Supplementary Material (ESI) for *MedChemComm*

Electronic Supplementary Information

Enhanced *in vitro* and *in vivo* uptake of a hydrophobic model drug coumarin-6 in the presence of cucurbit[7]uril

Xiaoqing Miao,[a] Ye Li,[a] Ian Wyman,[b] Simon M. Y. Lee,[a] Donal H. Macartney,[b] Ying Zheng,[a,*] and Ruibing Wang[a,*]

[a]*State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Taipa, Macao, China*

[b]*Department of Chemistry, Queen's University, Kingston, ON, K7L 3N6, Canada*

Materials and Instrumentation

Madin-Darby canine kidney Ⅱ (MDCK) cell line was obtained from American Type Culture Collection (ATCC). Wild type zebrafish was used as the *in vivo* model. Cucurbit[7]uril, Coumarin-6 (98%), Tween-80, agarose (low gelling temperature), N-phenylthiourea, MS-222 (tricaine methane sulfonate), thiazolyl blue tetrazolium bromide, paraformaldehyde, glucose, NaHCO3, Hank's balanced salt solution (HBSS; powder form), chlorpromazine, methyl-β-cyclodextrin (MβCD), filipin, nystatin, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), brefeldinA, and monensin were purchased from Sigma Aldrich (St. Louis, MO, USA). DMEM culture solution, penicillin, streptomycin, trypsin, EDTA, L-glutamine, phosphate buffered saline (PBS) and fetal bovine serum were supplied by GIBCO (Carlsbad, CA, USA). All of these reagents were of analytical grade or better and used as received.

Fluorescence measurements were performed with 10-mm quartz cells on a Lumina Fluorescence Spectrometer (Thermo Scientific). The fluorescence microscopy of was performed with a Zeiss LSM 710 CLSM (Carl Zeiss, Jena, Germany) equipped with argon laser (488 nm) at 63x oil objective lens. Images were processed using the LSM 710 software. UV-Visible absorption for MTT assay was measured on a UV-visible spectrophotometry (SpectraMax M5 Microplate Reader, Molecular Devices, USA). Imaging of zebrafish was done with fluorescence microscope (Olympus IX81 Motorized Inverted Microscope, Tokyo, Japan) equipped with a digital camera (DP controller, Soft Imaging System, Olympus).

MDCK cell culture

MDCK cells were seeded in high glucose DMEM, supplemented with 10% FBS and 1% penicillin–streptomycin in a humidified atmosphere containing 5% CO_2 at 37 ℃ in 75 cm^2 plastic flasks. The medium was refreshed every 2 days. Cells were passaged at 70%–90% confluency using 0.25% (w/v) trypsin–0.02% (w/v) ethylene diamine tetraacetic acid (EDTA) solution.

For endocytosis and exocytosis studies, MDCK cells were grown in culture flasks containing DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. MDCK cells were digested with 0.25% trypsin/0.02% EDTA and seeded in 12-well sterile plate at 1×10^5/mL.

Cytotoxicity study of CB[7] with MDCK cell line

MDCK cells were seeded in 96-well tissue culture plates (flat bottom, NUNC, Denmark) at a density of 5×10^3 cells in 100 μL of medium per well and incubated for 24 h. Cultures were incubated with different concentrations of CB[7]. After 2.5 h incubation, the medium was replaced with fresh medium containing 1 mg/mL of Thiazolyl Blue Tetrazolium Bromide (MTT) for 1 h. The supernatant was removed, and the MTT formazan crystals were dissolved in 100 μL of DMSO per well. Finally, the plates were shaken for 30 min and the absorbance was measured using a multiplate reader (SpectraMax M5 Microplate Reader, Molecular Devices, USA) at 570 nm. Cell growth was calculated by comparing the absorbance of treated versus untreated cells as control group.

Quantification of C6 taken up by MDCK cells and larval tissues: standard curves

A fluorescence method has been established and validated for quantitative determination of the C6 concentrations in MDCK and larval tissues.

MDCK cell model: MDCK cells were grown in 12-well plate and cells were rinsed with cold PBS for three times. After scraping from plate wells, cells were then collected and ultrasonic 1 hour to form single cell suspensions. Cell suspensions (100 μl) were treated by 100 μl different concentration of C6 dissolved methanol (to precipitate protein in cell). After vortexing for 2 min, the cell suspensions were centrifuged at 13000 rpm for 20 min, the supernatants were set as standard samples. The calibration curve between the peak area ratios of C6 fluorescent intensity (Y) and C6 concentrations (X) ranging from 7.8 to 500 ng/mL($y=3.117x-38.898$, $r^2=0.9988$) exhibited a satisfactory linearity. The quantity of C6 during the C6 uptake experiments with MDCK cell model was calculated by comparing with the standard curve. The experiment was performed three times to obtain S.D.

Zebrafish model: for calibration standards, zebrafish (n=30 per concentration points) were collected, dried, weighted and homogenized in water/methanol (1:1 v/v). After centrifugation at 13000 rpm for 30 min, they were mixed with different concentration of C6 dissolved in water/Methanol (1:1 v/v), respectively. The calibration curve of C6 was satisfactorily linear in the concentration range of 4-800 ng/ml ($y=11.83x+10.56$, $r^2=0.9999$). The quantity of C6 during the C6 uptake experiments with zebrafish model was calculated by comparing with the standard curve. The experiment was performed three times to obtain S.D.

Statistical significance of the results was analyzed using either a two-tailed independent samples t-test or a one-way ANOVA. Values of $p < 0.05$ were considered to be statistically significant.

Detection C6 content in endocytosis process

MDCK cells were grown in 12-well plate and incubated with 300ng/mL of C6@CB[7] complex at 37 ℃ for 5 min, 15 min, 30 min and 60 min, respectively. After that, cells were rinsed with cold PBS for three times to abort endocytosis. Scraped from plate wells, cells were then collected and ultrasonic 1 hour to form single cell suspensions. Methanol was added to the cell suspension to solubilize the C6 and impurities, after vortex for 2 min, cell suspension were centrifuged at 13000rpm for 20min. Then, the mean intracellular fluorescence intensity of

supernatant was measured by fluorescence spectrophotometry. The cellular uptake was expressed as the fluorescence quantification normalized to total cellular protein concentration which was determined by the BCA protein assay method. The results were expressed as the amount (ng) of drug per mg of total cellular protein.

Cellular uptake and intracellular tracking of C6 by CLSM

For the detection of C6 uptake in MDCK cells, 300ng/mL C6 (suspension, tween-80 solubilized, or in the presence of 0.80 mM CB[7]) was added in 12 well plate with cell confluency to be 70%–90% and incubated in dark at 37 ℃ for 5, 15, 30 and 60 min, respectively. After the dispersion was aspirated and the uptake process was aborted by cold HBSS, fixed with PFA (4.0% in PBS pH=7.4) for 15 min at room temperature. Mount coverslip with a drop of mounting medium (glycerin: PBS pH 7.4=1:1) and sealed the coverslip with nail polish to prevent drying and movement under microscope and stored in dark at 4 ℃ until the detection by CLSM.

Mechanisms detection of endocytosis and exocytosis

Pathway studies with endocytosis/exocytosis inhibitors

For the endocytosis pathway detection, various inhibitors listed in Table 1 at the given concentrations were pre-incubated with MDCK cells that seeded in 12-well plate for 30 min. After the aspiration of pre-incubation solutions, 300ng/mL of C6@CB[7] complex HBSS dispersion and different agents with the same concentrations were added and further incubated at 37 ℃ for 30min. The test was aborted by aspirating the dispersions and rinsing cells with cold PBS for three times. Then cells were scraped and centrifuged according to the method of 2.4.2, the supernatant sample was measured by fluorescence spectrophotometry.

Table 1 Regulators and their concentrations used in mechanism study.

Inhibitors	Function	Final concentration
Hypertonic sucrose	Clathrin mediated route	0.45 M
methyl-β-cyclodextrin (MβCD)	Depletion of cholesterol	10 mM
Nystatin	lipid raft/caveolae-mediated route	30 μM
Filipin	lipid raft/caveolae-mediated route	0.5 μg/mL
Monensin	Inhibitor of endosome maturation. Block the transportation of macromolecules from Golgi complex to PM.	25 μM
BrefeldinA	Inhibitors of different transport function of Golgi complex, block transport from ER to Golgi complex	25 μg/mL
Chlorpromazine	Inhibitor of clathrin-mediated endocytosis	30μM
5-(N-ethyl-N-isopropyl)-amiloride(EIPA)	Inhibitor of macropinocytosis	20 μM

In order to evaluate the pathways of exocytosis, the same inhibitors employed in endocytosis process were added. The MDCK cell monolayer incubated with 300ng/mL C6@CB[7] complex at 37 ℃ for 30min, then inhibitors dissolved in HBSS listed in Table 1 were added to re-incubate with cells for another 30min at 37 ℃ during exocytosis process. The intracellular intensities of

C6@CB[7] complex was measured by fluorescence spectrophotometry.

Uptake of C6@CB[7] complex in zebrafish model

Zebrafish Maintenance and breeding

Adult zebrafish were maintained at 26 ℃ in a 10:14 hour light:dark cycle. Embryos were obtained via natural mating and cultured in embryo medium (E3 medium) (13.7 mM NaCl, 540 μM KCl, 25 μM Na2HPO4, 44 μM KH2PO4, 300 μM CaCl2, 100 μM MgSO4, 420 μM NaHCO3, pH 7.4) at 28.5 ℃. In order to inhibit pigment formation, embryos were treated with 1-phenyl-2-thiourea (PTU) from 24 hours post fertilization. Ethical approval for the animal experiments was granted by the Animal Research Ethics Committee, University of Macau, Macau SAR, China.

Imaging of zebrafish incubated with C6

The zebrafish (8 days post fertilization) was incubated with C6@CB[7] complex or C6 solubilized by Tween 80 with different concentration (100, 200, 400, 800 ng/ml) or different time intervals at 28 ℃. After incubation, zebrafish were washed by E3 medium and anesthetized with 0.04% MS-222, then placed in 1% low-melting agarose. The zebrafish were imaged by fluorescence microscopy (Olympus IX81 Motorized Inverted Microscope).

Detection C6 content in larval tissues

After incubation with C6@CB[7] or C6 solubilized by Tween 80, zebrafish (30 live animals per replicate and three replicates per treatment) were washed and collected in the 1.5 ml pre-weighed polypropylene tubes. The whole body dried with silica gel over night. All the samples were weighted and homogenized in 500μL water/Methanol (1:1 v/v). After centrifugation at 13000 rpm for 30 min, the supernatant were measured via fluorescence spectrophotometry exiting C6 at 467 nm and measuring the emission fluorescence at 502 nm. The quantity of C6 is calculated by comparing with a standard curve (described above). The experiment was performed three times to obtain S.D.

Fluorescence spectra of extracted supernatant from zebrafish

The larval zebrafish (30/well) with egg water (E3 medium) in 12-well plate were incubated with C6@CB[7] (300 ng/ml) for 0.5 and 1 h. The control group was only added E3 medium. After incubation, the embryos were washed carefully and homogenized using a pestle in 300 μl egg water. After the centrifugation at 13000 rpm for 30 min, fluorescence intensities of the supernatant were recorded using fluorescence spectrometer. The fluorescence spectra were recorded in a wavelength range of 480 nm to 680 nm with an excitation wavelength of 471 nm.

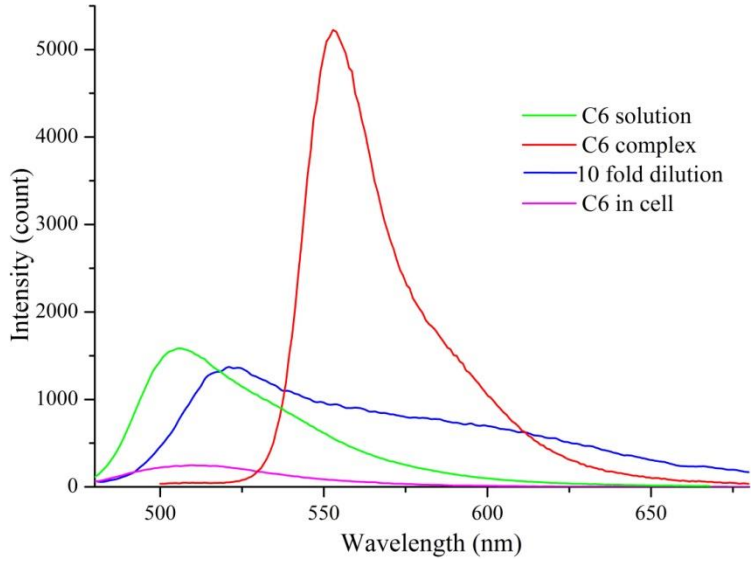

Fig. S1. Fluorescence spectra (Ex=471 nm) of C6 solution (C6 dissolved in methanol), C6@CB[7] complex, 10 fold dilution of the complex with HBSS and in the MDCK cell (the supernatant from cell disruption after incubated the complex with MDCK cell for 60 min).

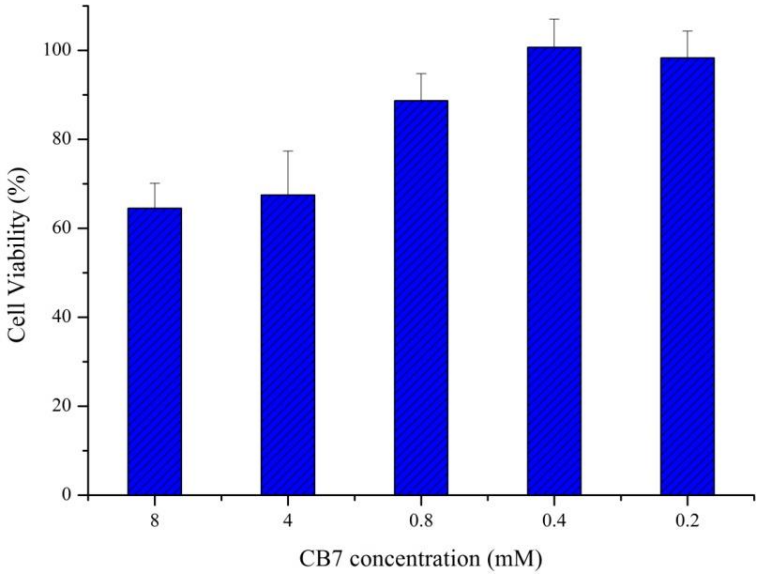

Fig. S2. Concentration-dependent cytotoxicity of CB[7] on MDCK cell for 1 h. Data are presented as the means ± S.D. (%) from three independent experiments.

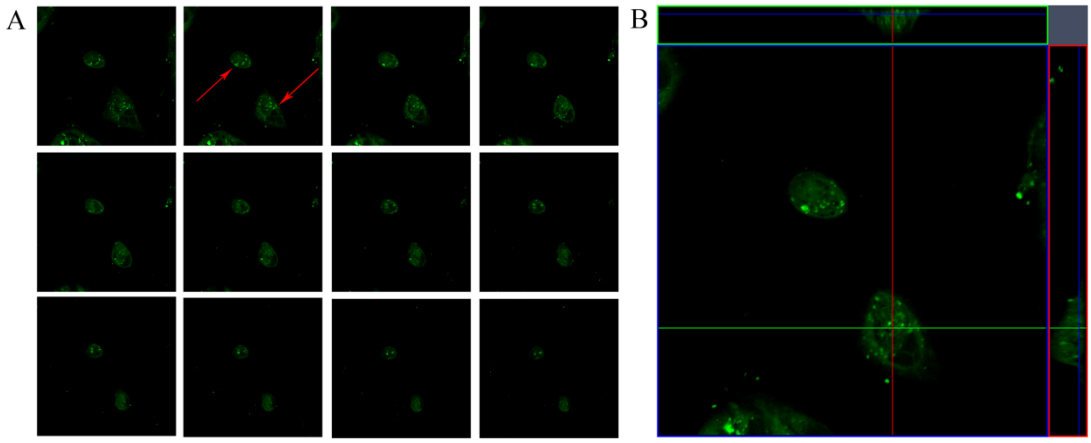

Fig S3. CLSM images of MDCK cell lines incubated with 300 nm/mL C6 (in the presence of 0.80 mM of CB[7]) for 60min from apical side to basolateral side (A) z-axis, (B) x, y axis. A z-position variation video was taken and attached in the supplementary section.

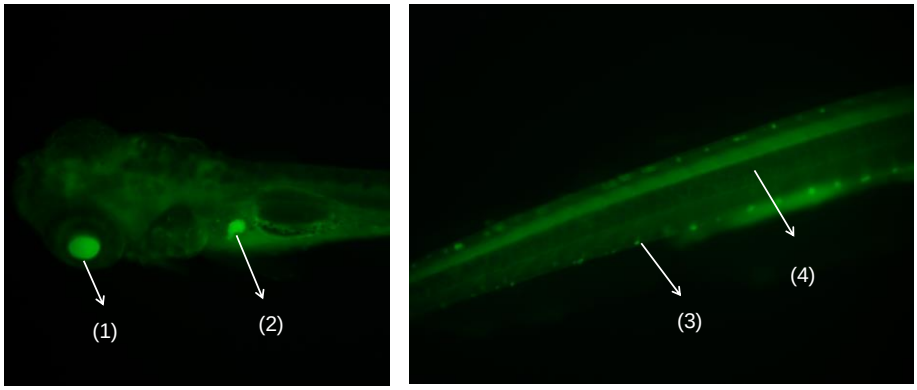

Fig. S4. Fluorescence micrographs (20×) of zebrafish after incubation with C6@CB[7] (C6 concentration of 300 ng/ml in the presence of 0.80 mM CB[7]) for 30 min. Fluorescence was observed in the (1) eyes, (2) bile ducts and liver, (3) iridophores, (4) microvascular.

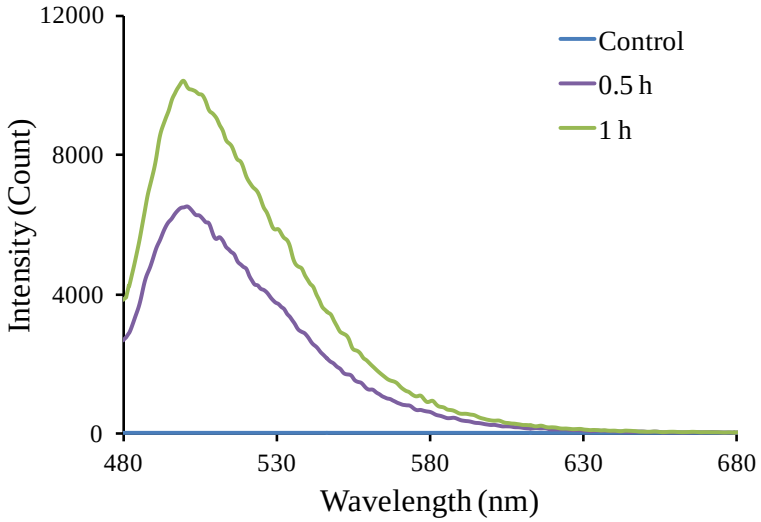

Fig. S5. Fluorescence spectra (Ex=471 nm) of C6 (300ng/mL, in the presence of 0.80 mM CB[7]) after uptake into the zebrafish for 0.5 h and 1 h, the control group was C6 suspended in E3 medium that showed no uptake.