

**An Amphipathic Cell Penetrating Peptide Aids Cell Penetration of
Cyclosporin A and Increases Its Therapeutic Effect in an *in Vivo* Mouse
Model for Dry Eye Disease**

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Table of contents

I. Experimental procedure

Peptide synthesis.

Binding affinity measurement.

Dynamic Light Scattering (DLS) and size exclusion analysis (SEC).

Parallel membrane permeability assay (PAMPA).

Synthesis of 5-TAMRA-CsA and NF-CsA.

Cell line and cell culture.

Flow Cytometry.

Confocal microscopy.

Measurement of mRNA levels of cytokines in HCE-2 by qRT-PCR analysis.

Mouse model of dry eye and experimental procedures.

II. Supporting Figure:

Figure S1. HPLC chromatograms and mass spectrometry data of 5-TAMRA-CsA and NF-CsA.

Figure S2. Job's plot analysis of binding of CsA and 5-TAMRA-labeled LK-3.

Figure S3. Increase of CsA cell uptake by LK-3.

Figure S4. Cytotoxicity of CsA/LK-3 in immune-stimulatory HCE-2 cells.

Figure S5. Efficacy of CsA/LK-3 *in vivo* mouse model for DED.

Figure S6. Concentration dependent cell penetration of CsA in the absence and presence of LK-3 in HCE-2 cells.

Figure S7. HPLC chromatogram of CsA.

III. Supporting Table:

Table S1. Peptide sequences and mass spectrometry data.

Table S2. Primers used for real time PCR

EXPERIMENTAL PROCEDURE

Peptide synthesis. Peptides, LK-3 and LK-2 (reduced Cys monomer of LK-3), were synthesized using previously described procedures.¹

Binding affinity measurement. Fluorescence intensities were measured using an LS-55 fluorescence spectrometer (Perkin Elmer). Fluorescence emission spectra of 100 nM of 5-TAMRA-labeled LK-3 were obtained in the range of 560 to 650 nm (slit width 7.0 nm) by excitation at 550 nm (slit width 3.5 nm) using 200 nm/min scan speed. Measurements were performed in PBS buffer. Fluorescence intensity changes at 580 nm were plotted against concentrations of CsA. The concentrations of CsA are 0, 200, 400, 600, 800, 1000, 3000, 5000, and 15000 nM, respectively. The binding constant (K_d) value was calculated by using global fitting using a one-site-specific binding model in Prism 6.

Dynamic Light Scattering (DLS) and size exclusion analysis (SEC). The hydrodynamic diameter of complex of CsA and LK-3 was determined by using DLS. Measurements were acquired with Zetasizer Nano-ZS (Malvern Instruments). Size exclusion analysis was performed using bio SEC-5 column (5 μ m, 150 \AA , 7.8 \times 300 mm) with HPLC (Agilent HPLC 1260 series instrument) system. Sodium phosphate buffer (150 mM, pH 7.4) was used as the mobile phase. The flow rate was set as 1.0 mL/min.

¹H-NMR experiment. Solutions of CsA (2 mM) in CD₃CN and LK-3 (0.5 mM) in D₂O were prepared and each solution was clearly soluble at RT. The same volume of each solution was combined and confirmed that the mixture was transparent. CsA (1 mM) in CD₃CN/ D₂O (1:1) solution was also prepared as a control. ¹H-NMR spectra were recorded at 500 MHz on a AVANCE™ III (Bruker, German). Spectra were aligned and overlaid using TopSpin (version 4.06) software.

Water solubility of CsA. Solubility of CsA was measured at RT, following the method described in the previously published paper² with slight modifications. Saturated CsA solutions were prepared in 100 μ L of distilled water in the presence and absence of LK-3 (200 μ M) or LK-2 (400 μ M). Each sample was centrifuged for 10 min at 13,000 rpm and 50 μ L of the

supernatant was carefully transferred to a sample vial. The concentrations of supernatants were analyzed using a HPLC system (Agilent 1260 series). The sample injection volume was 20 μL . Calibration curve and experimental conditions are shown in Figure S7.

Parallel membrane permeability assay (PAMPA). Corning Gentest Pre-Coated PAMPA Plate system was used following the manufacturer's protocol with slight modifications. An artificial membrane was prepared by loading lecithin solution on membrane supports, because the LK-3 is retained on the PVDF membrane and most of the solute in the receptor well is transferred to the donor well by osmotic pressure. A 1% (w/v) solution of lecithin (TCI, 51779-95-4) in dodecane (Sigma, 112-40-3) was prepared and sonicated for 5 min before use. The dodecane solution (5 μL) was carefully added to the membrane supports in the bottom of the acceptor plate without contacting the pipet tip to the membrane. A CsA solution was prepared at 50 μM in 5% DMSO/PBS (pH 7.4) and loaded with 300 μL of solution on the donor plate in the presence and absence of 100 μM of LK-3. The acceptor plates were prepared by adding of 5% DMSO/PBS (200 μL to each well). The acceptor plate was placed on the donor plate slowly and capped. The entire plate was placed in a container with a wet paper to prevent solvent evaporation and incubated at R.T for 16 h. The concentrations of CsA of each well were calculated using HPLC traces. The obtained P_e value of CsA is compatible to the reported one previously.³ Calibration curve and experimental conditions are shown in Figure S7.²

Synthesis of 5-TAMRA-CsA and NF-CsA. 5-TAMRA conjugated CsA was synthesized by using rink amide MBHA resin (Merckmillipore). Briefly, 20 mg of Rink amide MBHA resins (0.52 mmol/g, 0.010 mmol) were swelled using *N,N*-dimethylformamide (DMF, 2 mL) for 5 min at RT. Fmoc was deprotected using 20% piperidine in DMF (2 mL) via microwave irradiation using a programmed protocol (5 W, 35 $^{\circ}\text{C}$, ramping time for 1 min and holding for 2 min) on SPS Microwave Peptide Synthesizer (Discover, CEM). Then, resins were washed with DMF and methylene chloride. To a Fmoc-deprotected resin was added a solution of Fmoc-Cys(Mmt)-OH (6 eq), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 6 eq), and diisopropylethylamine (DIPEA, 6 eq) in DMF (2 mL) and amide coupling reaction was performed via microwave irradiation using a programmed protocol (5 W, 35 $^{\circ}\text{C}$, ramping time for 2 min and holding for 5 min) on SPS Microwave Peptide Synthesizer. Completion of the reaction was confirmed by TNBS test. Fmoc deprotection was carried out

as described above. Then, to Cys coupled resins was added a solution of 5-Carboxytetramethylrhodamine (5-TAMRA) fluorescence dye (Merckmillipore, 1.2 eq), *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, 1.2 eq), Hydroxybenzotriazole (HOBt, 1.2 eq), and DIPEA (2.4 eq) in DMF (1 mL) and agitated continuously for 2 h at RT. For fluorescence NF conjugation, a solution of 5-(6)-carboxynaphthofluorescein N-succinimidyl ester (Carbosynth, 1.2 eq) and DIPEA (2.4 eq) in DMF was added and agitated for 2 h at RT. CsA chloroacetic acid ester (**1**) was prepared using previously reported procedure.⁴ On resin S_N reaction was performed by deprotection of Mmt group and followed by substitution reaction with **1** but only failed. 5-TAMRA-Cys-NH₂ and NF-Cys-NH₂ were separately cleaved from resins with 1 mL of cleavage cocktail (trifluoroacetic acid (TFA) : triisopropylsilane (TIS) : H₂O = 95 : 2.5 : 2.5). Each of the crude product was precipitated by diethyl ether and HPLC purified (2.3 mg, y. 40%). 5-TAMRA-Cys-NH₂ (2.3 mg) was dissolved in 0.2 mL of DMF. To 5-TAMRA-Cys-NH₂ solution in DMF was added a solution of CsA chloroacetic acid ester (**1**, 11 mg, 2 eq), NaI (1.3 mg, 2 eq), and 17 μL of 0.5 M tris(2-carboxyethyl)phosphine (TCEP, 2 eq) and the reaction mixture was stirred overnight at 60 °C. 5-TAMRA conjugated CsA was purified using HPLC (1.8 mg, y. 23%). NF-Cys-NH₂ (2.8 mg) was dissolved in 0.2 mL of DMF. To NF-Cys-NH₂ solution in DMF was added a solution of CsA chloroacetic acid ester (**1**, 12.4 mg, 2 eq), NaI (1.5 mg, 2 eq), and 19 μL of 0.5 M tris(2-carboxyethyl)phosphine (TCEP, 2 eq) and the reaction mixture was stirred overnight at 60 °C. NF conjugated CsA was purified using HPLC (1.5 mg, y. 17%). HPLC chromatograms and MALDI-MS data are shown in Figure S1. The concentration of each fluorescence conjugated CsA DMSO stock solutions was determined by measuring the absorbance of the solutions at 555 nm in water and 598 nm in 100 mM Tris HCl (pH 10.0), respectively ($\epsilon_{555} = 89,000 \text{ M}^{-1}\text{cm}^{-1}$ for 5-TAMRA-CsA and $\epsilon_{595} = 49,000 \text{ M}^{-1}\text{cm}^{-1}$ (Buffer pH 10.0) for NF-CsA).

Cell line and cell culture. HCE-2 cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in EpiLife® Medium containing 10% of Human Corneal Growth Supplement (HCGS) and 1% penicillin/streptomycin/amphotericin B (all from ThermoFisher Scientific) and incubated at 37 °C in a humidified atmosphere with 5% CO₂. *In vitro* stimulation of HCE-2 cells was performed with 25 μg/mL of poly (I:C) (Sigma P9582) and 1 μg/mL of LPS (Sigma L3129).

Flow Cytometry. HeLa (or HCE-2) cells (5.0×10^4 cells/well) were seeded on 24 well plates and incubated for 24 h. Cells were incubated further in the presence of 5-TAMRA-CsA or NF-CsA for 2 h, then harvested using trypsin-EDTA solution and suspended in 0.3 ml PBS to measure the fluorescence intensities (PI filter for 5-TAMRA and FITC filter for NF) by a flow cytometer (BD Accuri™ C6, BD Bioscience).

Confocal fluorescence microscopy

Imaging of live HeLa cells treated with 5-TAMRA-CsA. HeLa cells (2.0×10^4 cells/well) were grown on 8 well Lab-Tek chamber slides (Thermo). Each fluorescence of 5-TAMRA dye and Hoechst 33342 was visualized using a Confocal LSM 710 system (Zeiss) equipped with argon and helium-neon lasers (Carl Zeiss). Images were captured using AxioCam camera equipped with the appropriate wavelength of filters and using identical settings and exposure times.

Immunostaining of fixed HeLa cells treated with CsA. HeLa cells grown on a 8 well chamber slide (Thermo) were pretreated with 400 nM of CsA in the presence and absence of LK-3 for 2 h and fixed by adding 4% paraformaldehyde (PFA) solution in PBS. After fixation, the cells were permeabilized with 0.2% Triton X-100 for 10 min. After blocking for 30 min at room temperature with 1% Bovine Serum Albumin (BSA) in PBS containing 0.1% Tween 20, cells were incubated overnight at 4 °C with rabbit anti-cyclosporin A (369494, US Biological Life Sciences, 5 µg/mL in PBS containing 0.1% Tween 20 and 1% BSA,) as primary antibody and Texas Red® conjugated mouse anti-rabbit IgG (sc-3917, Santa Cruz Biotechnology, 1:100 diluted in PBS containing 0.1% Tween 20 and 1% BSA) for 1 h at room temperature. Nuclei were stained with 1 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) in PBS containing 0.1% Tween 20 and 1% BSA for 10 min. Fluorescence images were analyzed using a confocal laser-scanning microscope (Confocal LSM 710 system (Zeiss)).

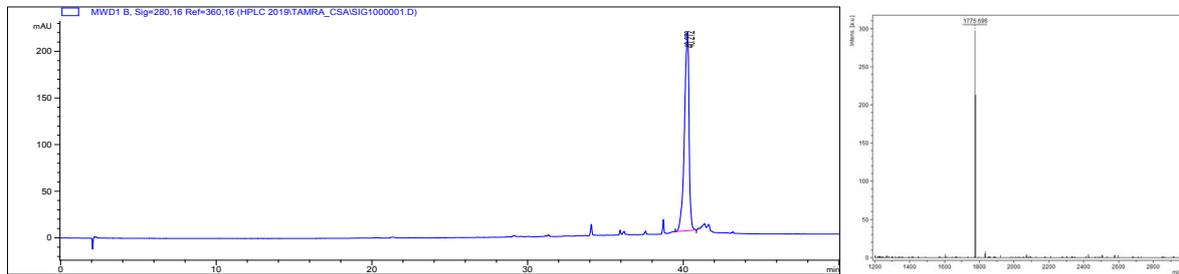
Measurement of mRNA levels of cytokines in HCE-2 by qRT-PCR analysis. mRNA expressions of IL-6 and IL-8 were assessed by RT-PCR. RNA preparation, cDNA synthesis and quantitative real-time PCR were performed as described previously.⁵ Gene of GAPDH was used as the control. The sequences of primer sets were shown in Table S2.

Mouse model of dry eye and experimental procedures. All mice were used in guidelines approved by the Chonnam National University Medical School Research Institutional Animal

Care and Use Committee. All experimental procedures are the same as those reported previously.^{6, 7} The protocol used for the animal experiments was approved by the Research Institutional Animal Care and Use Committee of Chonnam National University Medical School (CNU IACUC-H-2018-46).

Figure S1. HPLC Chromatograms and MS spectra of the purified CsA derivatives. Fluorescence derivatives were confirmed with using RP-HPLC (Agilent HPLC 1260 series instrument). A Zorbax C₁₈ column (3.5 μm, 4.6 × 150 mm) was used as the stationary phase. For the mobile phase, buffer A (water with 0.1% v/v TFA) and buffer B (MeOH with 0.1% v/v TFA) were used as a gradient. The gradient conditions are as follows: 5 min, 5% B followed by linear gradient 5-70% B over 25 min, 70-100% B over 15 min, and, 10 min, 100% B. The flow rate was 1.0 mL/min. MS of peptides were measured using a Voyager™ MALDI-TOF mass spectrometer (Applied Biosystems).

5-TAMRA-CsA (>90% purity), MS [M+H]⁺: 1774.98 (calcd), 1775.70 (obsd).



NF-CsA (>95% purity), MS [M+Na]⁺: 1842.90 (calcd), 1844.36 (obsd).

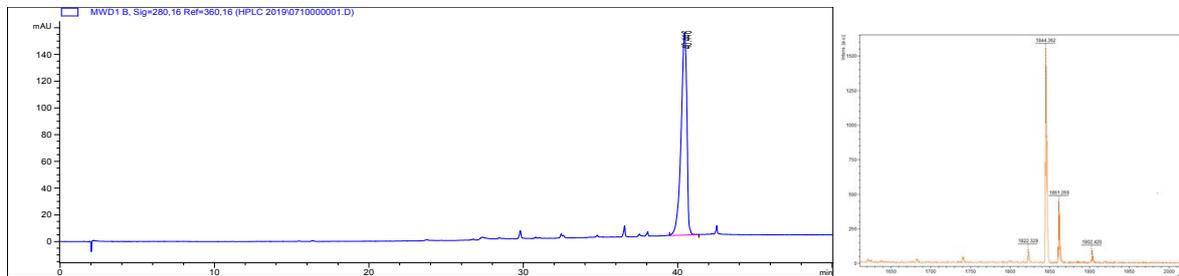


Figure S2. Job's plot analysis of binding of CsA and 5-TAMRA-labeled LK-3. The data shows 1:1 apparent binding.

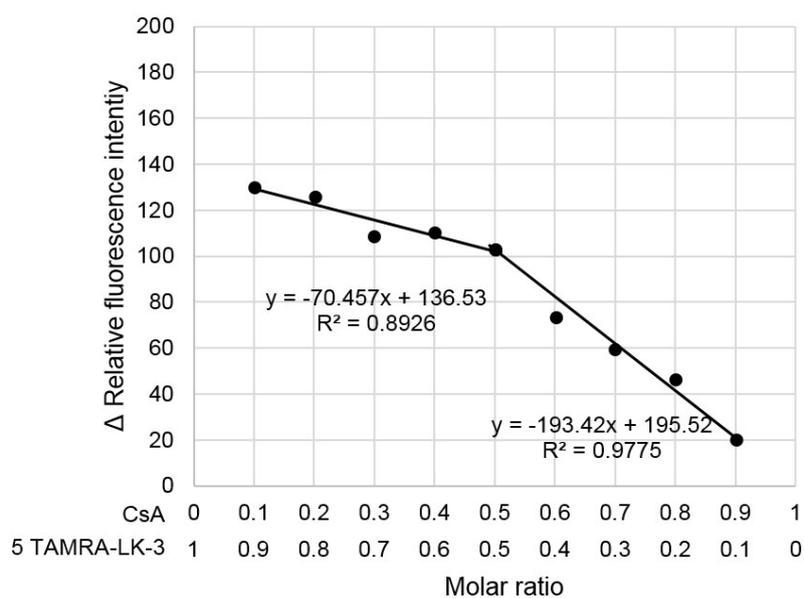


Figure S3. Increase of CsA cell uptake by LK-3. a) Flow cytometry of cell penetrating efficiency of 5-TAMRA-CsA. HeLa cells were incubated with 5-TAMRA-CsA at various concentrations in the absence (●) and presence (■) of 750 nM of LK-3. The fluorescence intensities of the cells were measured and the percentage of fluorescence positive cells were plotted against the concentrations of 5-TAMRA-CsA incubated with cells. The percentage of fluorescence positive cells was determined by placing a horizontal marker on the right side of the control histogram plot (Fig. 4a) using a software (BD Accuri™ C6 software 1.0.264.21) b) Fold increase of concentration dependent CsA HeLa cell uptake by LK-3. Fold increase in cell uptake of CsA was calculated by dividing the mean fluorescence intensity of cells in the presence of 750 nM of LK-3 divided by that in the absence of LK-3. c) Cell penetration of 5-TAMRA-R9 in the presence of endocytosis inhibitors as inhibitor control experiments. Cells were pre-incubated at 37 °C for 30 min with 5 µg/mL of chlorpromazine (CPZ), 25 µg/mL of nystatin (Nys), or 15 µg/mL of 5-(N-ethyl-N-isopropyl)amiloride (EIPA). After 30 min incubation with inhibitors, cells were incubated with 1 µM of 5-TAMRA-R9 for 2 h. **P ≤ 0.01; ***P ≤ 0.001; NS, not significant compared to control. Data show that the inhibitions of R9 cell penetration in the presence of endocytosis inhibitors are similar to the previous publication.⁸ d) Mean fluorescence intensity (MFI) of the cells incubated with NF-CsA. e) Mean fluorescence intensity (MFI) of the cells incubated with 5-TAMRA-CsA. f) Confocal laser scanning microscope (CLSM) images of live HeLa cells treated with 5-TAMRA-CsA (400 nM, red) in the presence and absence of LK-3 (750 nM) or EIPA (15 µg/mL). The nucleus was stained with Hoechst 33442 (blue). g) Immunocytochemistry of HeLa cells treated with CsA (400 nM) in the presence and absence of LK-3 (750 nM), immunostained with anti-CsA antibody (red). DMSO was used as a negative control for immunostaining. Nucleus was visualized by DAPI. Scale bar = 10 µm.

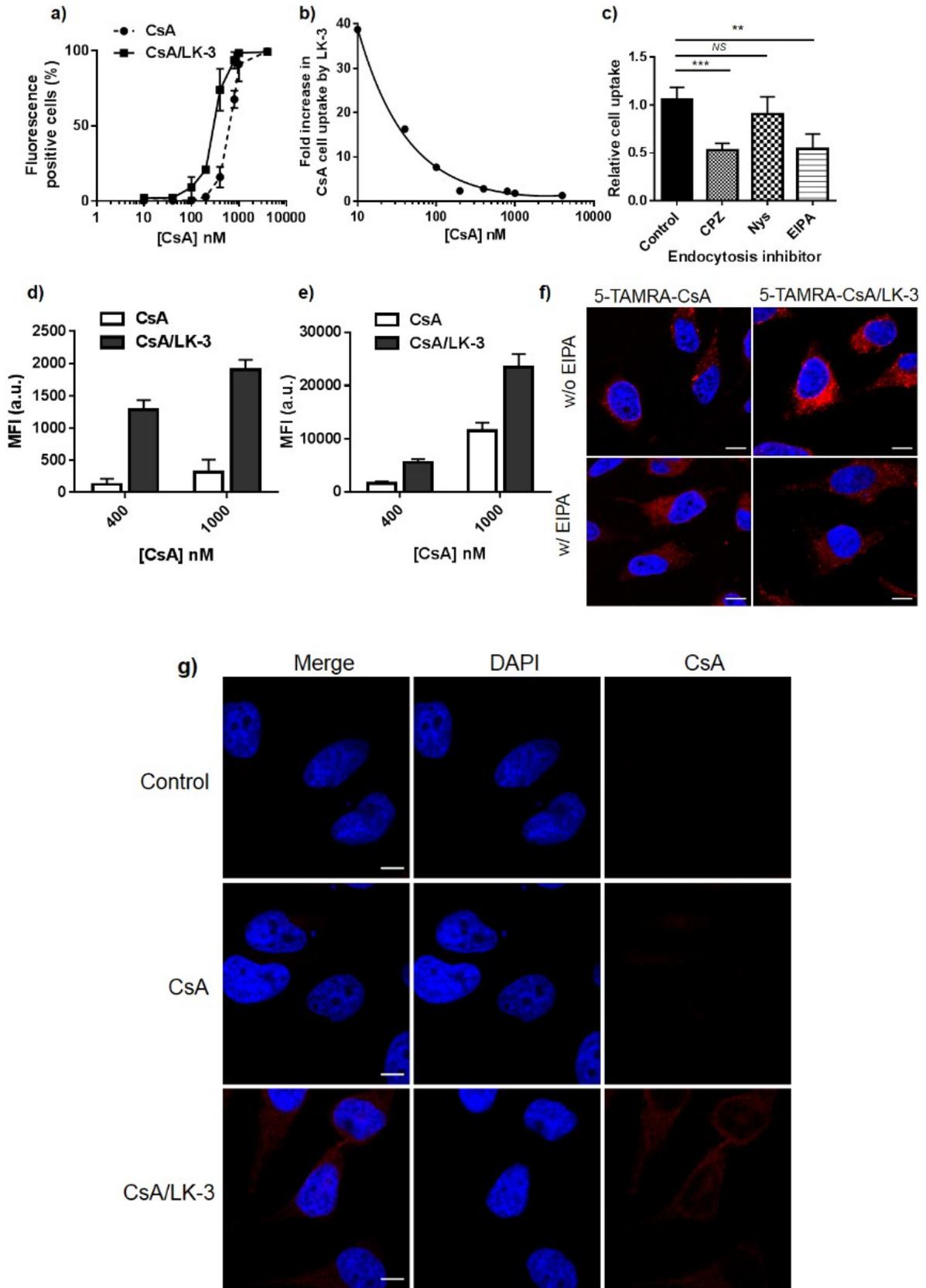


Figure S4. Cytotoxicity of CsA/LK-3 in immune-stimulatory HCE-2 cells (Fig. 5). The trypan blue exclusion method was used to measure cell viability (n = 4). All data show not significant difference from control data confirmed by unpaired Student's t-test.

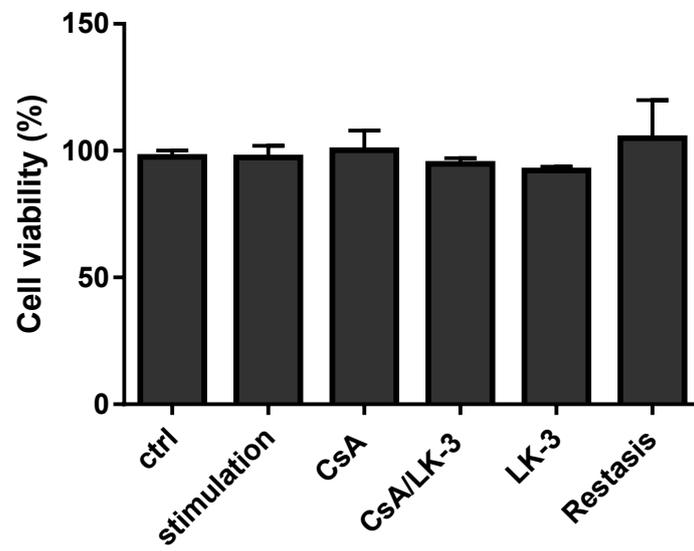


Figure S5. Efficacy of CsA/LK-3 *in vivo* mouse model for DED. a) conjunctival goblet cells (left) and representative figures (right), and b-c) flow cytometry analysis of populations (%) of CD4⁺/CCR5⁺ cells in cornea (b) and conjunctiva (c) are shown. *P<0.05 compared with the experimental dry eye (EDE) group, †P<0.05 compared with LK-3 only group. n = 12 per group.

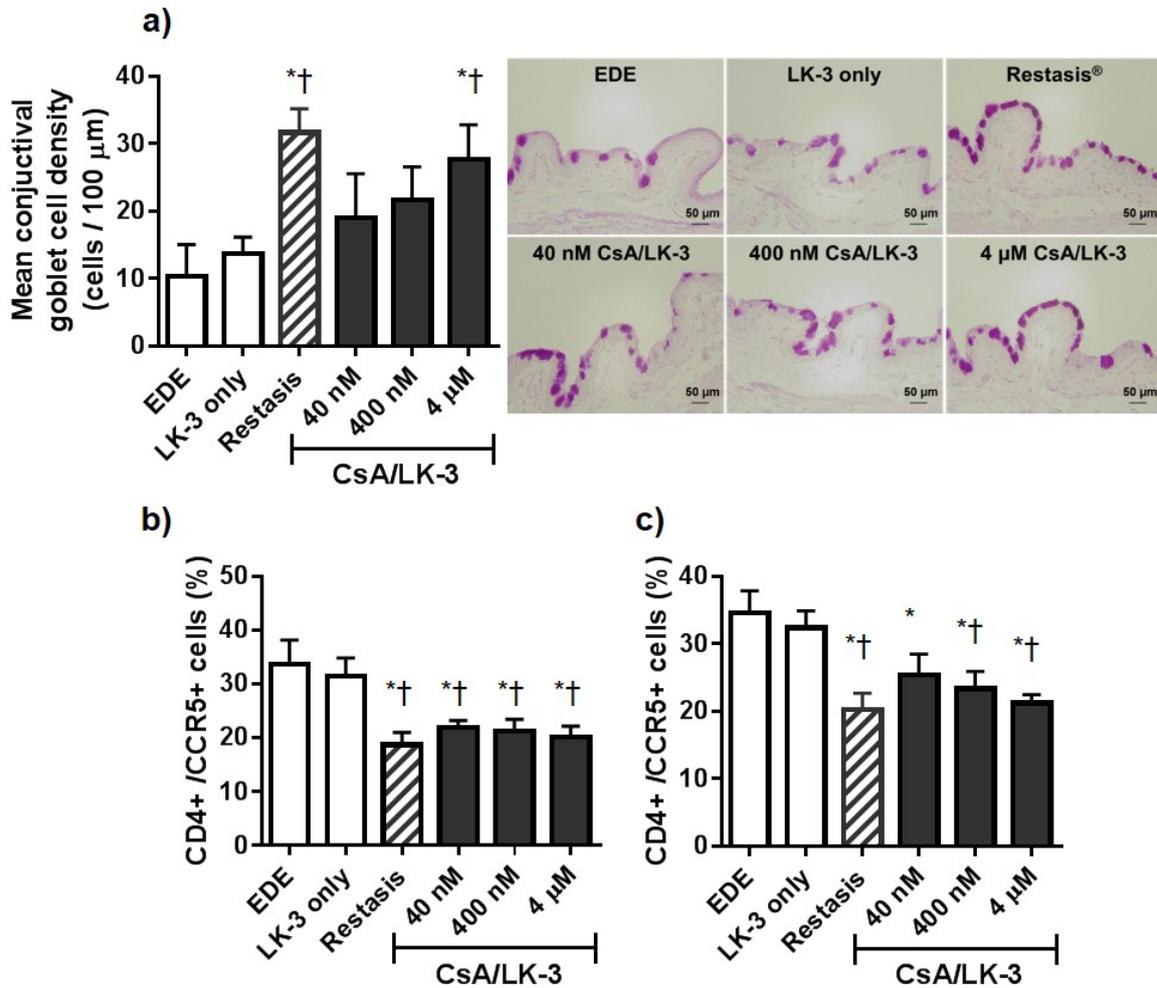


Figure S6. Concentration dependent cell penetration of CsA in the absence (●) and presence (■) of LK-3 in HCE-2 cells.

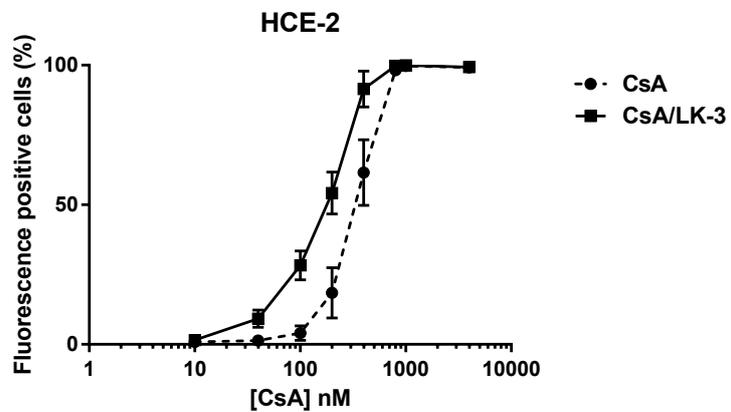


Figure S7. HPLC chromatogram of CsA. RP-HPLC (Agilent HPLC 1260 series instrument) was used equipped with a Zorbax C₁₈ column (3.5 μm, 4.6 × 150 mm) thermostated at 65 °C as the stationary phase. The mobile phase consisted of 64% acetonitrile, 34% water and 3% methanol. The flow rate was 2.3 mL/min. a) The representative chromatogram of CsA. The retention time of CsA is 5.7 min. b) Calibration curve for CsA concentration measurement. The concentration of CsA was measured using the peak area obtained from the HPLC chromatogram.

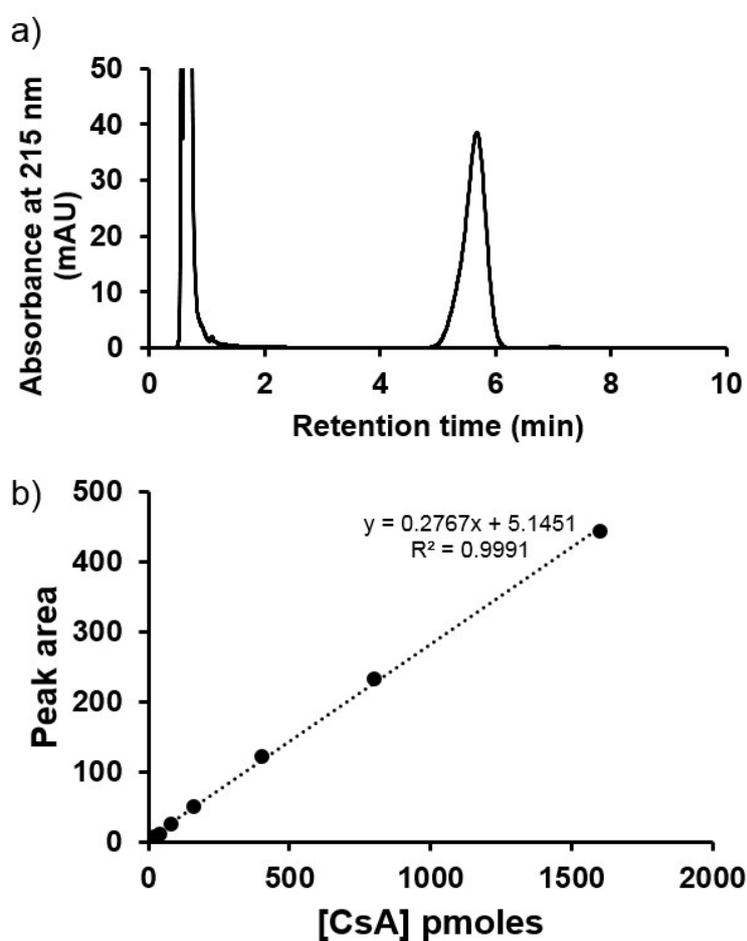


Table S1. Peptide sequences and mass spectrometry data.^[a]

Peptide	Sequence	MS (calcd)	MS (obsd)
LK-2	LKKLCKLLKKLCKAG	1841.2	1840.7
LK-3 ^[b]	(LKKL <u>C</u> KLLKKL <u>C</u> KAG) ₂	3677.4	3678.8

^[a]All mass spectrometric data correspond to [M+H]⁺ peaks. ^[b]Two helices are connected by bis-disulfide bonds.

Table S2. Primers used for real time PCR

Primers	Sequence
IL-6 forward	5'-CCCTGACCCAACCACAAAT -3'
IL-6 reverse	5'- ACATTTGCCGAAGAGCCC -3'
IL-8 forward	5'- AATAGCAAAATTGAGGCCAAGG -3'
IL-8 reverse	5'- GCTAAATTGACTTTATGGCAAATT -3'
GAPDH forward ^[a]	5'-TCGCTCTCTGCTCCTCCTGTTC-3'
GAPDH reverse ^[a]	5'-CGCCAATACGACCAAATCC-3'

^[a]GAPDH is used as a control.

Reference

1. Hyun, S.; Lee, Y.; Jin, S. M.; Cho, J.; Park, J.; Hyeon, C.; Kim, K.-S.; Lee, Y.; Yu, J., Oligomer Formation Propensities of Dimeric Bundle Peptides Correlate with Cell Penetration Abilities. *ACS Central Science* **2018**, *4* (7), 885-893.
2. Ismailos, G.; Reppas, C.; Dressman, J. B.; Macheras, P., Unusual solubility behaviour of cyclosporin A in aqueous media. *J. Pharm. Pharmacol.* **1991**, *43* (4), 287-289.
3. Yang, N. J.; Hinner, M. J., Getting Across the Cell Membrane: An Overview for Small Molecules, Peptides, and Proteins. In *Site-Specific Protein Labeling: Methods and Protocols*, Gautier, A.; Hinner, M. J., Eds. Springer New York: New York, NY, 2015; pp 29-53.
4. Rothbard, J. B.; Garlington, S.; Lin, Q.; Kirschberg, T.; Kreider, E.; McGrane, P. L.; Wender, P. A.; Khavari, P. A., Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. *Nat. Med.* **2000**, *6* (11), 1253-7.
5. Hyun, S.; Choi, Y.; Lee, H. N.; Lee, C.; Oh, D.; Lee, D.-K.; Lee, C.; Lee, Y.; Yu, J., Construction of histidine-containing hydrocarbon stapled cell penetrating peptides for in vitro and in vivo delivery of siRNAs. *Chemical Science* **2018**, *9* (15), 3820-3827.
6. Cheon, Y. I.; Ying, L.; Rujun, J.; Min, A.; Won, C.; Chul, Y. K., Comparison of 0.1%, 0.18%, and 0.3% Hyaluronic Acid Eye Drops in the Treatment of Experimental Dry Eye. *J. Ocul. Pharmacol. Ther.* **2018**, *34* (8), 557-564.
7. Choi, J. H.; Li, Y.; Jin, R.; Shrestha, T.; Choi, J. S.; Lee, W. J.; Moon, M. J.; Ju, H. T.; Choi, W.; Yoon, K. C., The Efficiency of Cyclosporine A-Eluting Contact Lenses for the Treatment of Dry Eye. *Curr. Eye Res.* **2019**, *44* (5), 486-496.
8. Yamashita, H.; Kato, T.; Oba, M.; Misawa, T.; Hattori, T.; Ohoka, N.; Tanaka, M.; Naito, M.; Kurihara, M.; Demizu, Y., Development of a Cell-penetrating Peptide that Exhibits Responsive Changes in its Secondary Structure in the Cellular Environment. *Sci. Rep.* **2016**, *6*, 33003.