

Cytosolic antibody delivery by lipid-sensitive endosomolytic peptide

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Abstract

One of the major obstacles in intracellular targeting using antibodies is their limited release from endosomes into cytosol. Here, we report an approach for delivering bioactive proteins, including antibodies, into cells using endosomolytic peptides derived from the cationic and membrane-lytic spider venom peptide M-lycotoxin. The delivery peptides were developed by introducing one or two glutamic acid residues into the hydrophobic face. One peptide with substitution of leucine at position 17 with glutamic acid (L17E) was shown to enable marked cytosolic liberation of antibodies (IgGs) from endosomes. We also demonstrate that the predominant membrane perturbation mechanism of this peptide is the preferential disruption of negatively charged membranes (endosomal membranes) over neutral membranes (plasma membranes) and that the endosomolytic peptide promotes uptake by inducing macropinocytosis. The fidelity of this approach was further confirmed through intracellular delivery of a ribosome inactivation protein (saporin), Cre recombinase and IgG delivery which achieved specific labelling of cytosolic proteins and subsequent suppression of glucocorticoid receptor-mediated transcription. Intracellular delivery using exosomes was also demonstrated.

Introduction

Recent advances in molecular and cell biology have shed light on various key proteins in cells, which is important for developing new therapies.¹ Antibodies are the supreme molecules for molecular recognition and targeting.^{2,3} Enabling the access of antibodies to the cytosol provides a clearer understanding of the localisation and behaviour of target proteins. Modulating the molecular interplay involving these proteins also provides information on their biological significance, along with their feasibility as therapeutic targets. Although approaches for intracellular delivery of antibodies and other bioactive proteins have been previously reported (*e.g.*, references 4-10), practical approaches based on novel concepts are still needed at levels of efficacy that achieve sufficient interactions with cellular targets without cytotoxicity.

One of the major obstacles in intracellular targeting by antibodies is their limited release from endosomes into the cytosol. Antibodies are taken up by cells via endocytosis, which is a process involving the physiological uptake of extracellular materials that are delivered into cells by encapsulation into vesicular compartments named endosomes.¹¹ Without their release from vesicular compartments into the cytosol, antibodies cannot interact with their target molecules, and may eventually be degraded in these compartments.

To overcome this issue, numerous approaches, including the use of pH-sensitive membrane-perturbing peptides and polymers, have been reported.¹²⁻²⁰ These molecules are designed to switch their structures and lyse endosomal membranes, in accordance with the pH decrease in endosomal compartments. However, current approaches are inadequate to achieve the complete release of antibodies from

endosomes.^{2,3} This not only reduces the targeting efficacy of antibodies, but also results in inaccurate signals during in-cell analysis and diagnostics. Therefore, there is a high demand for novel strategies that achieve ~100% release of endosome-encapsulated antibodies into the cytosol.

We hypothesised that the reason for the incomplete endosomal escape of antibodies is insufficient perturbation of endosomal membranes by currently used endosomolytic agents. Most of currently available pH-sensitive membrane-lytic peptides and polymers are designed to preferentially perturb endosomal membranes by the protonation of carboxylates at an endosomal acidic pH.¹²⁻¹⁵ GALA is a representative peptide in this class (WEAALAEALAEALAEHLAEALAEALEALAA-amide).¹² This peptide has a net negative and potentially amphiphilic structure, and its hydrophilic face comprises a series of glutamate (Glu) residues. Negative charges on Glu residues in the extracellular region prevent peptide interaction with membranes (no lysis). The protonation of Glu at an acidic pH allows a less charged peptide to form a helical structure and interact with and perturb membranes. However, the lytic activity of neutral (or negatively charged) peptides is much less intense than that of cationic haemolytic peptides.²¹⁻²³ In addition, cell surfaces have an overall negative charge due to the abundance of proteoglycans and sialic acids, and the cellular uptake efficacy of negatively charged molecules is lower than that of positively charged ones.¹³ Without incorporation into endosomes, these pH-sensitive peptides do not have lytic activity.

Therefore, we employed cationic, amphiphilic peptides with strong membrane-lytic activity as a starting material for the design of endosomolytic peptides (Fig. 1a). Cationic haemolytic peptides often have basic amphiphilic helical structures. For

membrane perturbation, the hydrophobic interaction of the peptides with membranes is critical.^{21,22} The insertion of negatively charged Glu into a hydrophobic face should effectively attenuate lytic activity, while retaining the ability to adsorb to the cell surface. The facilitated entrapment of peptides into endosomes and the eventual protonation of Glu should recover the membrane-lytic activity of the peptides. Due to the high cytotoxicity of these lytic peptides, only minor attention has been paid to their utilisation for intracellular delivery, although the targeting of cationic amphiphilic peptides to tumour cells and to their mitochondrial membranes reportedly results in tumour cell death.²³

Recent studies have revealed that endosomal membranes are rich in negatively charged lipids.^{24,25} This confers another advantage for cationic peptides over neutral or anionic peptides in that they have a higher affinity for perturbing endosomal membranes. Although this difference in the charged states of membranes provides important information for designing an intracellular antibody delivery system, very few studies have focused on this issue.

In this report, we describe the impact of our approach of using attenuated, membrane-lytic peptides for the cytosolic targeting of bioactive proteins including saporin (ribosome-inactivating protein), Cre gene recombinase, and antibodies (IgGs). The applicability of this system to the cytosolic delivery of exosome-encapsulated bioactive proteins is also demonstrated.

Results and Discussion

Design strategy of endosomolytic peptides and their selection

Our design consisted of simply introducing Glu into natural membrane-lytic peptides (Fig. 1a). (i) The perturbation activity of the peptides on cell surfaces (i.e., plasma membranes) causing cytotoxicity is abrogated by the appropriate introduction of negatively charged Glu into the hydrophobic face of the amphiphilic helix (Fig. 1a, panels [1] and [2]). (ii) The extent of Glu substitution should be as small as possible to retain cationic features. This helps the peptides adsorb on cell surfaces, leading to efficient entrapment and accumulation into endosomes (Fig. 1a, panel [2]). We expected that (iii) the protonation of Glu carboxylate, under endosomal acidic pH, should recover the net hydrophobicity of the peptide and perturbation activity on membranes (Fig. 1a, panel [3]). In addition, (iv) the cationic feature of the peptides should enhance the electrostatic interaction with endosomal membranes (abundant negatively charged lipids) compared to that with plasma membranes (rich in neutral lipids) (Fig. 1a, panel [3]). Thus, we expected that the synergistic effects of (iii) and (iv) would effectively perturb the endosomal membranes, thereby (v) facilitating the escape of antibodies from endosomes and their entry into the cytosol to target cytosolic proteins (Fig. 1a, [4]).

To establish the validity of this approach, nine cationic, amphiphilic peptides with haemolytic activity were selected as candidates for the design templates (see Supplementary Table 1). For convenience in peptide preparation, those not exceeding 25 residues in length were selected. The perturbation activity of these peptides on cell membranes was evaluated using the WST-1 cell proliferation assay²⁶ after treatment with peptides for 2 h. M-lycotoxin, derived from the venom of the wolf spider *Lycosa*

carolinensis,²⁷ showed the lowest half-maximal effective concentration (EC₅₀) of 1.36 μ M. Thus, we selected it as the design template for Glu substitution (Fig. 1a).

To reduce the cationic charges, we introduced one or two glutamic acids (Glu, E) into the peptide sequence (Table 1). M-lycotoxin has a potential amphiphilic helical structure (Fig. 1a, [2], left). Five lysines in the sequence and its N-terminus amino function should confer cationic charges and haemolytic activity to the peptide. Five mono-substituted analogues (L6E, L9E, A13E, L17E, and Q21E) and four di-substituted analogues (L6E/G10E, L9E/A13E, A13E/L17E, and L17E/Q21E) of wild-type (WT) M-lycotoxin were designed so that one or two amino acid residues in the hydrophobic face were replaced by Glu residues (Table 1, Supplementary Fig. 1). This simple substitution yielded dramatic attenuation of the plasma membrane perturbation activity of the peptides. EC₅₀ values of these analogues, as determined using the WST-1 assay, were >40 μ M (*i.e.*, >~30-fold reduction in cytotoxicity), with the exception of Q21E (3.54 μ M) (Supplementary Table 2).

Next, we evaluated the ability of these peptides to promote the cytosolic release of endocytosed biomacromolecules. HeLa cells were incubated with dextran (10 kDa) labelled with Alexa Fluor 488 (Alexa488-dextran, 250 μ g/mL) as a model macromolecule in medium for 1 h, and the cellular distribution of Alexa488-dextran signal was analysed using confocal laser scanning microscopy (CLSM) after 3 h (Fig. 1b; see also Supplementary Fig. 2 with differential interference contrast [DIC] images). In the absence of peptides, Alexa488-dextran yielded only punctate, dot-like signals in the cells, indicative of the predominant encapsulation of Alexa488-dextran into endosomes (Fig. 1b, top left panels). In contrast, one of these Glu-substituted analogues

(L17E) showed significant stimulation of cytosolic release of endocytosed molecules, as intended. Almost 50% of the cells co-incubated with L17E (40 μ M) showed spread signals of Alexa488-dextran in the cytosol and in the nucleus (Fig. 1b, top right panels), indicating the effective cytosolic release of Alexa488-dextran. Almost 20% and 5% of A13E- and L9E-treated cells, respectively, had cytosolic/nuclear Alexa488-dextran signals (Fig. 1b, top panels). For disubstituted peptides, ~30% of cells yielded cytosolic/nuclear localization of Alexa488-dextran signals when treated with L17E/Q21E, followed by ~15% of cells treated with A13E/L17E (Fig. 1b, bottom right panels). On the other hand, when the cells were similarly treated with GALA, as an example of a pH-sensitive lytic peptide bearing Glu residues, no significant cytosolic Alexa488-dextran signals were observed (Fig. 1b, bottom left panels).

The fidelity of our design concept was further confirmed using two analogues of L17E. One was the D-amino acid version of L17E (D-L17E) (Table 1). If L17E requires some protein machinery (e.g., receptor and transporter) to induce cytosolic release, its enantiomer should have less activity. However, D-L17E similarly yielded marked cytosolic distribution of dextran, suggesting that the targets of action of L17E are not proteins but membranes (Supplementary Fig. 3a, left). The other analogue was L17D, in which leucine (Leu) at position 17 is substituted for aspartate (Asp) (Table 1), which also bears a carboxylate in its side chain. L17D also yielded significant cytosolic dextran signals (Supplementary Fig. 3a, right). However, the proportion of cells bearing diffuse cytosolic distribution of dextran was approximately 20%. The β -COOH group in Asp has a lower pK_a (3.65) than γ -COOH in Glu (4.25),²⁸ and the side chain of Asp is

missing one methylene compared to the Glu side chain. The increase in hydrophilicity should make L17D less effective than L17E.

Next, we analysed the core sequence of L17E responsible for dextran release. The very low level of dextran cytosolic release by insertion of Glu at positions 6 (L6E) and 9 (L9E) was suggestive of the importance of the N-terminus in membrane interaction. Thus, we prepared L17E analogues with a C-terminal deletion of positions 23–25 (L17EΔ[23–25]) and 20–25 (L17EΔ[20–25]) (Table 1). While the former peptide yielded diffuse cytosolic distribution of dextran in 15% of cells, the latter only yielded 2% (Supplementary Fig. 3b), indicating that positions 1–22 comprise the minimum sequence for exerting the proper function of L17E. Interestingly, although there were considerable differences in the efficacies in dextran cytosolic release between L17E and the C-terminal deleted peptides L17EΔ(23–25) and L17EΔ(20–25), the cytotoxicities of WTΔ(23–25) and WTΔ(20–25) [*i.e.*, C-terminus-truncated versions of the WT corresponding to L17EΔ(23–25) and L17EΔ (20–25)] were almost similar (EC_{50} , $<3\ \mu\text{M}$) to that of WT ($1.36\ \mu\text{M}$) (Supplementary Table 3). Therefore, peptide chain length may play a particular role for promoting cytosolic release of endosomal encapsulates.

Notably, serum only yielded marginal effects on the cytosolic release of dextran (cells showing cytosolic distribution in total cells: $\sim 50\%$ versus $\sim 55\%$ in the presence and absence of serum, respectively), although it has often been claimed that its presence decreases the efficacy of intracellular delivery^{14,15} (Supplementary Fig. 3c). No considerable cytotoxicity accompanied L17E treatment. The cell viability with L17E treatment, as determined by the WST-1 assay, was almost 90% when the cells were treated with $40\ \mu\text{M}$ L17E in serum-free medium for 1 h or serum-supplemented medium

for 24 h (Supplementary Fig. 4a and b, respectively). Prolonged treatment of the cells with L17E (40 μ M) for 4 days showed no observable effect on cell growth and proliferation (Supplementary Fig. 4c).

The applicability of L17E to cytosolic delivery was also confirmed in cells other than human cervical carcinoma HeLa cells, such as human colon adenocarcinoma SW480 cells, African green monkey kidney fibroblast-like COS-7 cells, and human umbilical vein endothelial cells (HUVECs), while only a marginal effect was observed in mouse embryonic fibroblasts (NIH3T3) (Supplementary Fig. 5).

Intracellular fate of L17E was analysed by CLSM using C-terminal Alexa488-labeled L17E (L17E-Alexa488). HeLa cells were treated with 20 μ M L17E-Alexa488 for 1 h. After washing and medium change, the signals of internalized L17E-Alexa488 were analysed by CLSM (Supplementary Fig. 6a, left). Although there was some cytosolic L17E-Alexa488 labelling, punctate L17E-Alexa488 signals were predominantly observed, suggesting that considerable amount of L17E was kept in endosomes. While L17E effectively perturbs endosomal membranes to release endosomal contents into the cytosol, L17E itself is rather hydrophobic and may stay with membranes than penetrate into cytosol. The L17E-Alexa488 signals became less apparent over time (Supplementary Fig. 6a), suggesting possible intracellular degradation of L17E. After 24 hours, signals were mostly observed at the perinuclear area, colocalising with lysosome marker, LysoTracker (Supplementary Fig. 6b).

Additionally, based on the fluorescent intensity of Alexa488 in cell lysates, it was estimated that only 0.5%, or less, of total L17E-Alexa488 administrated into the culture media was actually taken up by the cells (see Supplementary Information for

experimental details). Thus, L17E is taken up into endosomes via non-specific manner; more efficient cytosolic delivery systems may be established by the enhancement of recruitment of L17E into endosomal compartments.

Cytosolic delivery of bioactive proteins using L17E

The applicability of this approach for the cytosolic delivery of bioactive proteins by L17E was confirmed using saporin (30 kDa), a membrane-impermeable ribosome inactivation protein (Fig. 2a).²⁹ The applicability of this toxin conjugated with monoclonal antibodies to tumour targeting has been reported.³⁰ When HeLa cells were treated with 10 µg/mL saporin in the presence of L17E for 1 h, ~80% cell death was obtained. However, in the absence of L17E, cell death was only observed in ~15% of cells (Fig. 2a, right).

The cytosolic delivery of bioactive proteins by L17E was further confirmed by gene recombination assay using Cre protein (38 kDa), an enzyme that catalyses recombination between the specific DNA sequences called *loxP* sites.³¹ We used cells with plasmid encoding the *loxP-DsRed-loxP-EGFP* gene, in which red-fluorescent protein DsRed is constitutively expressed while EGFP expression is suppressed by the stop codon at the end of the *DsRed* gene (Fig. 2b, left). Once Cre is introduced into the cells, the protein will facilitate site-specific gene recombination to remove the DNA region between the two *loxP* sites, leading to conditional expression of EGFP (Fig. 2b, left). When cells were treated with extracellularly prepared Cre protein (5 µM) in the presence of L17E (40 µM), significant expression of EGFP was observed in the DsRed-

expressing cells (Fig. 2b, right, bottom panels), suggesting that recombination reaction was induced by L17E-mediated delivery of Cre. In contrast, no significant EGFP signals were observed after treatment with Cre in the absence of L17E (Fig. 2b, top right panels). These results collectively indicate that L17E allows delivery of proteins into the cytosol and preserving the protein's bioactivity.

Targeting cytosolic proteins via intracellularly delivered antibodies using L17E

Hydrodynamic radii of antibodies (IgGs) are estimated to be ~5 nm, which is about twice as large as proteins of ~30 kDa.³² Therefore, more effective perturbation of endosomal membranes is needed to achieve efficient (complete) cytosolic release of IgG from endosomes. We were very pleased to find that L17E allowed marked cytosolic targeting of antibodies (Supplementary Fig. 3a, top panels and 7a). The cytosolic distribution of Alexa488-labeled IgG (polyclonal, from human serum) was observed for almost 40% of cells, suggesting the cytosolic liberation of Alexa488-labeled IgG. There was no significant diffused cytosolic appearance of Alexa488-labeled IgG in the absence of L17E (Fig. 3a, lower panel). Additionally, use of high sensitivity GaAsP detector allowed observation of significant cytosolic localization of Alexa488-labeled IgG signals even at decreased amount of Alexa488-labeled IgG (50 µg/mL; i.e., 1/20 amount that was employed for Fig. 3a) for 30 min in the presence of 40 µM L17E (Supplementary Fig. 7b).

Next, the ability of exogenous antibodies to target cellular proteins in the presence of L17E was demonstrated (Fig. 3b). As a model of cytosolic targets,

monomeric DsRed fluorescent protein bearing a His₆ recognition sequence (DsRed-His₆) was expressed in HeLa cells. The N-terminal 20-amino-acid sequence of neuromodulin (mem)³³ was fused with the protein as a membrane localisation sequence. DsRed-His₆ without the mem sequence showed diffuse distribution throughout the cells (Fig. 3b-ii, center). On the other hand, distinct signals of mem-DsRed-His₆ were observed on plasma membranes (e.g., boxed areas in Fig. 3b-i, top panels, center), in addition to punctate staining, suggestive of anchoring of mem-DsRed-His₆ onto the cytosolic face of plasma membranes and intracellular vesicular compartments. Significant co-localisation of monoclonal anti-His₆-IgG (labelled with fluorescein isothiocyanate [FITC]) with mem-DsRed-His₆, anchored on plasma membranes, was observed upon incubation with L17E (Fig. 3b-i, upper panels, right, boxed area), indicative of actual targeting of the intracellular protein by intracellularly delivered IgG. In the absence of L17E, anti-His₆-IgG yielded punctate signals. No significant anti-His₆-IgG signals co-localised with mem-DsRed-His₆ on plasma membranes were observed (Fig. 3b-i, bottom right panels, boxed area), suggesting that no substantial amount of anti-His₆-IgG were released from endosomes to interact with the target molecules in the cytosol. Note that, although there were some overlaps of the anti-His₆-IgG signals with mem-DsRed-His₆ signals in perinuclear areas, these might be due to anti-His₆-IgG inside endosomal membranes and mem-DsRed-His₆ on the cytosolic face of endosomal compartments and are thus deceptive. Even when the amount of anti-His₆-IgG was reduced to 1/20 of that used in Fig. 3b, same tendencies of cellular localization of anti-His₆-IgG were observed using high sensitivity GaAsP detector (Supplementary Fig. 8) as in the case of Supplementary Fig. 7b. Collectively, these results further supports the usefulness of this approach for intracellular targeting.

Intracellularly delivered IgG also blocked intracellular signalling and transcription. The glucocorticoid receptor (GR) is a nuclear receptor that is activated by steroids including dexamethasone (Dex) (Fig. 3c). The unbound receptor resides in the cytosol. After the receptor is bound to ligands, the activated GR complex translocates to the nucleus and initiates the transcription of glucocorticoid-responsive genes, including that for metallothionein 1E (MT1E). The possible contribution of MT1E to tumorigenesis has been suggested.³⁴

We used real-time quantitative PCR to analyse the mRNA expression level of MT1E after the stimulation of HeLa cells with Dex. Upon stimulation with 100 nM Dex, the cellular mRNA level of MT1E became 5.3-fold higher than that in unstimulated cells (Fig. 3c, first and second bars from the left). When the cells were treated with monoclonal anti-GR-IgG in the presence of L17E prior to Dex stimulation, the increase in MT1E mRNA expression decreased to <60% of that in the absence of anti-GR-IgG treatment (Fig. 3c, third bar from the left), indicating significant suppression of Dex-induced transcription by the antibody. On the other hand, upon pretreatment of cells with nonspecific IgG (human, polyclonal) instead of GR-specific anti-GR-IgG, no significant suppression was observed (Fig. 3c, right bar). Anti-GR-IgG successfully recognised and interacted with the cytosolic GR, inhibiting Dex-induced nuclear translocation of GR. In the absence of L17E, anti-GR-IgG had no effect on MT1E mRNA expression (Supplementary Fig. 9). Thus, the feasibility of using L17E for cytosolic antibody delivery was demonstrated.

Stimulation of cytosolic delivery of exosomal contents using L17E

Exosomes have been considered a promising vehicle for the intracellular delivery of biomacromolecules including bioactive proteins and nucleic acids. However, the low efficacy of cytosolic release of exosome-encapsulated molecules is an obstacle to achieving this goal.³⁵ We herein showed that L17E may have the ability to promote the endocytic uptake and cytosolic delivery of exosome-encapsulated proteins.

First, the stability of exosomes upon L17E-treatment was confirmed in terms of FITC-dextran leakage from exosomes. Exosomes encapsulating FITC-dextran (4 kDa) were incubated with L17E (40 μ M) for 1 h at 37°C. The release of FITC-dextran into PBS was analysed after separating the exosomes by ultracentrifugation (Supplementary Table 4). Results showed that in both presence and absence of L17E, only ~4% of FITC-dextran was released. This confirms the absence of marked perturbation effect of L17E for exosomal membranes.

After confirming that L17E is unable to perturb exosome membranes, the ability of L17E to release exosome-encapsulated biomacromolecules into the cytosol was then evaluated. HeLa cells were treated with FITC-dextran-encapsulated exosomes in the presence of L17E (40 μ M) for 2 h. CLSM analysis showed significant cytosolic and nuclear distribution of dextran (Supplementary Fig. 10). This showed the effectiveness of L17E-mediated cytosolic delivery of exosomal content. We also confirmed incorporation of exosomes into endosomes through the CLSM analysis of exosomes bearing CD63 -green fluorescence protein fusion protein (CD63-GFP-exosomes). CD63 is an exosome-associated membrane protein. CD63-GFP-exosomes were isolated from the cells that express CD63-GFP fusion proteins. Treatment of cells

with CD63-GFP-exosomes in the presence of L17E yielded significant signals of CD63-GFP-exosomes in the cells, showing colocalisation with lysosome marker (i.e., LysoTracker) (Supplementary Fig. 11). Taken together, exosome-encapsulated cargos were released into the cytosol from endosomal compartment in the presence of L17E.

Feasibility of L17E for intracellular delivery of exosome-encapsulated bioactive proteins was further demonstrated using saporin. Exosomes containing saporin were treated with L17E (40 μ M) in medium for 1 h, and cytotoxicity was evaluated using the WST-1 assay after 48 h (Fig. 3d, left). While the treatment with saporin-encapsulated exosomes in the presence of L17E led to significant cell death, exosomes were not toxic to cells in its absence, indicating the potential of L17E in exosome-mediated intracellular delivery (Fig. 3d, right graph).

Biophysical study on the perturbation of membranes by L17E: No apparent pH-sensitive membrane perturbation observed for L17E

We inserted a negatively charged Glu into the potentially hydrophobic face of WT M-lycotoxin to attenuate the membrane interaction of the peptide and membrane-lytic activity. Possible protonation of the Glu residues under reduced pH in endosomes should achieve hydrophobicity and positive charges of the peptides, by which the selective perturbation of endosomal membranes would be expected. To validate our design strategy, we conducted dye-leakage assays using artificial membrane systems (Fig. 4a).³⁶

Large unilamellar vesicles (LUVs) containing 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) and its quencher, *p*-xylene-bis-pyridinium bromide (DPX), were treated with peptides at varying concentrations. Increases in fluorescence intensity by the liberation of ANTS from LUVs were analysed to evaluate the membrane-lytic activity.³⁶ Considering that the outer leaflet of cell surface membranes (plasma membranes) is predominantly composed of neutral lipids and that endosomal membranes are rich in negatively charged lipids,^{24,25} 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was employed as a plasma membrane model lipid. Combinations of lipids of POPC and a negatively charged lipid, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), were employed for endosome model membranes (Fig. 4a).³⁷ L17E induced little dye leakage from POPC LUVs at pH 7.4 with a half-maximal effective peptide/lipid ratio ($[P/L]_{50}$) > 0.3 (filled circle in Fig. 4a-i). On the other hand, L17E showed >25 times higher perturbation activity against POPC/POPG (3:1) LUVs at pH 5.0, yielding a $[P/L]_{50}$ value of 1.2×10^{-2} (open square). This difference in perturbation activity supported the endosome-selective perturbation of L17E to stimulate the cytosolic release of endocytosed biomacromolecules very well. However, surprisingly, L17E had perturbation activity against POPC/POPG (3:1) LUVs at pH 7.4 (filled square, $[P/L]_{50} = 1.0 \times 10^{-2}$) comparable to that at pH 5.0 (shown above). Therefore, L17E had little pH dependence, but had specific perturbation ability to negatively charged membranes, yielding a marked contrast to conventional pH-dependent endosomolytic peptides and polymers.

To determine the reason for this lack of pH dependence, methods of membrane perturbation were analysed using WT peptide (Fig. 4a-ii). The WT peptide also showed

higher perturbation for negatively charged membranes than for neutral membranes. However, a certain pH dependence was also observed for the WT peptide, showing higher perturbation activity at pH 7.4 than at 5.0, against POPC/POPG (3:1) and POPC (100%) LUVs (Fig. 4a-ii). Thus, the apparent lack of pH dependence in the membrane perturbation ability of L17E may be explained as follows. Glu is in its charged state at pH 7.4, and insertion of this charged residue into the WT sequence should yield a considerable decrease in the membrane perturbation activity. Alternatively, WT has lower membrane-lytic activity at pH 5.0 (Fig. 4a-ii), and the partial protonation of Glu at pH 5.0 (making the peptide more hydrophobic) should yield a smaller effect of Glu insertion on membrane perturbation than at pH 7.4. L17E may thus achieve similar membrane perturbation at pH 5.0 to that at pH 7.4, yielding a pH-independent method of membrane perturbation of L17E (see Supplementary Fig. 12a). The same explanation would be applicable to L17E/Q21E and Q21E peptides (Supplementary Fig. 13). Thus, L17E can be considered to have attenuated membrane-lytic activity compared to WT. One might wonder whether a similar endosomolytic effect could be obtained using a lower concentration of WT. Cell-penetrating peptides may also have the ability to stimulate the endosomal escape of biomacromolecules. However, treatment with either 1 μ M WT or 40 μ M R8 (a representative cell-penetrating peptide) yielded no significant cytosolic appearance of Alexa488-dextran (Supplementary Fig. 14).

Compared with L17E, L17D had inferior ability to release endosome-trapped Alexa488-dextran into the cytosol (Supplementary Fig. 3a). This parallels the dye leakage assay results (Supplementary Fig. 12b). L17D induced dye leakage from POPC/POPG (3:1) LUVs at pH 5.0 with a half-maximal effective peptide/lipid ratio

$([P/L]_{50}) > 0.3$ (open square in Supplementary Fig. 12b), which is considerably lower than that of L17E ($[P/L]_{50} = 1.2 \times 10^{-2}$). As described above, Asp is more hydrophilic than Glu, therefore, substitution of Leu to Asp on the WT peptide results to weaker membrane perturbation activity compared to Glu substitution (Supplementary Fig. 12b).

Because amphiphilic helical structures are the canonical conformation for yielding membrane perturbation, many conventional pH-sensitive endosomolytic peptides have been designed to have an enhanced helical structure at a reduced pH. However, different from these peptides, no enhanced helical structure was observed for L17E at a reduced pH (Fig. 4b). While the WT peptide showed a typical helical structure, the circular dichroism (CD) spectrum of L17E in the presence of POPC/POPG (3:1) LUVs at pH 5.0 was suggestive of a very weak helical structure (Fig. 4b-i), although this peptide showed considerable lytic activity under these conditions. Analysed by molecular ellipticity at 222 nm ($[\theta]_{222}$) as a measure of helical content,³⁸ a rather small decrease in helical content was observed at the reduced pH ($[\theta]_{222}$ at pH 7.4 and 5.0: -6.1×10^3 and -4.9×10^3 deg cm² dmol⁻¹, respectively) (Fig. 4b-ii). No significant concentration effects were observed on the secondary structure of L17E, regardless of whether or not the peptide concentration induced membrane lysis (P/L ratio of $0.5\text{--}2.0 \times 10^{-2}$) (Supplementary Fig. 15). L17E also had a very low level of helical structure in the presence of POPC LUVs at pH 7.4 (Fig. 4b-iii). Therefore, L17E should have its membrane perturbation effects due to the electrostatic interaction with negatively charged membranes, without the need for amphiphilic helical structure formation. The insertion of a single Glu residue into the hydrophobic face effectively acts as a safety catch of the membrane perturbation ability of the peptide.

Inhibitor studies: L17E has a physiological effect to promote cellular uptake by inducing macropinocytosis

To further evaluate the fidelity of our design strategy, the involvement of endocytosis in L17E-mediated cytosolic release was confirmed by the treatment of cells at 4 °C (at this temperature, endocytosis does not occur)³⁹ (Fig. 5a and Supplementary Fig. 16). No significant Alexa488-dextran signal was observed in the cells under this treatment. Sodium azide treatment leaves the cells in an ATP-depleted state and thus inhibits endocytosis.⁴⁰ This treatment also diminished the number of cells with diffuse appearance of cytosolic dextran, further supporting the involvement of endocytosis in the L17E-mediated cytosolic release of biomacromolecules (Fig. 5b-i).

More detailed studies suggested the involvement of macropinocytosis in this process (Fig. 5b-ii). Macropinocytosis is a fluid-phase form of endocytosis driven by actin organisation,⁴¹ and often plays a role in the cellular entry of viruses and cationic cell-penetrating peptides.^{42,43} Significant suppression in the L17E-mediated cytosolic diffusion of Alexa488-dextran was observed by treatment with EIPA [=5-(*N*-ethyl-*N*-isopropyl)amiloride], a representative macropinocytosis inhibitor (Fig. 5b-ii).³⁹ L17E may also have the ability to induce macropinocytosis. Increased uptake of dextran (also employed as an extracellular fluid marker) is a criterion defining macropinocytosis. In fact, ~40% increase in Alexa488-dextran uptake was attained by L17E treatment regardless of the absence or presence of serum (Fig. 5c-i and ii, respectively). The enhanced uptake of exosomes was also observed in the presence of L17E (Fig. 5d). L17E has a positively charged structure even after the insertion of Glu. The cationic

feature of this peptide not only helps L17E to adsorb on cell surfaces, but may also contribute to induce macropinocytosis, as in the case of cationic cell-penetrating peptides.^{39,43} This eventually leads to enhanced cellular uptake of the molecular cargo, together with L17E, to accelerate their cytosolic targeting. Treatment with clathrin-mediated endocytosis inhibitors including phenylarsine oxide (PAO),⁴⁴ monodansylcadaverine (MDC),⁴⁵ and dynasore⁴⁶ did not prevent the cytosolic distribution of dextran, suggesting that clathrin-mediated endocytosis does not significantly contribute to this process (Supplementary Fig. 17).

Conclusions

In this report, we present a novel concept for the design of endosomolytic peptides that effectively deliver biomacromolecules, including antibodies, into the cytosol (summarised in Fig. 5e). Here, the introduction of a single negatively charged Glu residue into the potentially hydrophobic face of M-lycotoxin, a cationic membrane-lytic peptide, effectively acted as a safety catch for modulating the lytic activity of this peptide, selectively perturbing endosomal membranes. The membrane-lytic activity is induced by the electrostatic interaction of the positively charged peptide with negatively charged endosomal membranes. Unlike existing pH-sensitive lytic peptides, L17E does not have significant pH-dependent membrane lytic activity. In contrast to the WT peptide, no enhanced helical structure is needed for L17E-mediated membrane lysis. This cationic feature of L17E not only facilitates the cytosolic release of endocytosed biomacromolecules in terms of *physicochemical* peptide-membrane interactions, but should also stimulate the *physiological* uptake of cargo via the induction of macropinocytosis. Although many endosome-lytic peptides and polymers have been reported, to the best of our knowledge, this is the first demonstration of peptides that can achieve enhanced cellular uptake via the induction of macropinocytosis, leading to efficient cytosolic delivery. Thus, our approach should be useful for designing systems that achieve the effective cytosolic delivery of biomacromolecules.

In our approach, biomacromolecules are endocytosed into cells via non-specific manners, without using specific machineries to capture and to accumulate these biomacromolecules into endosomes. Improvements of the system should be achieved by the greater recruitment of cargos of interests together with endosomolytic peptides into

endosomal compartments. Developing peptides with higher endosomolytic activity to achieve similar extent of endosomolytic activity with using less of the peptide is also a practical approach, which is under study in our laboratory.

Methods

Cellular uptake of 10 kDa dextran and microscopic observation

HeLa cells were seeded into 35 mm glass-bottom dishes (Iwaki), and the cells were allowed to reach 80–90% confluence in 24 h. These cells were washed twice with serum-free α -minimum essential medium [α -MEM(-)], and then incubated with 250 μ g/mL dextran (10 kDa) labelled with Alexa Fluor 488 (Alexa488-dextran) (Invitrogen, Carlsbad, CA, USA) in the presence of a peptide (40 μ M) in α -MEM(-) for 1 h at 37°C. Cells were washed with α -MEM(-) and incubated in serum-supplemented α -MEM [α -MEM(+)] for 3 h at 37°C. Cellular uptake of dextran was analysed in live cells using an FV1000 Olympus confocal laser scanning microscope. The numbers of the cells showing diffuse cytosolic signal of dextran among 250-700 cells were counted and the percentages were used as a measure of endosomolytic activity for each sample.

Recognition of membrane-anchored DsRed-His₆ proteins by intracellularly delivered anti-His₆ antibody

HeLa cells plated on 35 mm glass-bottom dishes (1.5×10^5 cells) were transfected with expression vectors for DsRed-His₆ and mem-DsRed-His₆ (DsRed-His₆-pCI and mem-DsRed-His₆-pCI, respectively) using Lipofectamine LTX (Invitrogen), in accordance with the manufacturer's instructions. After incubation for 46 h, the cells were treated with anti-6 $\times$ His tag antibody (FITC) (ab1206; Abcam) (500 μ g/mL) in the presence or absence of L17E (40 μ M) in α -MEM(-) for 1 h at 37°C, followed by incubation for another 30 min in α -MEM(+). The localisation of fluorescently labelled antibody in live

cells was analysed by CLSM. In addition, the ability of anti-6× His tag antibody (FITC) to correctly recognise His-tagged proteins in cells was confirmed by CLSM, for which the cells were fixed in 4% paraformaldehyde and permeabilised with 0.5% Triton X-100 prior to staining with the antibody (Supplementary Fig. 18)

Inhibition of MT1E mRNA induction by intracellularly delivered anti-glucocorticoid receptor antibody

HeLa cells were seeded in a 24-well plate (Iwaki) in Dulbecco's Modified Eagle Medium without phenol red [DMEM(-)] supplemented with 10% charcoal-stripped fetal bovine serum (Wako) [DMEM(+)] so that the cells were 80–90% confluent in 24 h. Cells were washed twice with phosphate-buffered saline (PBS) and then incubated with 300 µg/mL anti-GR antibody (BuGR2) (Invitrogen) in the presence of L17E (40 µM) in PBS supplemented with 1 mM MgCl₂ and 1 mM CaCl₂ (PBS[+]) for 30 min at 37°C. Cells were washed twice with PBS and then incubated with 100 nM dexamethasone (Dex) in serum-free medium for 60 min. After incubation, cells were washed twice and additionally incubated for 2 h in DMEM(+). Cells were washed with PBS twice and total RNA was extracted using the RNeasy Mini Kit (Qiagen), in accordance with the instructions of the manufacturer. cDNA was synthesised from total RNA using a PrimeScript RT reagent kit (Takara Bio), and real-time PCR was performed with a 7300 Real-Time PCR System (Applied Biosystems) using PowerUp SYBR Green PCR Master Mix (Applied Biosystems).

ANTS/DPX leakage assay

Peptides were incubated with 134 μ M LUVs in 10 mM MES containing 150 mM NaCl (pH 7.4 or 5.0) for 1 h at 37°C. Fluorescence intensity was measured at an excitation wavelength of 355 nm and emission of 535 nm using a Wallac 1420 Victor2 Microplate Reader (PerkinElmer). The percentage of ANTS released was calculated as follows: % ANTS released = $100 \times ([F_{\text{obs}} - F_0] / [F_{\text{max}} - F_0])$. F_{obs} represents the observed fluorescence intensity. F_{max} is the maximum fluorescence intensity after adding Triton X-100 at a final concentration of 0.1% (v/v). F_0 represents the initial fluorescence intensity.

Data availability statements

All available data can be obtained by contacting the corresponding author.

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Author contribution

M.A. designed and executed the most of the experiments. H.-H. Y., F. M., and A.G. were involved in peptide design, evaluation of the endosomal escape, and discussion of peptide-membrane interaction. Y.K. and I.N. were involved in experiments using saporin and exosomes, respectively. T. T.-N. was involved in statistical analysis of the experimental data. K.S. was involved peptide synthesis and the biophysical studies. S.F. directed the project, designed the experiments, analysed the data and wrote the paper with T.T.

Competing financial interests

The authors declare no competing financial interests.

Figure Legends

Figure 1. Design strategy of endosomolytic peptides and their selection. (a) Design concept and working hypothesis of endosomolytic peptides. A cationic, amphiphilic peptide with strong membrane lytic activity was selected as a design template of endosomolytic peptides (panel [1]). Its lytic activity was attenuated by insertion of negatively charged Glu residue(s) into the hydrophobic face while retaining net positive charges of the peptide to facilitate its cell surface adsorption and endocytic uptake (panel [2]). Reduced pH in endosomes may make Glu more protonated and less hydrophilic. Endosomal membranes are abundant in negatively charged lipids, increasing the interaction with cationic, lytic peptides (panel [3]). Thus, the peptide preferentially perturbs endosomal membranes to attain the cytosolic release of antibodies with sufficient efficacy to target cytosolic proteins [4]. Note that although the endosomolytic peptide obtained in this study (L17E) turned out to have a low helical structure, peptides were illustrated in this figure to promote an understanding of the design concept. Helical wheel projection of L17E is given in panel 2. (b) Marked cytosolic distribution of Alexa488-dextran (10 kDa) facilitated by L17E, indicating the fidelity of our design concept. HeLa cells were treated with Alexa488-dextran (10 kDa) (250 $\mu\text{g/mL}$) in the presence of peptides (40 μM) in $\alpha\text{-MEM}(-)$ for 1 h, prior to washing with and incubation in $\alpha\text{-MEM}(+)$ for 3 h. The inset in each picture represents a magnified view of the boxed area. Scale bars =100 μm .

Figure 2. Cytosolic delivery of bioactive proteins using L17E. (a) Enhanced cytosolic effect of a ribosome inactivation protein (saporin) by co-administration with L17E.

HeLa cells were treated with saporin (10 $\mu\text{g/mL}$) in the presence of L17E (40 μM) in $\alpha\text{-MEM(-)}$ for 1 h, prior to washing with and incubation in $\alpha\text{-MEM(+)}$ for 6 h. Then, the cell viability was analysed using the WST-1 assay. (b) Cre/LoxP recombination assay system and successful gene targeting via intracellular delivery of Cre recombinase with the help of L17E. Cells were treated with Cre protein (5 μM) in the presence of L17E peptide (40 μM) for 1 h prior to protein washout and incubation for another 24 h.

Figure 3. Targeting cytosolic proteins via intracellularly delivered antibodies using L17E. Efficient cytosolic release of IgGs (a) and targeting of membrane-anchored proteins (b) in the presence of L17E. (a) Cells were treated with Alexa488-labeled human IgG (1 mg/mL) in the presence of L17E (40 μM) in $\alpha\text{-MEM(-)}$ for 1 h. After cell wash, cells were further incubated in $\alpha\text{-MEM(+)}$ for 30 min, prior to CLSM analysis. In (b), (i) significant co-localisation of monoclonal anti-His₆-IgG (labelled with FITC) with mem-DsRed-His₆, anchored on plasma membranes, was observed upon incubation with L17E (upper panels). The successful targeting of the cytosolic protein by IgG suggests that a significant fraction of the IgG retained its functional structure even in a reducing cytosolic environment rich in glutathione⁴⁷ (see also reference 48), although IgG is composed of four peptide chains cross-linked via disulphide bridges. In the absence of L17E, anti-His₆-IgG yielded punctate signals, and no significant anti-His₆-IgG signals were observed on plasma membranes co-localised with mem-DsRed-His₆ (lower panels). (ii) Anti-His₆-IgG incubated with cells expressing DsRed-His₆ (without bearing a mem sequence) showed diffuse appearance of Anti-His₆-IgG throughout the cells (without localising on membranes). (c) Blocked signalling and

transcription by intracellularly delivered antibody in the presence of L17E. Cells were incubated with 300 $\mu\text{g/mL}$ anti-glucocorticoid receptor antibody in the presence of L17E (40 μM) in PBS(+) for 30 min at 37°C. Then, the mRNA levels of MT1E mRNA upon stimulation with Dex were analysed. Results are presented as mean $\pm$ standard error (SE) (n=9). **p<0.01, ***p<0.001 (one-way ANOVA followed by Dunnett's post-hoc test). (d) L17E also enhances the efficacy of exosome-mediated intracellular delivery. HeLa cells were treated with exosomes containing saporin for 1 h. Then, FBS was added to the sample and incubated for 48 h. Results are presented as means $\pm$ SE (n=4).

Figure 4. Biophysical study on the perturbation of membranes by L17E. (a) (i) Preferable perturbation of negatively charged membranes by L17E, regardless of pH (liposome leakage assay). (ii) WT M-lycotoxin also shows preferential perturbation of negatively charged membranes, but produces greater perturbation at a neutral pH than at an acidic pH. The membrane perturbation ability of the WT peptide against respective membranes was in the following order: POPC/POPG (3:1) LUVs at pH 7.4 (filled square, $[\text{P/L}]_{50} = 3.3 \times 10^{-4}$) > POPC/POPG (3:1) LUVs at pH 5.0 (open square, $[\text{P/L}]_{50} = 1.8 \times 10^{-3}$) > POPC LUVs at pH 7.4 (filled circle, $[\text{P/L}]_{50} = 7.9 \times 10^{-3}$) >> POPC LUVs at pH 5.0 (open circle, $[\text{P/L}]_{50} > 0.3$). The $[\text{P/L}]_{50}$ value of L17E against POPC/POPG (3:1) LUVs at pH 5.0 (1.2×10^{-2}) (open circle in Fig. 4a-i) was comparable to that of WT against POPC LUVs at pH 7.4 ($[\text{P/L}]_{50} = 7.9 \times 10^{-3}$) (filled circle in Fig. 4a-ii), suggesting that L17E successfully has perturbation ability in endosomes and leads to the cytosolic release of endosomal encapsulates. (b) CD spectra

of M-lycotoxin (open circle) and L17E (filled circle) in 10 mM MES containing 150 mM NaCl in the presence of 2.67 mM POPC/POPG (3:1) LUVs at pH 5.0 (i) and 7.4 (ii) (P/L ratio, 1.5×10^{-2}), and 1 mM POPC (100%) at pH 7.4 (iii) (P/L ratio, 2.0×10^{-2}), respectively. CD spectra of L17E suggest that the peptide has only a very weak helical structure. CD spectra of WT, Q21E, L17E, L9E, and L17E/Q21E in the presence of POPC/POPG (3:1) LUVs at pH 5.0 and 7.4 are also given in Supplementary Fig. 15b.

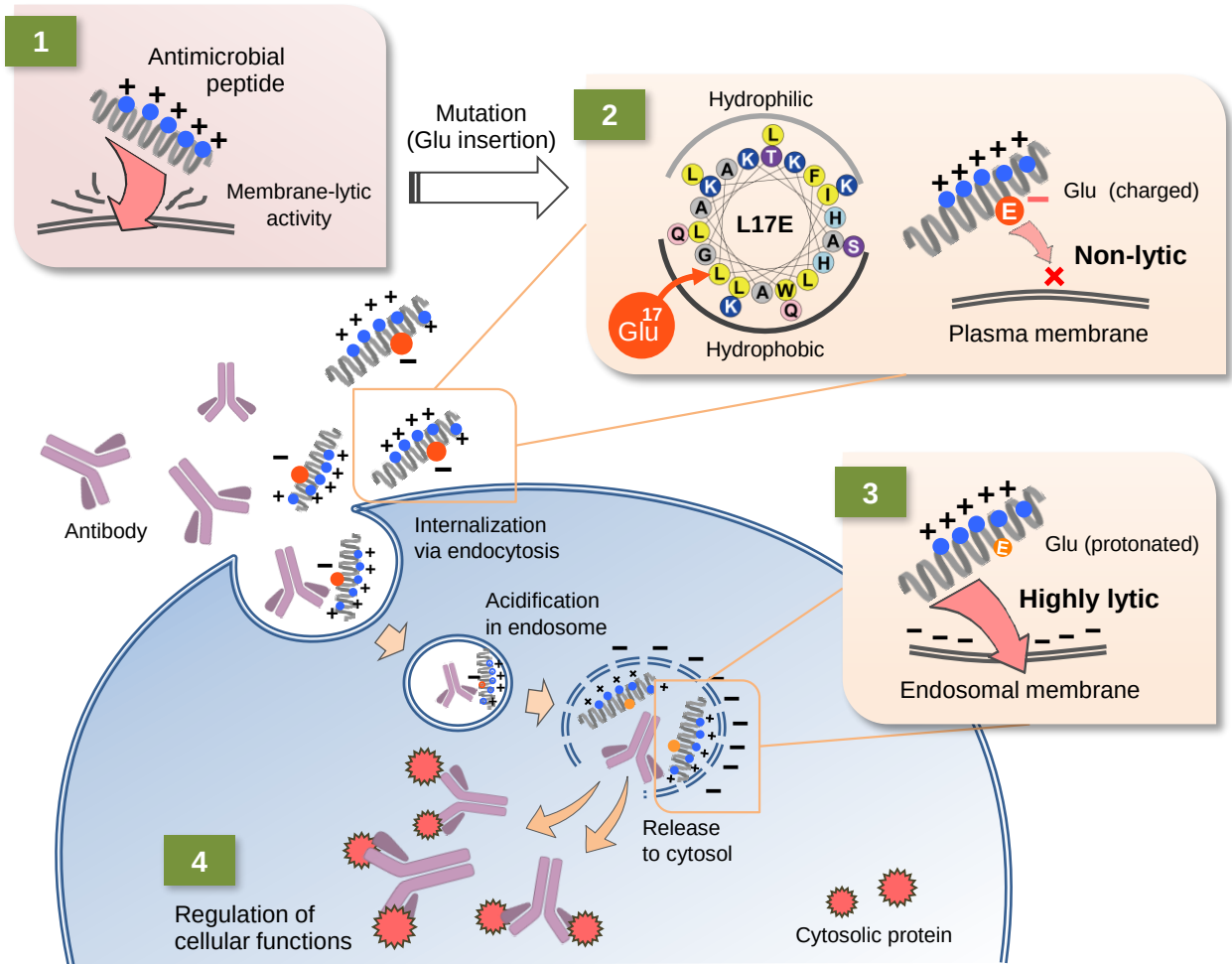
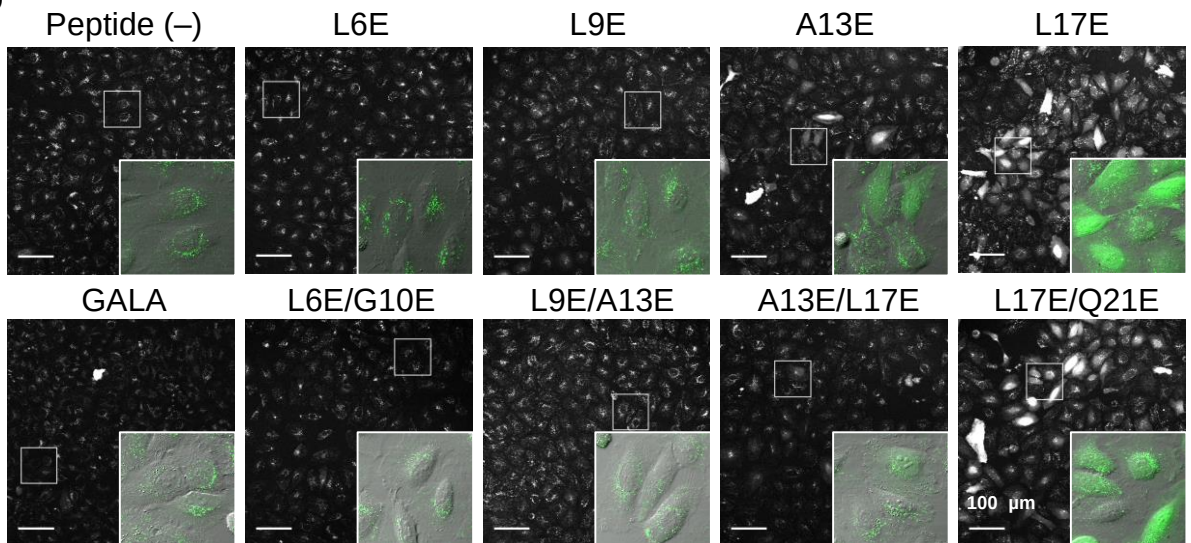
Figure 5. Inhibitor studies: L17E has a physiological effect to promote cellular uptake by inducing macropinocytosis. (a) Absence of diffuse cytosolic appearance of Alexa488-dextran (10 kDa) when cells were treated at 4°C for 30 min in the presence of L17E, suggesting the involvement of endocytosis in L17E-mediated cytosolic delivery. (b) Decrease in the numbers of cells showing diffuse cytosolic Alexa488-dextran signals by the treatment of (i) metabolic (NaN_3) and (ii) macropinocytosis (EIPA) inhibitors. Results are presented as mean $\pm$ SE (n=3). ***p<0.001 ((i) unpaired Student's *t*-test, (ii) unpaired Student's *t*-test with Welch's correction). (c) Stimulated Alexa488-dextran (10 kDa) uptake by the treatment of cells with L17E in serum-free (i) and serum-supplemented (ii) α -MEM for 1 h. Results are presented as mean $\pm$ SE (n=3). *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA followed by Dunnett's post-hoc test). (d) Increased exosome uptake by L17E. HeLa cells were treated with exosomes containing FITC-labelled dextran in α -MEM(–) for 1 h and then in α -MEM(+) for 11 h. Results are presented as mean $\pm$ SE (n=3). **p<0.01, ***p<0.001 (one-way ANOVA followed by Dunnett's post-hoc test). (e) Proposed mechanism of action of L17E: (i) Insertion of a Glu residue in the M-lycotoxin sequence led to attenuation of the membrane-lytic

activity. (ii) Induction of macropinocytosis by the peptide led to enhanced cellular uptake of L17E and biomacromolecules (e.g., antibodies). (iii) L17E showed lipid-sensitive disruption of endosomal membranes. However, different from conventional endosomolytic peptides and polymers, L17E had no apparent pH-sensitivity in membrane lysis. (iv) Antibodies and other proteins were effectively released into the cytosol with the help of L17E to have their bioactivities and functions.

Table 1. Sequences of M-lycotoxin and its analogues

Peptide	Sequence
WT	IWLTALKFLGKHA AKHLAKQQLSKL-amide
L6E	IWLTA EKFLGKHA AKHLAKQQLSKL-amide
L9E	IWLTALKF EGKHA AKHLAKQQLSKL-amide
A13E	IWLTALKFLGKH EAKHLAKQQLSKL-amide
L17E	IWLTALKFLGKHA AKHEAKQQLSKL-amide
Q21E	IWLTALKFLGKHA AKHLAKQ ELSKL-amide
L6E/G10E	IWLTA EKFL EKHA AKHLAKQQLSKL-amide
L9E/A13E	IWLTALKF EGKHE AKHLAKQQLSKL-amide
A13E/L17E	IWLTALKFLGKH EAKHEAKQQLSKL-amide
L17E/Q21E	IWLTALKFLGKHA AKHEAKQ ELSKL-amide
D-L17E	iwltalkflgkhaakheakqqlskl-amide
L17D	IWLTALKFLGKHA AKHD AKQQLSKL-amide
L17EΔ(23-25)	IWLTALKFLGKHA AKHEAKQQ L-amide
L17EΔ(20-25)	IWLTALKFLGKHA AKHEAK -amide

(Lowercase letters denote D-amino acids)

a**b****Figure 1**

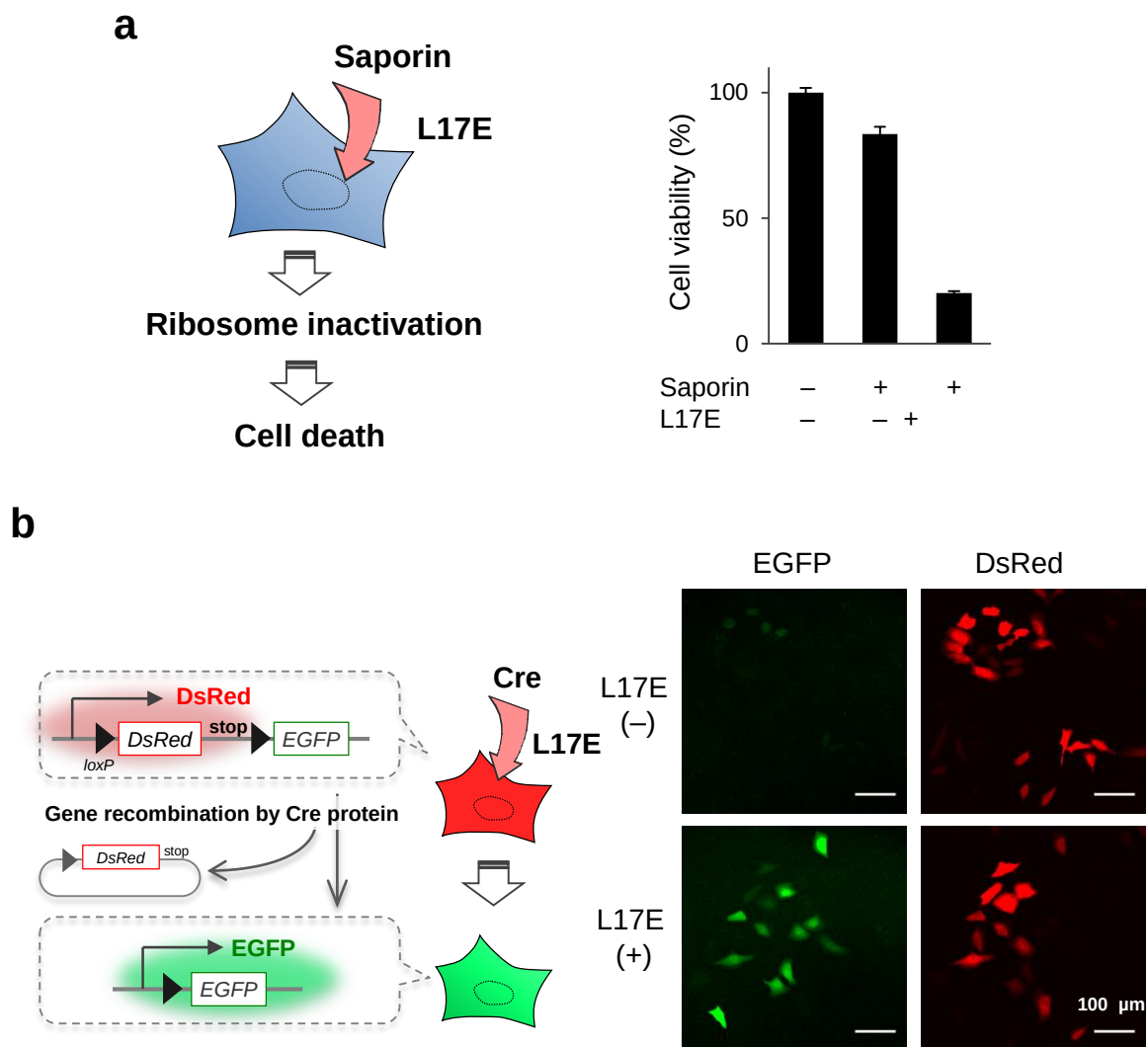


Figure 2

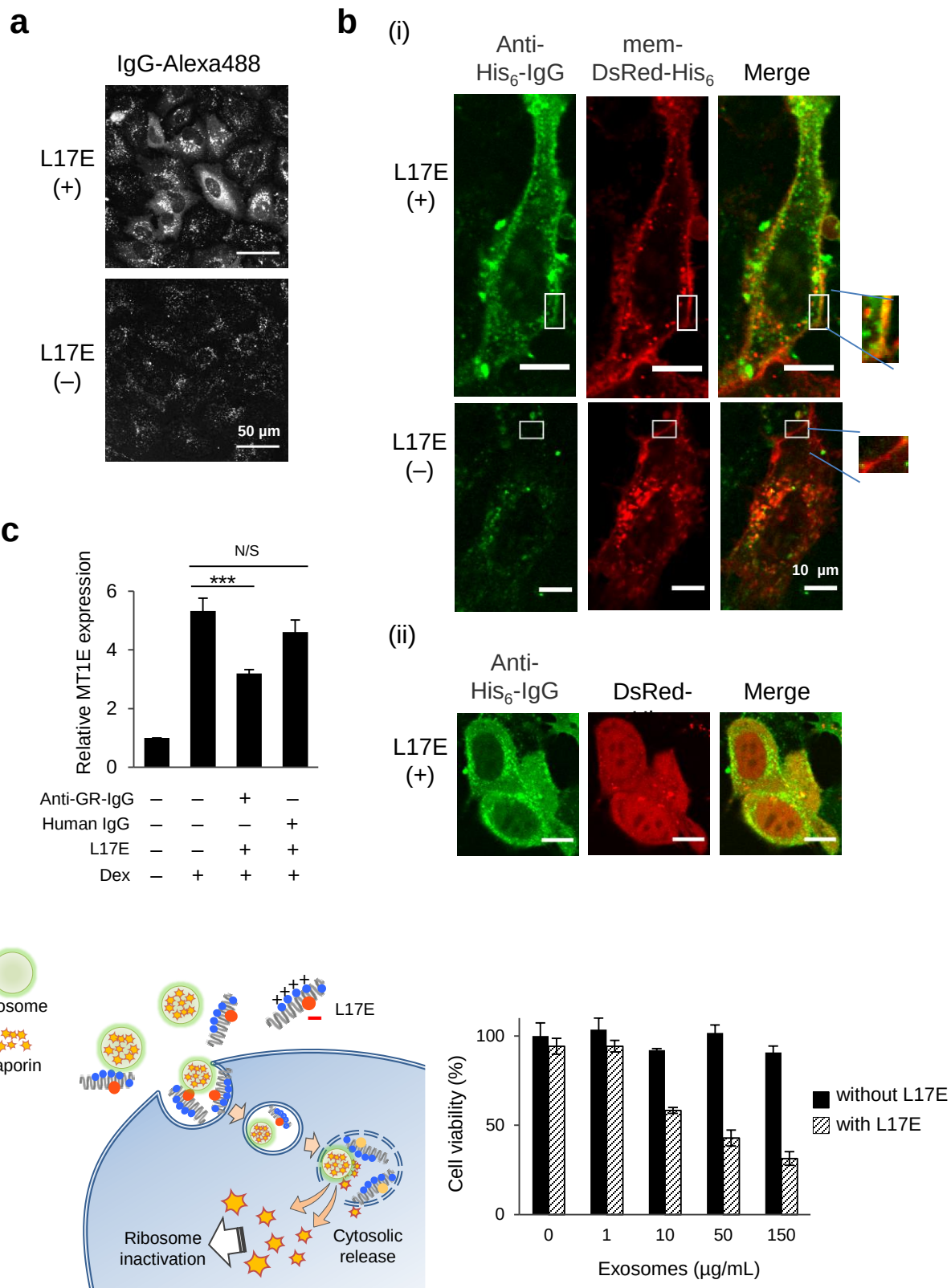
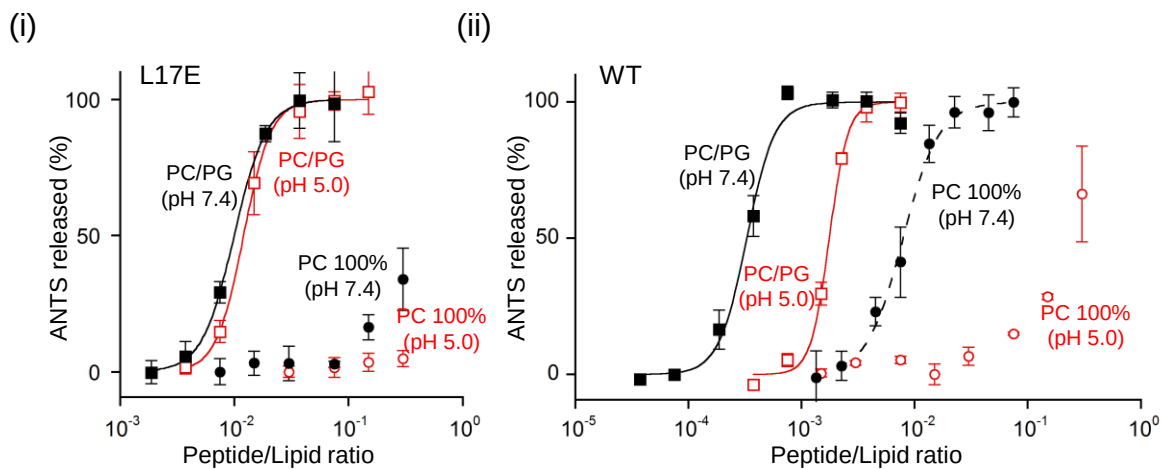
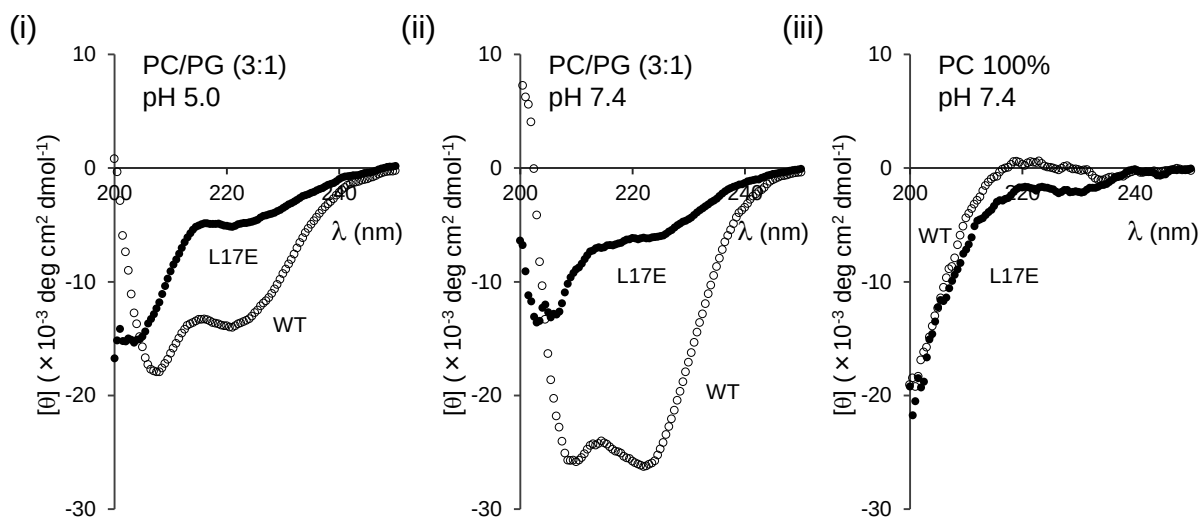


Figure 3

a**b****Figure 4**

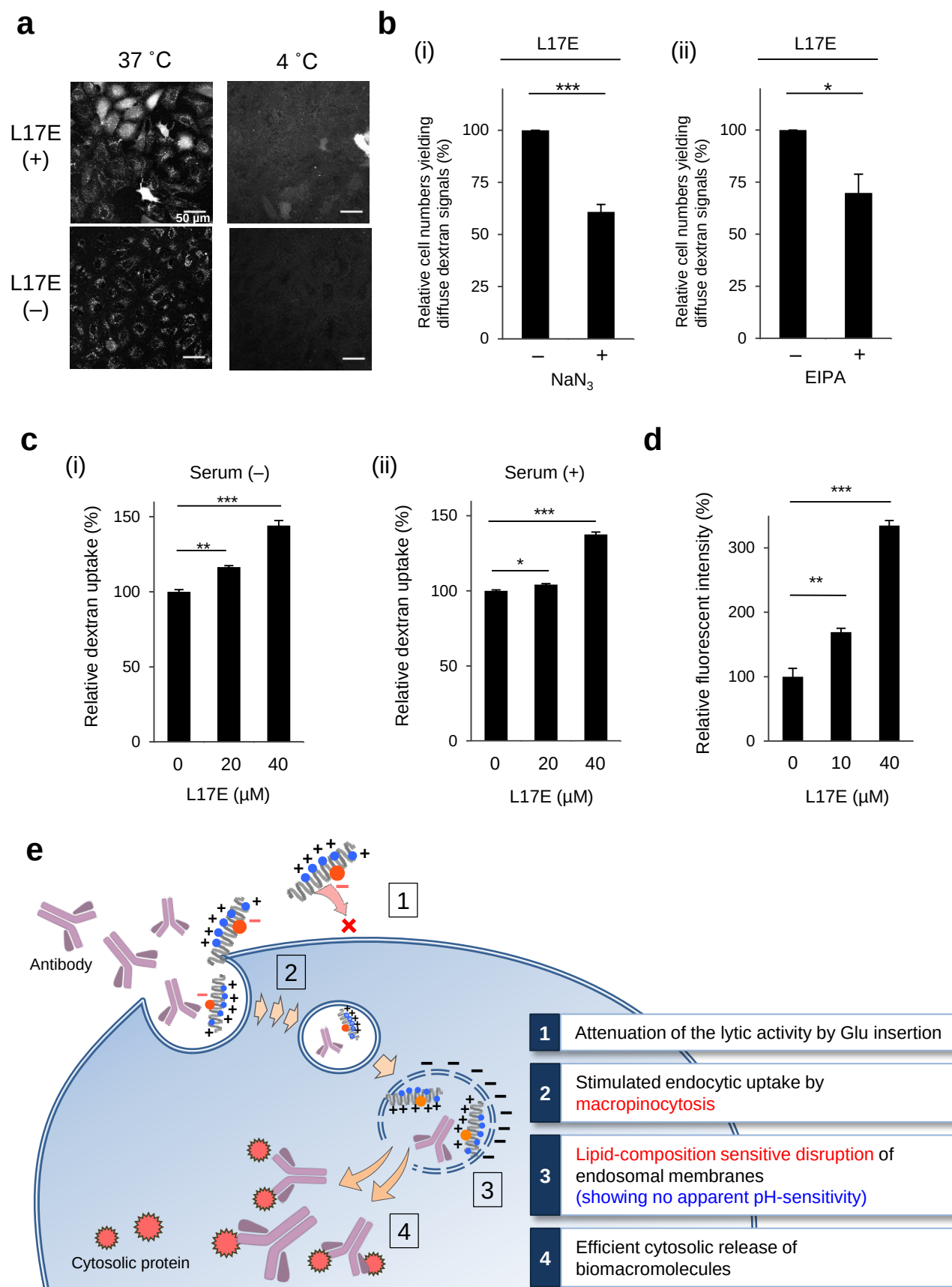


Figure 5