conditions.

Intracellular Distribution of R8-β-CyD To reveal whether R8-β-CyD enters Npc1-trap-CHO cells, we evaluated the intracellular distribution of Alexa568-labeled R8-β-CyD. As shown in Fig. 3, the cellular uptake of Alexa568-R8-β-CyD in Npc1-trap-CHO cells was observed at 24 h after incubation. Additionally, Alexa568-R8-β-CyD was co-localized with endolysosomes stained by LysoTracker®. Meanwhile, the intracellular levels of β-CyD and HP-β-CyD labeled with tetramethylrhodamine isothiocyanate (TRITC-β-CyD and TRITC-HP-β-CyD) in Npc1-trap-CHO cells were not observed (Supplementary Fig. 1). These results indicate that R8-β-CyD distributed in endolysosomes after the cellular uptake into Npc1-trap-CHO cells. Meanwhile, it is possible to affect the intracellular distribution of R8-β-CyD by the introduction of Alexa568 due to changes of molecular weight and/or hydrophobicity of the fluorescent probe. Therefore, to reveal the intracellular distribution of R8-β-CyD precisely, a mass imaging system should be necessary to detect R8-β-CyD directly.

To investigate whether R8-β-CyD is endocyted in Npc1-trap-CHO cells, we next examined the cellular uptake of Alexa568-R8-β-CyD under the treatment at 4°C, in which endocytosis is inhibited. As shown in Fig. 4, the fluorescence derived from Alexa568 in Npc1-trap-CHO cells was significantly inhibited at 4°C, but not at 37°C. In cellular uptake of arginine-rich CPPs, macroinocytosis is found to be an important pathway.16–20 In addition, to induce the macroinocytosis by arginine-rich CPPs, the formation of divalent hydrogen bonds and electrostatic interactions between the arginines and sulfates in glycosaminoglycans (GAGs) are important to accumulate CPPs on plasma membranes.14 Recently, Takechi-Haraya et al. reported that R8 interacted with GAGs and highly internalized into CHO cells.23 Indeed, the cellular uptake of Alexa568-R8-β-CyD was inhibited by the presence of amiloride, an inhibitor of macroinocytosis in Npc1-trap-CHO cells (Fig. 5). Therefore, the R8 moieties of R8-β-CyD are likely to interact with GAGs and endocyted via the macroinocytosis pathway. Collectively, these findings indicate that R8-β-CyD was endocyted in Npc1-trap-CHO cells, possibly via macroinocytosis.

Effects of R8-β-CyD on Intracellular Cholesterol Levels To evaluate the cholesterol-decreasing ability of R8-β-CyD, the effects of R8-β-CyD on cholesterol levels in Npc1-trap-CHO cells were examined. Here, to detect the cholesterol levels in the cells, we used Filipin III, as a specific binder to unesterified cholesterol. The fluorescence intensity derived from Filipin III was detected by a fluorescence microscope after treatment with β-CyDs for 24 h (Fig. 6). As shown in Fig. 6A, the treatment with 10 µM HP-β-CyD and R8-β-CyD for 24 h lowered the fluorescent intensity derived from Filipin III in Npc1-trap-CHO cells. In the results of quantification of

![Fig. 2. Cytotoxic Activity of R8-β-CyD in Npc1-Trap-CHO Cells after Treatment for 24h](image)

Npc1-trap-CHO cells (5×10⁴ cells/well) were incubated with 150 µL of medium (FBS (−)) containing R8-β-CyD for 24 h at 37°C. After washing once with PBS, the cells were incubated with 100 µL of fresh HBSS and 10 µL of WST-1 reagent for 30 min at 37°C. The absorbance at 450 nm against a reference wavelength of 655 nm was measured. Each value represents the mean±S.E.M. of 6–8 experiments.

![Fig. 3. Intracellular Distribution of R8-β-CyD in Npc1-Trap-CHO Cells](image)

Npc1-trap-CHO cells (5×10⁴ cells/well) were incubated with or without 100 nM R8-β-CyD for 24 h. After washing once with PBS, the cells were incubated with 100 nM LysoTracker® for 15 min. The cells were washed once with PBS, fixed with 4% paraformaldehyde, and observed by an In Cell Analyzer 6000. The images reflect similar results from three separate experiments.
the fluorescence intensity, the significant lowering effect of R8-β-CyD was shown, compared to that of HP-β-CyD (Fig. 6B). In addition, this cholesterol-lowering effect of R8-β-CyD was in a concentration and time dependent manner (Figs. 7, 8). Therefore, these results indicate that R8-β-CyD lowered the cholesterol levels in Npc1-trap-CHO cells through R8-mediated endocytosis.

Recently, Rosenbaum et al. demonstrated that endocytosis of β-CyDs was responsible for cholesterol reduction in NPC mutant cells. 24,25 Therefore, a macropinocytosis of R8-β-CyD through cell-penetrating peptides of R8 was found to be crucial to lower the cholesterol accumulation in Npc1-trap-CHO cells. Actually, the cholesterol-lowering effect of R8-β-CyD was much higher than that of HP-β-CyD, which cannot enter cells due to lacking of CPPs, in Npc1-trap-CHO cells (Fig. 6). However, the role of R8-β-CyD endocytosed in cholesterol-lowering effect still remains unclear. Therefore, to reveal the detail mechanism of cholesterol-lowering effect of R8-β-CyD, further elaborate studies on not only cholesterol trafficking system but also an interaction with endolysosomal membranes are necessary.

Autophagy is one of the bulk degradation systems of cyto-

Fig. 4. Effects of Endocytosis Inhibition to R8-β-CyD in Npc1-Trap-CHO Cells

Npc1-trap-CHO cells (1×10⁴ cells/35 mm dish) were treated with 10 μM R8-β-CyD in medium (FBS (−)) for 1 h at 37°C (A) or at 4°C (B). The cells were washed once with PBS, fixed, and observed by an In Cell Analyzer 6000. The images reflect similar results from three separate experiments.

Fig. 5. Effect of Amiloride, a Macropinocytosis Inhibitor, on Cellular Uptake of R8-β-CyD in Npc1-Trap-CHO Cells

Npc1-trap-CHO cells (5×10⁴ cells/35 mm dish) were pretreated with 1 mg/mL of amiloride for 30 min. Then, the cells were incubated with 10 μM Alexa568-R8-β-CyD in medium (FBS (−)) for 1 h at 37°C. The experiments were performed independently three times.

Fig. 6. Effects of R8-β-CyD on Cholesterol Levels in Npc1-Trap-CHO Cells

Npc1-trap-CHO cells (5×10⁴ cells/well) were treated with 10 μM HP-β-CyD or 10 μM R8-β-CyD for 24 h. (A) The cells were washed once with PBS, fixed, and observed by an In Cell Analyzer 6000. (B) The fluorescence intensities were determined by a BZ-II analyzer. The experiments were performed independently three times. Each value represents the mean±S.E.M. of 3 experiments. *p<0.05, compared with Npc1-trap-CHO. †p<0.05, compared with HP-β-CyD.
plasmic protein aggregates and subcellular organelles, and is crucial for the regulation of various diseases. In addition, a basal autophagy plays a pivotal role in the constitutive turnover of cytoplasmic components for maintaining cellular function.\(^26\)\(^{-}\)\(^29\) Indeed, the impairment of basal autophagy has been reported in various neurodegenerative diseases and lysosomal storage disorders including NPC.\(^30\),\(^31\) Therefore, an amelioration of impaired autophagy is still a challenging issue for the treatment of NPC. Recently, Tamura and Yui reported that HP-\(\beta\)-CyD markedly increased the number of LC3-positive puncta and the p62 levels in NPC1 patient-derived fibroblasts, indicating that autophagic flux was further perturbed.\(^32\) In sharp contrast, the intracellular \(\beta\)-CyD, which was delivered by the biocleavable polyrotaxane system, significantly reduced the number of LC3-positive puncta and the p62 levels in NPC1 patient-derived fibroblasts, indicating the amelioration of impaired autophagy.\(^32\) Therefore, the intracellular R8-β-CyD...
CONCLUSION

In the present study, we successfully prepared R8-β-CyD and examined its cholesterol-lowering effect in Npc1-trap-CHO cells. As the results, R8-β-CyD was endocytosed via a cell-penetrating peptide of R8 and reduced the cholesterol accumulation in Npc1-trap-CHO cells. Therefore, R8-β-CyD may be a promising therapeutic for ameliorating cholesterol accumulation in NPC.

Acknowledgments This work was funded by the Japan Agency of Medical Research and Development (AMED). We thank Dr. K. Higaki and Dr. K. Ohno for the donation of the Npc1-trap-CHO cells.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Carstea ED, Morris JA, Coleman KG, Loftus SK, Zhang D, Cummings C, Gu J, Rosenfeld MA, Pavan WJ, Kritzman DB, Nagle J, Polymeropoulos MH, Sturley SL, Ioannou YA, Higgins ME, Comly M, Cooney A, Brown A, Kanekri CR, Blanchette-Mackie EJ, Dwyer NK, Neufeld EB, Chang TY, Liscum L, Strauss JF 3rd, Ohno K, Zeugler M, Carri M, Sokol J, Markie D, O'Neill RR, van Diggelen OP, Ellender M, Patterson MC, Brady RO, Vanier MT, Pentchev PG, Tagle DA. Niemann–Pick C1 disease gene: homology to mediators of cholesterol homeostasis. Science, 277, 228–231 (1997).

2) Naureckiene S, Sleat DE, Lackland H, Fensom A, Vanier MT, Wadstrom T, Jadot M, Lobel P. Identification of HE1 as the second gene of Niemann–Pick C disease. Science, 290, 2298–2301 (2000).

3) Vanier MT. Niemann–Pick disease type C. Orphanet J. Rare Dis., 5, 16 (2010).

4) Matsuo M, Togawa M, Hirabaru K, Mochinaga S, Narita A, Adachi M, Egashira M, Irie T, Ohno K. Effects of cyclodextrin in two patients with Niemann–Pick Type C disease. Mol. Genet. Metab., 108, 76–81 (2013).

5) Higaki K, Ninomiya H, Sugimoto Y, Suzuki T, Taniguchi M, Niwa H, Pentchev PG, Vanier MT, Ohno K. Isolation of NPC1-deficient Chinese hamster ovary cell mutants by drug target mutagenesis. J. Biochem., 129, 875–880 (2001).

6) Sugimoto Y, Ninomiya H, Ohaki Y, Higaki K, Davies JP, Ioannou YA, Ohno K. Accumulation of cholesterin and GM1 ganglioside in the early endosome of Niemann-Pick C1-deficient cells. Proc. Natl. Acad. Sci. U.S.A., 98, 12391–12396 (2001).

7) Szente L, Szijártó J. Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development. Adv. Drug Deliv. Rev., 36, 17–28 (1999).

8) Uekama K, Otagiri M. Cyclodextrins in drug carrier systems. Crit. Rev. Ther. Drug Carrier Syst., 3, 1–40 (1987).

9) Davidson CD, Ali NF, Micsenyi MC, Stephney G, Renault S, Dobrenis K, Ory DS, Vanier MT, Walkley SU. Chronic cyclodextrin treatment of murine Niemann-Pick C disease ameliorates neuronal cholesterol and glycosphingolipid storage and disease progression. PLoS ONE, e6951 (2009).

10) Liu B, Turley SD, Burns DK, Miller AM, Repa JJ, Dietschy JM. Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the npe1–/– mouse. Proc. Natl. Acad. Sci. U.S.A., 106, 2377–2382 (2009).

11) Porter FD, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE, Olzese D, Sidhu R, Dietzen DJ, Fu R, Wassif CA, Yanjain NM, Marso SP, House J, Vite C, Schaffer JE, Ory DS. Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann–Pick C1 disease. Sci. Transl. Med., 2, 56ra81 (2010).

12) Ward S, O’Donnell P, Fernandez S, Vite CH. 2-Hydroxypropylyl-β-cyclodextrin raises hearing threshold in normal cats and in cats with Niemann–Pick type C disease. Pediatr. Res., 68, 52–56 (2010).

13) Soga M, Ishitsuka Y, Hamasaki M, Yoneda K, Furuya H, Matsuo M, Ihn H, Fusaki N, Nakamura K, Nakagata N, Endo F, Irie T, Era T. HPGCd outperforms HPBCD as a potential treatment for Niemann–Pick disease type C during disease modeling with iPSC cells Stem Cells, 33, 1095–1088 (2015).

14) Futaki S. Membrane permeable peptide vectors: chemistry and functional design for the therapeutic applications. Adv. Drug Deliv. Rev., 60, 441 (2008).

15) Nakase I, Akita H, Kogure K, Graslund A, Langel U, Harashima H, Futaki S. Efficient intracellular delivery of nucleic acid pharmaceuticals using cell-penetrating peptides. Acc. Chem. Res., 45, 1132–1139 (2012).

16) Kaplan IM, Wadia JS, Dowdy SF. Cationic TAT peptide transduction domain enters cells by macropinocytosis. J. Control. Release, 102, 247–253 (2005).

17) Nakase I, Hirose T, Tanaka G, Tadokoro A, Kobayashi S, Takeuchi T, Futaki S. Cell-surface accumulation of flock house virus-derived peptide leads to efficient internalization via macropinocytosis. Mol. Ther., 17, 1868–1876 (2009).

18) Nakase I, Niwa M, Takeuchi T, Sonomura K, Kawabata N, Koike Y, Takehashi M, Tanaka S, Ueda K, Simpson JC, Jones AJ, Sugiuira Y, Futaki S. Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. Mol. Ther., 10, 1011–1022 (2004).

19) Nakase I, Tadokoro A, Kawabata N, Takeuchi T, Kato H, Hiramoto K, Negishi M, Nomizu M, Sugiuira Y, Futaki S. Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. Biochemistry, 46, 492–501 (2007).

20) Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat. Med., 10, 310–315 (2004).

21) Auzély-Valéry R, Perly B, Tache O, Zemb T, Jéhan P, Guénot P, Dalibert J-F, Djouani-Piland F. Cholesterol-oligosaccharides: synthesis and insertion into phospholipid membranes. Carbohydr. Res., 318, 82–90 (1999).

22) Maeda Y, Motomaya K, Higashi T, Horikoshi Y, Takeo T, Nakagata N, Kurauchi Y, Katsuki H, Ishitsuka Y, Kondo Y, Irie T, Furuya H, Era T, Arima H. Effects of cyclodextrins on GM1-gangliosides in fibroblasts from GM1-gangliosidosidosis patients. J. Pharm. Pharma col., 67, 1133–1142 (2015).

23) Takechi-Haraya Y, Nadai R, Kimura H, Nishitsuji K, Uchimura K, Sakai-Kato K, Kawakami K, Shigenaga A, Kawakami T, Otaka A, Hojo H, Sakashita N, Saito H. Enthalpy-driven interactions with sulfated glycosaminoglycans promote cell membrane penetration of arginine-rich peptides. Biochim. Biophys. Acta, 1858, 1339–1349 (2016).

24) Rosenbaum AI, Maxfield FR. Niemann–Pick type C disease: molecular mechanisms and potential therapeutic approaches. J. Neurochem., 116, 789–795 (2011).

25) Rosenbaum AI, Zhang G, Warren JD, Maxfield FR. Endocytosis of β-cyclodextrin is responsible for cholesterol reduction in Nie-
mann–Pick type C mutant cells. Proc. Natl. Acad. Sci. U.S.A., 107, 5477–5482 (2010).

26) Elrick MJ, Yu T, Chung C, Lieberman AP. Impaired proteolysis underlies autophagic dysfunction in Niemann–Pick type C disease. Hum. Mol. Genet., 21, 4876–4887 (2012).

27) Ishibashi S, Yamazaki T, Okamoto K. Association of autophagy with cholesterol-accumulated compartments in Niemann–Pick disease type C cells. J. Clin. Neurosci., 16, 954–959 (2009).

28) Ordonez MP, Roberts EA, Kidwell CU, Yuan SH, Plaisted WC, Goldstein LS. Disruption and therapeutic rescue of autophagy in a human neuronal model of Niemann–Pick type C1. Hum. Mol. Genet., 21, 2651–2662 (2012).

29) Sarkar S, Carroll B, Buganim Y, Maetzel D, Ng AH, Cassady JP, Cohen MA, Chakraborty S, Wang H, Spooner E, Ploegh H, Gsponer J, Korolchuk VI, Jaenisch R. Impaired autophagy in the lipid-storage disorder Niemann–Pick type C1 disease. Cell Reports, 5, 1302–1315 (2013).

30) Lieberman AP, Puertollano R, Raben N, Slaugenhaupt S, Walkley SU, Ballabio A. Autophagy in lysosomal storage disorders. Autophagy, 8, 719–730 (2012).

31) Nixon RA. The role of autophagy in neurodegenerative disease. Nat. Med., 19, 983–997 (2013).

32) Tamura A, Yui N. β-Cyclodextrin-threaded bio cleavable polyrotaxanes ameliorate impaired autophagic flux in Niemann–Pick type C disease. J. Biol. Chem., 290, 9442–9454 (2015).