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Electronic Supplementary Information for

Precise ricin A-chain delivery by Golgi-targeting carbon dots

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Experimental section

1. Materials and instruments.

RTA was purchased from Sigma-Aldrich (USA, L9514). Chlorpromazine was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Sodium azide (SA), 2-deoxy-Dglucose (2-DG), cytochalasin D, nocodazole, and genistein were purchased from Sigma-Aldrich (USA). CFSE and cell-counting kit-8 (CCK-8) were obtained from Dojindo Laboratories. Callein-AM/PI was purchased from Biwu Biotechnology Co., Ltd (Chongqing, China). The rabbit polyclonal antibody against RTA was purchased from Abcam (USA). Alexa Fluor 488 conjugated goat anti-rabbit IgG was purchased from Proteintech (USA). Lyso-tracker red, Golgi-tracker red, ER-tracker red, and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Beyotime Institute of Biotechnology. All reagents and solvents were analytical grade or better and used directly without further purification.

Absorption spectra were measured with an UV-3600 spectrophotometer (Shimadzu, Japan). TEM images of CDs and CDs-RTA are photographed by a high resolution transmission electron microscope (Tecnai G2 F20 STWIN, FEI Company, USA). The cell viability was measured using a microplate reader (Bio-Tek, USA).

Dynamic light scattering data and zeta-potential were measured using a Zetasizer (Nano ZS, Malvern, Worcestershire, UK). Confocal fluorescent images were acquired using an Olympus IX-81 inverted microscope equipped with an Olympus IX2-DSU confocal scanning system and a Rolera-MGi EMCCD camera (Olympus, Japan). Image-Pro Plus 6.0 (IPP) software (Media Cybernetics, USA) and Image J software were employed to analyze the fluorescence images. Ultrapure water obtained from a Millipore water purification system (18.2 MΩ•cm resistivity) was used in all experiments.

2. Preparation of the various CDs and CDs-RTA conjugates.

Golgi-targeting CDs were synthesized through a pyrolysis method with citric acid and L-cysteine as carbon sources, controlling the pyrolysis temperature according to our previous report.[1] Several other CDs with no Golgi apparatus-targeting properties were prepared respectively by using ciprofloxacin as the carbon source at 200 °C,[2] using hydroquinone and ethylenediamine as the precursors at room temperature in a self-exothermic method,[3] mixing hydroquinone with hydrogen peroxide at room temperature and further reacting with triethylenetetramine,[4] or through a simple hydrothermal route using spermine and m-phenylenediamine as precursors.[5]

CDs-RTA conjugates were prepared by simply mixing the protein with different concentrations of CDs in 1× PBS buffer and stirring gently overnight at room temperature,[6,7] the cell viability was used to determine the optimal concentration of the conjugates. The mixture of CDs and RTA (CDs+RTA) was obtained by just mixing the CDs and RTA with cell culture medium before each incubation.

3. Cellular viability measurement.

The CCK-8 assay was used to determine the cellular viabilities of HEp-2 cells treated with CDs, RTA, or CDs-RTA conjugates. HEp-2 cells in RPMI 1640 medium supplemented with 2% FBS were initially seeded in a 96-well plate at a density of 1×10^4 cells per well (100 μL per well) and incubated for 24 h at 37 °C and 5% CO_2. Following incubation with the required concentrations of CDs, RTA, or CDs-RTA conjugates for 24 h, the plates were washed twice with ice-cold PBS, and then 10 μL CCK-8 solution and 90 μL RPMI 1640 were added to every well and incubated at 37 °C for 20–30 minutes. The optical density (OD) of the mixture was measured at 450 nm with a Microplate Reader Model. The relative cytotoxicity was expressed as a percentage as:

$$V_{cells} = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100\%$$

Replicate experiments were performed (n=5).

4. Agarose Gel Electrophoresis.

The obtained CDs-RTA conjugates were characterized by 2% agarose gel electrophoresis. Briefly, free CDs (1.8 mg/mL) and CDs-RTA conjugates (1.8 mg/mL CDs–25 nM RTA) were conducted in 0.5×TBE at 100 V for 60 min. For UV imaging, the gel was excited using 365-nm light and collected photos by an ordinary camera.

5. Confocal fluorescence imaging analysis of live cells.

HEp-2 cells were plated at a density of 5.0×10^5 cells per dish on glass bottom cell culture dishes for 24 h. Then, 36 μg/mL CDs, 0.5 nM RTA, or 36 μg/mL CDs–0.5 nM RTA were added and the cells incubated at 37 °C with 5% CO_2 for a required time. Following incubation, the cells were then washed three times with PBS buffer. For CFSE staining, living cells were stained in green with 20 mM CFSE and incubated for 10 min at 37 °C; for calcein-AM/PI staining, living cells were stained in green with 2 μM calcein-AM, and dead cells were stained in red with 4.5 μM PI for 15 min at 37 °C. The maximum excitation/emission wavelengths of CFSE, calcein-AM and PI were 496/516, 490/515 and 535/617 nm.

6. CDs-RTA immunofluorescence assay.

HEp-2 cells were cultured in 35-mm glass-bottom dishes (Nest Biotechnology Co., LTD, USA) over 24 h. Next, the cells were incubated with 36 μg/mL CDs–0.5 nM RTA at 37 °C for required time, and washed three times with ice-cold PBS buffer. Following fixation with 4% paraformaldehyde for 20 min, permeabilization with 0.1% Triton X-100 for 10 min at room temperature, and blocking with 2% BSA for 1 h at 37 °C, the cells were incubated with rabbit polyclonal antibody against RTA for 1.5 h at 37 °C. Finally, the cells were incubated with Alexa Fluor 488 conjugated goat anti-rabbit IgG for 0.5 h at 37 °C. Following three washes with PBS, the immunofluorescence of RTA was obtained by the inverted microscope. Alexa Fluor 488 was excited at 470-490 nm and detected with a BA510-550 nm barrier filter.

7. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was used to verify the protein stability against enzymatic digestion. The 12% separation gel was prepared in 4 mL of 40% acrylamide/bis-acrylamide gel solution (29:1) supplemented with 10 mM $MgCl_2$, 2.5 mL 1.5 M tris buffer (pH 8.8), 0.1 mL 10% lauryl sodium sulfate (SDS), 0.1 mL 10% ammonium persulfate (APS) and 0.004 mL N,N,N',N'-tetramethylethylenediamine (TEMED), the final volume was filled with water to 10 mL. Then protease aqueous solution (0.05 mg/mL) was introduced into each free RTA or CDs-RTA samples with the same RTA concentration (8 μM), and the enzyme reaction was performed at 37 °C for 10 min. All samples were mixed with 2 × loading buffer and heated to 100 °C for 5 min, and then electrophoresis was conducted in 0.1% SDS electrophoretic buffer contain 25 mM tris and 0.192 M glycine at 80 V for 30 min and 120 V for 60 min. Bands were detected by staining with Coomassie Blue.

8. Confocal microscopy of the subcellular localization of CDs-RTA conjugates.

Two fine intersected grooves were carved along the 35-mm glass-bottom dishes and HEp-2 cells were cultured within these dishes with RPMI 1640 containing 2% FBS and 1% penicillin-streptomycin (2% medium) at 37 °C for 24 h. The medium was removed from the dishes and 2% RPMI medium containing 36 μg/mL CDs–0.5 nM RTA was added, and the cells were incubated at 37 °C for 1-12 h. The cells were then washed three times with HBSS (with Ca^{2+} and Mg^{2+}). For Lyso-Tracker Red staining, pre-warmed Lyso-Tracker Red (50 nM) was added and incubated for approximately 1 h under growth conditions. For Golgi-Tracker Red staining, 0.17 mg/mL of Golgi-Tracker Red were added. Following incubation at 4 °C for 30 min, the Golgi-Tracker Red medium was collected and the cells were washed three times with freshly cooled medium, followed by further incubation at 37 °C for 30 min in fresh medium. For ER-Tracker Red staining, ER-Tracker Red (1 μM) was added and incubated at 37 °C for 30 min. The dishes were washed twice with fresh ice-cold medium and the CDs and organelles were observed by confocal microscopy with exciting at 360-370 nm, 530-550 nm and detecting with a BA420-460 nm, BA575-625 nm barrier filter. Cells were then performed with the steps of immunofluorescence assay and obtained the immunofluorescence signal of RTA. Thus, the co-location data was achieved through the merging of the two images. The maximum excitation/emission wavelengths of CDs, Lyso Tracker Red, Golgi-Tracker Red, and ER-Tracker Red were 350/420, 577/590, 589/617, and 587/615 nm, respectively.

9. The loading amount and release dose of RTA.

The centrifugal ultrafiltration experiment was conducted to calculate the loading amount of RTA. The prepared CDs-RTA conjugates were filtrated using a 30K ultrafiltration centrifuge tube at 15000rpm for 5 min, and the absorption spectrum of original solution and filtrate was measured respectively. Based on the absorbance at 280 nm and according to Lambert-Beer's law, the loading amount of RTA in each CDs-RTA conjugates was obtained.

The release dose of RTA was calculated by the co-localization data between RTA and ER (RTA&ER), RTA and CDs (RTA&CDs). The RTA release dose was estimated as a percentage as:

$$Release\ dose = \frac{RTA\ \&\ ER}{RTA\ \&\ ER + RTA\ \&\ CDs} \times 100\%$$

The co-localization data of 10 h and 12 h was chose to calculate the release dose, when most of the RTA was separated from CDs and hold a good co-localization rate with ER, while only a part of RTA was still loaded on CDs.

10. The cytotoxicity of cellular uptake inhibitors.

The cytotoxicity of cellular uptake inhibitors against HEp-2 cells was determined using the CCK-8 assay. Briefly, HEp-2 cells (1×10^4 cells/well) were seeded in 96-well plates and incubated for 24 h. Genistein (50 μg/mL), Cyto-D (5 μM), chlorpromazine (10 μg/mL), or nocodazole (15 μM) were added and the cells further incubated for 6 h. The culture medium was subsequently replaced with 10 μL CCK-8 and 90 μL RPMI 1640 and the samples were incubated for an additional 20–30 min. The OD of the mixture was measured at 450 nm with a Microplate Reader Model.

11. Energy-dependent cellular uptake.

The energy-dependent cellular uptake experiments were performed at low temperature incubation (4 °C) and ATP-depleted environments (37 °C, treated with NaN_3 and 2-DG). HEp-2 cells were cultured in 35-mm glass-bottom dishes and incubated at 37 °C for 24 h. The cells were then pre-incubated at 4 °C or in serum-free medium supplemented with 10 mM SA and 50 mM 2-DG at 37 °C for 45 min, respectively. Following this, 36 μg/mL CDs–0.5 nM RTA were added and co-incubated for an additional 6 h. The cells were then immunofluorescence staining by primary antibody and the secondary antibody (green) and the

immunofluorescence imaging of RTA were observed using confocal microscopy. The mean fluorescence intensity was analyzed by Image J software in 5 images.

12. The effect of uptake inhibitors on endocytic pathways of CDs-RTA conjugates.

The endocytic pathways of CDs-RTA were investigated following the addition of specific pharmacological inhibitors and detected by confocal microscopy. HEp-2 cells were seeded in 35-mm glass-bottom dishes and incubated for 24 h. Genistein (50 μg/mL), the inhibitor for caveolae-mediated endocytosis; Cyto-D (5 μM), the inhibitors for macropinocytosis-mediated endocytosis; chlorpromazine (10 μg/mL), the inhibitors for clathrin-mediated endocytosis and nocodazole (15 μM), the inhibitor for microtubule transport were separately added into 2% medium and preincubated at 37 °C for 45 min.[8] Finally, 36 μg/mL CDs–0.5 nM RTA were added and co-incubated for an additional 6 h, and the cells were then immunofluorescence staining by primary antibody and the secondary antibody (green) and the immunofluorescence imaging of RTA were observed using confocal microscopy.

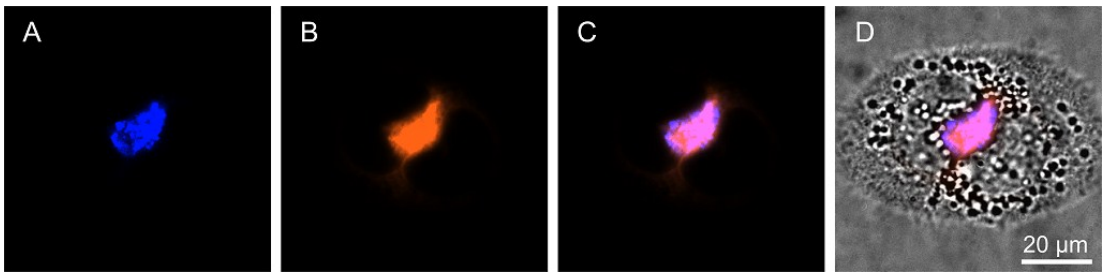

Fig. S1 Fluorescence colocalization assay of CDs with Golgi apparatus following 12 h of incubation with HEp-2 cells. The blue fluorescence signal from intracellular CDs overlapped well with the red signal from the Golgi stained by Golgi-Tracker Red. The overlapped portion appears in pink. (A) blue fluorescence image of CDs; (B) red fluorescence image of Golgi-Tracker Red; (C) overlapped fluorescence images of CDs and Golgi-Tracker Red; (D) merged fluorescence images of bright field, CDs and Golgi-Tracker Red. Scale bar, 20 μm.

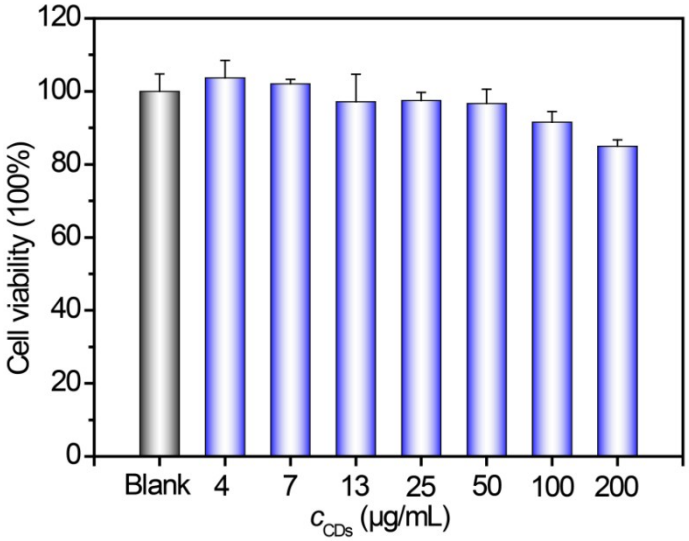

Fig. S2 Cell viability of the Golgi targeting CDs on HEp-2 cells.

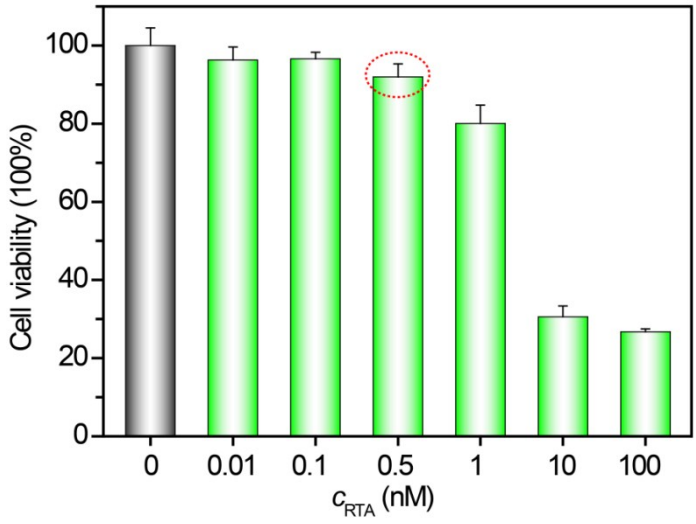

Fig. S3 Cell viability of RTA on HEp-2 cells.

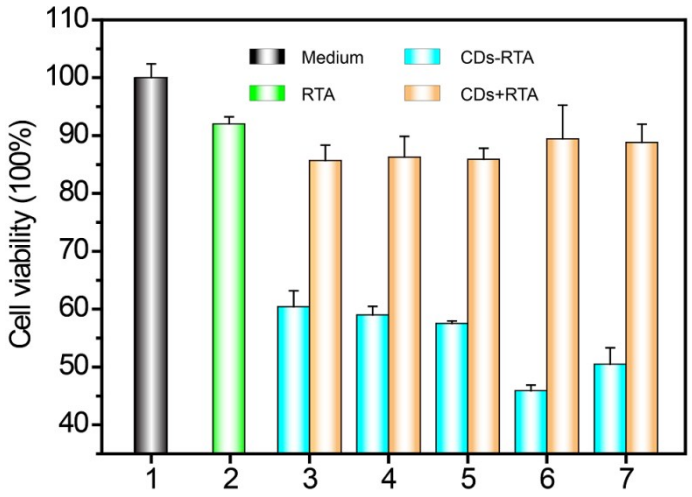

Fig. S4 The cytotoxicity test of CDs-RTA conjugates (the blue columns) and the mixture of CDs and RTA (the orange columns) with different CDs concentrations. The concentration of CDs in the conjugates: 3-7, 9, 18, 27, 36, 45 μg/mL. The mixture of CDs and RTA (CDs+RTA) was prepared by mixing the CDs (9-45 μg/mL) with RTA before incubation with cells. The concentration of RTA is 0.5 nM.

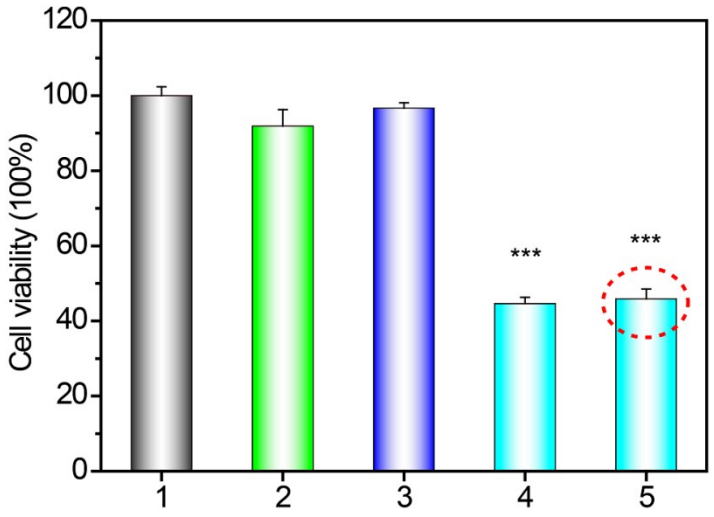

Fig. S5 The cell viability of CDs-RTA conjugates prepared at different conditions. 1, control; 2, 0.5 nM RTA; 3, 36 μg/mL CDs; 4, 36 μg/mL CDs-0.5 nM RTA prepared in ice bath; 5, 36 μg/mL CDs-0.5 nM RTA prepared in room temperature.

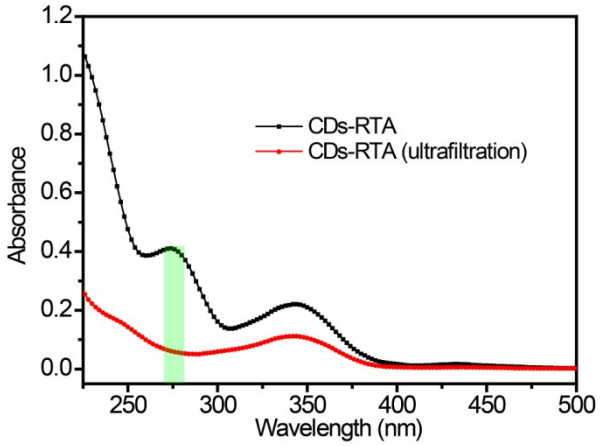

Fig. S6 UV absorption spectrum of CDs-RTA conjugates with and without ultrafiltration.

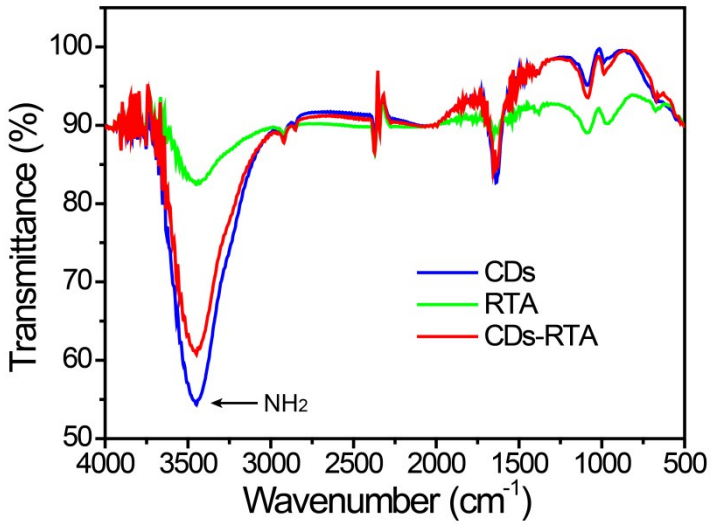

Fig. S7 The infrared spectrum of RTA, CDs and the CDs-RTA conjugates

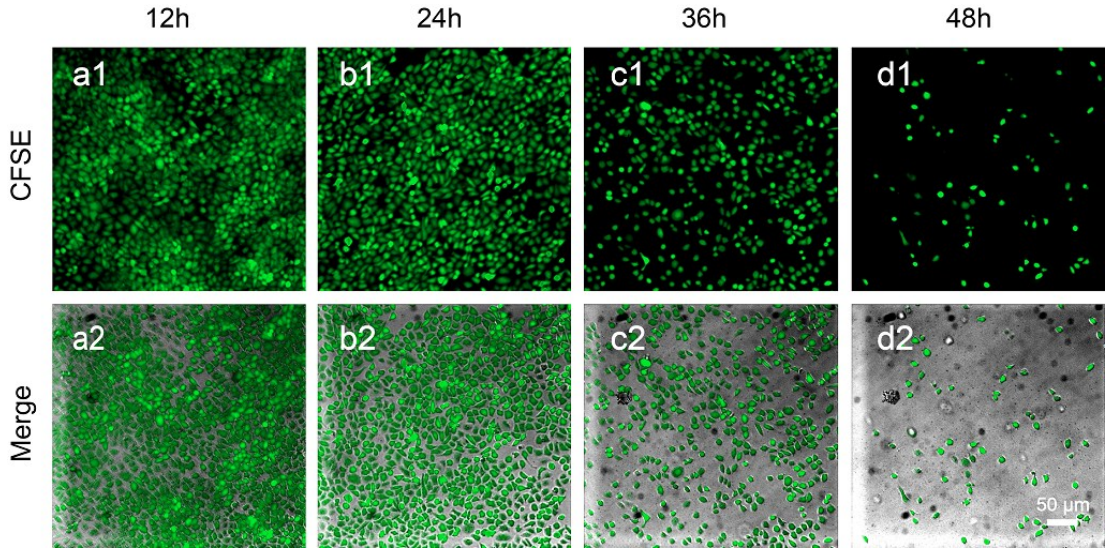

Fig. S8 Confocal microscopy images of HEp-2 cells incubated with 36 μg/mL CDs-0.5 nM RTA for various time: 12 h (a1 and a2); 24 h (b1 and b2); 36 h (c1 and c2); 48 h (d1 and d2). From top to bottom, CFSE fluorescence images and merged fluorescence images of bright field and CFSE images. Scale bar, 50 μm.

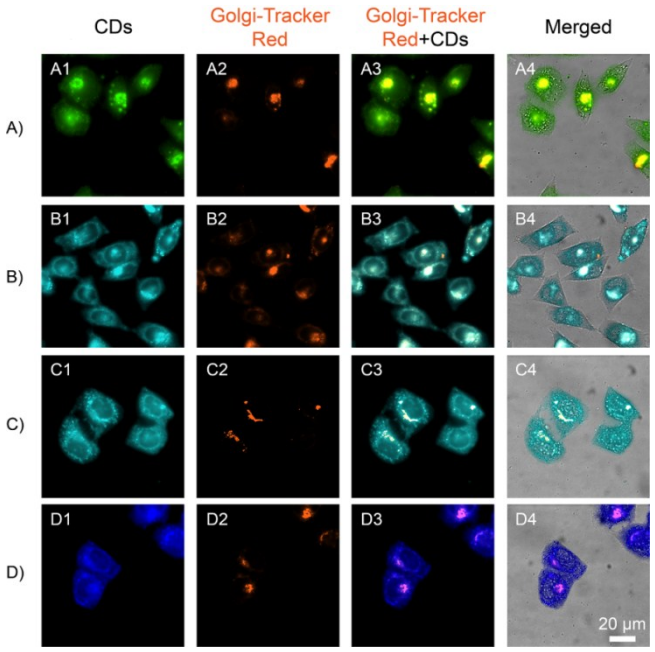

Fig. S9 Fluorescence colocalization assay of various CDs with Golgi apparatus following 12 h of incubation with HEp-2 cells. A), CDs prepared by using hydroquinone and ethylenediamine as the precursors at room temperature in a self-exothermic method; B), CDs prepared by mixing hydroquinone with hydrogen peroxide at room temperature and further reacting with triethylenetetramine; C), CDs prepared by using ciprofloxacin as carbon source at 200 °C; D), CDs prepared through a simple hydrothermal route using spermine and m-phenylenediamine as precursors. From left to right, the fluorescence images of various CDs; red fluorescence images of Golgi-Tracker Red; overlapped fluorescence images of CDs and Golgi-Tracker Red; merged fluorescence images of bright field, CDs and Golgi-Tracker Red. Scale bar, 20 μm.

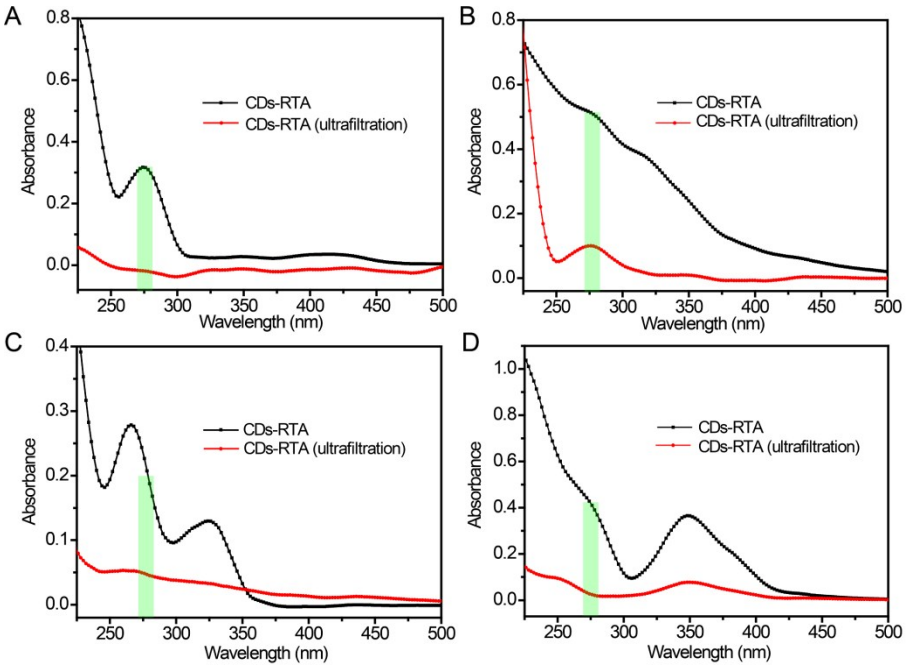

Fig. S10 UV absorption spectrum of various CDs-RTA conjugates prepared by CDs without Golgi-targeting properties with and without ultrafiltration (12000rpm/5min). A-D, CDs-RTA conjugates prepared by the CDs of A-D in Fig. S9. The loading amount in these conjugates was calculated as 0.46, 0.4, 0.38, 0.48 nM respectively, which was similar with the loading content of 0.43 nM RTA in CDs-RTA conjugates prepared by Golgi-targeting CDs.

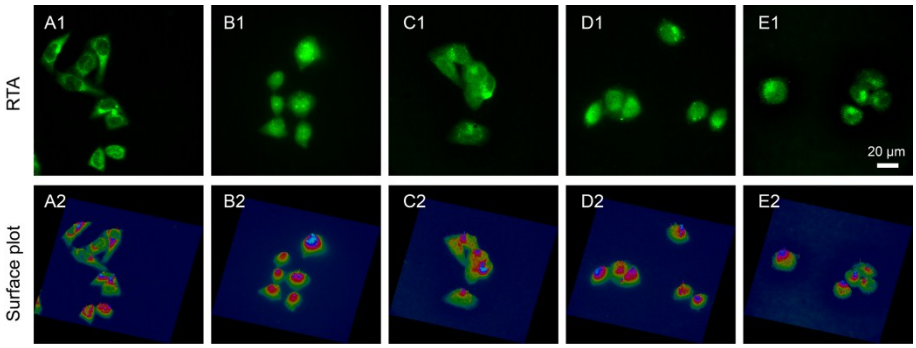

Fig. S11 Confocal microscopy images of HEp-2 cells following incubation with different CDs-RTA conjugates prepared by various CDs at 37 °C for 12 h. A, CDs-RTA conjugates prepared by Golgi-targeting CDs; B-E, CDs-RTA conjugates prepared by the CDs of A-D in Fig. S9. From up to down are immunofluorescence (A1-E1) and surface plot images (A2-E2) of intracellular RTA respectively. Scale bar, 20 μm.

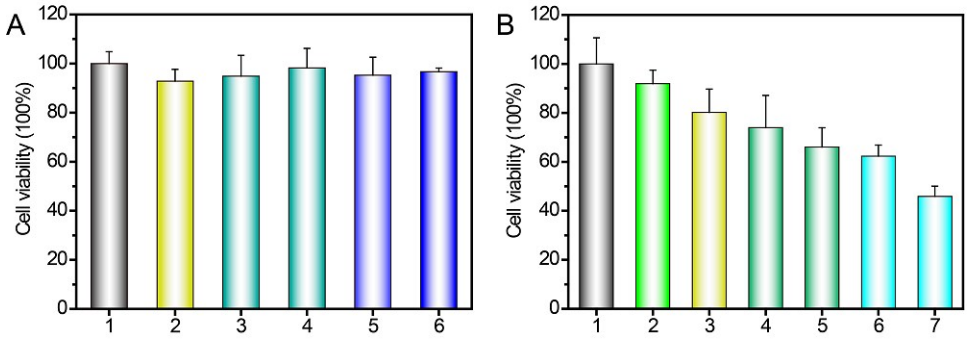

Fig. S12 Cytotoxicity comparison of different CDs and CDs-RTA conjugates. (A) The cytotoxicity test of diverse CDs. 1, control; 2-5, the CDs of A-D in Fig. S9; 6, the Golgi targeting CDs. The concentration of all the CDs are 36 μg/mL. (B) The cytotoxicity test of diverse CDs-RTA conjugates, 1, control; 2, 0.5 nM RTA; 3-7, CDs-RTA conjugates prepared by the CDs corresponding to Fig. S12 (A) of 2-6, all the concentration of conjugates is 36 μg/mL CDs-0.5 nM RTA.

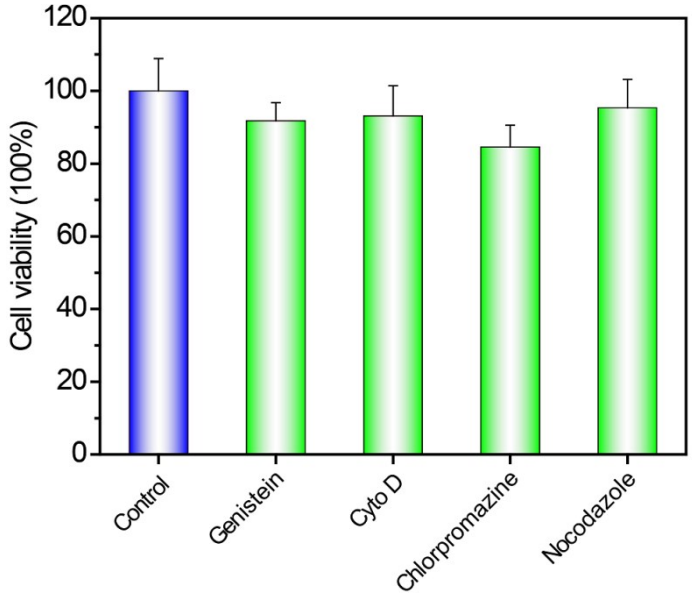

Fig. S13 The cytotoxicity of cellular uptake inhibitors. Concentrations: 50 μg/mL genistein; 5 μM Cyto D; 10 μg/ mL chlorpromazine; 15 μM nocodazde.

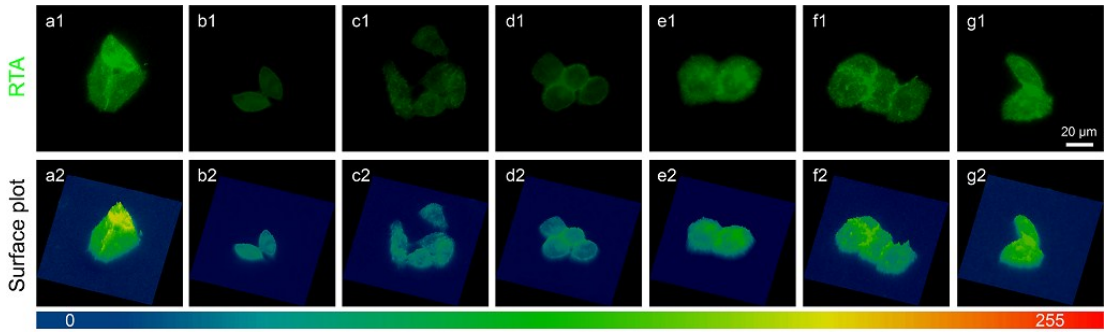

Fig. S14 Confocal microscopy images of HEp-2 cells following incubation with CDs-RTA conjugates at 37 °C (a1); 4 °C (b1); after pretreatment with NaN_3 and 2-DG (c1); genistein (d1); chlorpromazine (e1); Cyto D (f1) and nocodazde (g1) for 45 min and further incubated 6 h at 37 °C. From up to down are immunofluorescence (a1-g1) and surface plot images (a2-g2) of intracellular RTA respectively.

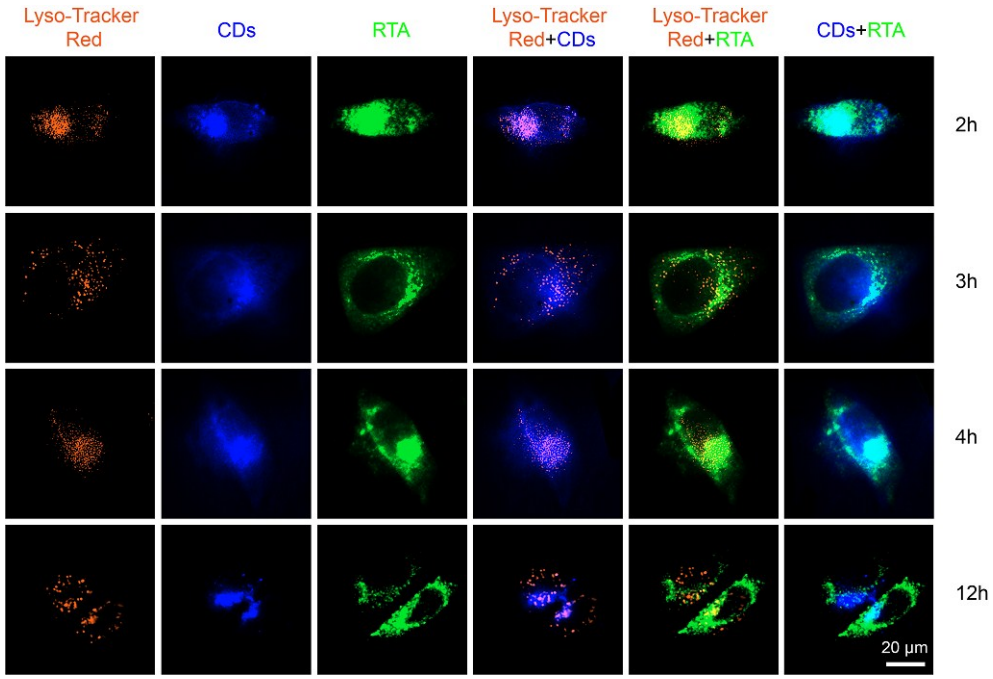

Fig. S15 Fluorescence colocalization assay of CDs-RTA with lysosome following 2, 3, 4 and 12 h of incubation. From left to right: fluorescence images of lysosome stained by Lyso-Tracker Red; fluorescence images of CDs; immunofluorescence images of RTA; merged images of lysosome and CDs; merged images of lysosome and RTA; merged images of CDs and RTA. Scale bar, 20 μm.

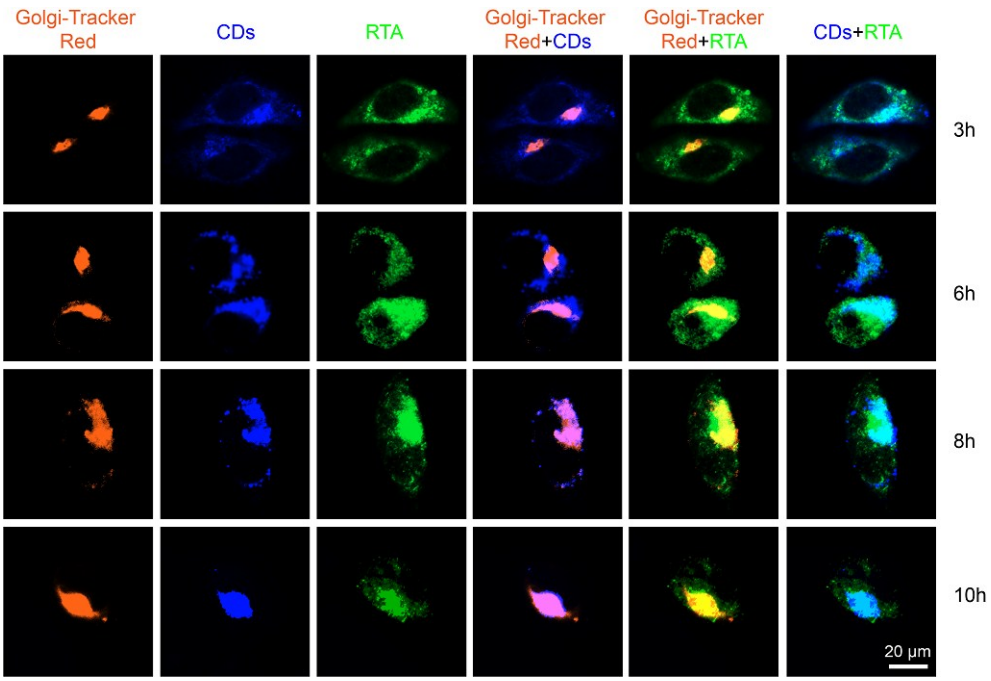

Fig. S16 Fluorescence colocalization assay of CDs-RTA with Golgi apparatus following 3, 6, 8 and 10 h incubation. From left to right: fluorescence images of Golgi stained by Golgi-Tracker Red; fluorescence images of CDs; immunofluorescence images of RTA; merged images of Golgi and CDs; merged images of Golgi and RTA; merged images of CDs and RTA. Scale bar, 20 μm.

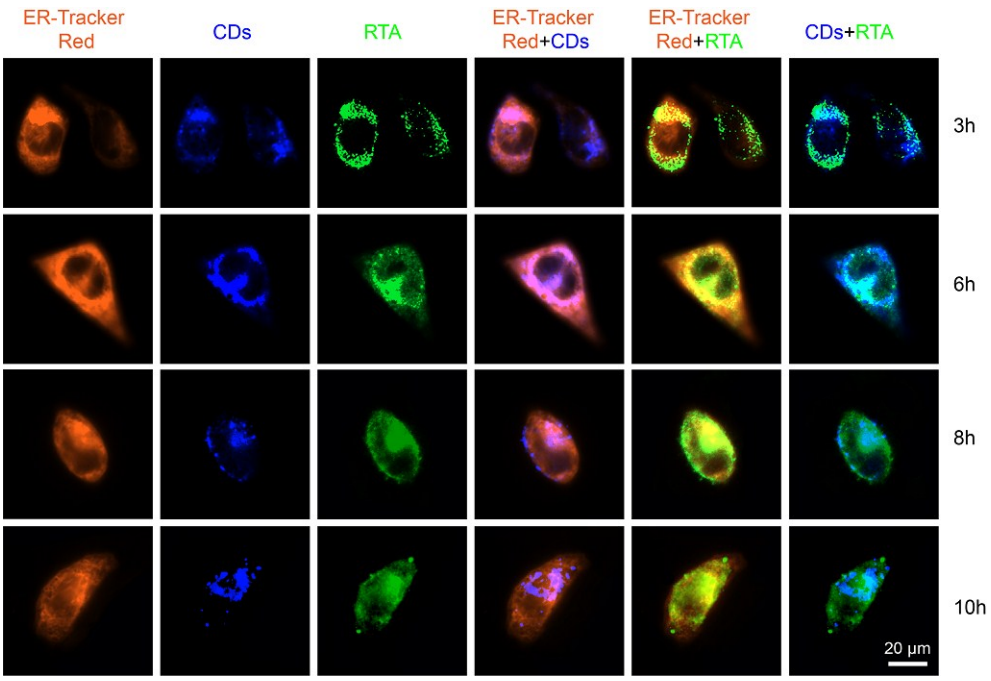

Fig. S17 Fluorescence colocalization assay of CDs-RTA with ER following 3, 6, 8 and 10 h incubation. From left to right: fluorescence images of ER stained by ER-Tracker Red; fluorescence images of CDs;

immunofluorescence images of RTA; merged images of ER and CDs; merged images of ER and RTA; merged images of CDs and RTA. Scale bar, 20 μm.

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