Controllable Drug Uptake and Non-genomic Response through Estrogen Anchored Cyclodextrin Drug Complex

Juan-Juan Yin, Stepan P. Shumyak, Christopher Burgess, Zhi-Wei Zhou, Zhi-Xu He, Xue-Ji Zhang, Shu-Ting Pan, Tian-Xin Yang, Wei Duan, Jia-Xuan Qiu, and Shu-Feng Zhou

1 Xiaolan Hospital, Southern Medical University, Zhongshan, Guangdong 528415, China
2 Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, Florida, 33612, USA
3 Guizhou Provincial Key Laboratory for Regenerative Medicine, Stem Cell and Tissue Engineering Research Center & Sino-US Joint Laboratory for Medical Sciences, Guiyang Medical University, Guiyang, Guizhou 550004, China
4 Research Center for Bioengineering and Sensing Technology, University of Science and Technology Beijing, Beijing 100083, China
5 Department of Oral and Maxillofacial Surgery, the First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi, China
6 Department of Internal Medicine, University of Utah and Salt Lake Veterans Affairs Medical Center, Salt Lake City, Utah, 84132, USA
7 School of Medicine, Deakin University, Waurn Ponds, Victoria 3217, Australia

Table of Content

Figure S1. The mass spectra of β-CD (a), β-CDNH₂ (b), α-CD (c), α-CDNH₂ (d), β-CDE₁ (e), β-CDPg (f), α-CDE₁(g), α-CDPg (h). .................................................................S2-4
Figure S2. The LC-MS spectrum of Ada-DOX .......................................................... S5
Figure S3. Solubility assay for CDE₁ . ........................................................................S6
Figure S4. The FTIR spectra of β-CD (a), β-CDNH₂ (b), E₁ (c), (β) CDE₁ (d) .................S7
Figure S5. The UV spectra of (β) CDE₁ and E₁ (insert) in water and the HNMR spectrum of Ada-DOX .....................................................................................................S8
Figure S6. The ¹H NMR spectrum CDE₁ (800 MHz, d6-DMSO) .....................................S9
Figure S7. The ¹³C NMR spectrum CDE₁ (100 MHz, d6-DMSO) .................................S10
Figure S8. The representative TEM images of CDE₁, a (10K), b (10K), c (40K); and the TEM images of CDE₁-Ada-DOX, d (10K), e (30K)..................................................S11
Figure S9. The cytotoxicity of Dox, Ada-Dox and CDE₁-Ada-DOX to MCF-7 cells (left) and 3T3 cells (Right) after 24 h drug exposure based on MTT assay...............................S12
Experimental ...........................................................................................................S13-18
References ..............................................................................................................S19
Figure S1. The mass spectra of β-CD (a), β-CDNH₂ (b), α-CD (c), α-CDNH₂ (d), β-CDE₁ (e), β-CDPg (f), α-CDE₁ (g), α-CDPg (h)
Figure S2. The LC-MS spectrum of Ada-DOX (a): LC chromatograph (b): MS spectrum
The assay was carried out using the μSol Solubility assay developed by pION. All liquid handling steps for μSol are performed on a TECAN Fedom EVO150 robot and analyzed by the pION’s μSol Evolution Software. The test compounds were dispensed into one 96-well sample stock plate at concentration of 10 mM in DMSO. 10 μl of each well was transferred into the 96-well sample plate then 1-propanol (190 μl) was added. 5 uL of the sample plate solution and PrismaTM HT buffer at pH= 7.4 (75 μl) were added to each well to measure the absorbance reference of each compounds. The UV spectra from 200–500 nm was measured for all wells and subtracted from the background (DMSO). 6 μl of each well of the sample stock was added into a deep 96 well plate containing 600 μl PrismaTM HT buffer in each well and the solutions were incubated (no shaking) for a period of 15 hours. 200 μl of the solutions were treated by a PVDF 0.2 μm filter plate (x2). To 75 μl of the filtered solutions was added 75 μl of 1-propanol to measure the samples absorbance. UV spectra measurement was determined using the Tecan infinite M-1000 pro microplate reader. Note: testing was realized in triplicate.
Figure S4. The Fourier transform infrared spectroscopy (FTIR) spectra of β-CD (a), β-CDNH$_2$ (b), E$_1$ (c), (β)CDE$_1$ (d)
Figure S5. (a) The UV spectra of (β)CDE$_1$ and E$_1$ (insert) in water; (b) The $^1$HNMR spectrum of Ada-DOX, (500MHz, CDCl$_3$)
Figure S6. The $^1$H-NMR spectrum CDE₁ (800 MHz, $d_6$-DMSO)
Figure S7. The $^1$C NMR spectrum CDE$_1$ (100 MHz, $d_6$-DMSO)
Figure S8. The representative TEM images of CDE₁, a (10K), b (10K), c (40K); and the TEM images of CDE₁-Ada-DOX, d (10K), e (30K)
Figure S9. The Cytotoxicity of Dox, Ada-Dox and CDE1-Ada-DOX to MCF-7 cells (left) and 3T3 cells (Right) after 24 h drug exposure based on MTT assay.
Experimental:

Synthesis and characterization

To construct the supramolecule, the mER-targeted drug carrier CDE\textsubscript{1} was prepared with α- and β-CDNH\textsubscript{2} being synthesized as the starting material. \(^1\)α- and β-CDOT were prepared by the reaction of α- or β-CD with \(p\)-toluenesulfonyl. Thereafter, α- and β-CDNH\textsubscript{2} were generated by a reaction of CDOTs and ammonium hydroxide for 3 days at 50°C. Finally, α- and β-CDE\textsubscript{1} were prepared by coupling of NaBH\textsubscript{4}-activated CDNH\textsubscript{2} and estrone, and the sample was purified through chromatographic separation on a CM sephadex C\textsubscript{25} ion-exchange column, preparative TLC, recrystallization in acetone three times, and dried in a vacuum oven overnight to obtain α-CDE\textsubscript{1} with a yield of 12.41% and β-CDE\textsubscript{1} with a yield of 10.80%. In addition, the guest molecule Ada-DOX was synthesized according to the reaction of Error! Reference source not found. where DOX was grafted to Ada molecule through amide bonding.

Synthesis of (β) CDE\textsubscript{1}: A mixture of β-CDNH\textsubscript{2} (200 mg, 0.177 mmol) and 4 equivalents of estrone (200 mg, 0.740 mmol) in anhydrous pyridine (or DMF) (3 mL) was flushed with N\textsubscript{2} and stirred vigorously at room temperature for 48 hours. The mixture was evaporated and re-dissolved in methanol, excessive sodium borohydride (NaBH\textsubscript{4}, a reducing agent) was then added, and the mixture was stirred overnight. At the end of the reaction, the product was extracted with water from ethyl acetate, purified by cation-exchange column chromatography with water, preparative TLC, and re-crystallization, and finally the product β-CDE\textsubscript{1} (30.4 mg, yield: 12.41%, \(R_f\)\textsubscript{β-CDE1/CDNH2} = 2.29; Solvents used: 1-propanol/ethyl acetate/water/Ammonium hydroxide=3:1:2:1) was produced.

Synthesis of α-CDE\textsubscript{1}: A mixture of α-CDNH\textsubscript{2} (17.7 mg, 0.018 mmol) and excessive estrone (45.5 mg, 0.17 mmol) in anhydrous pyridine (2 mL) was flushed with N\textsubscript{2} and stirred vigorously at room temperature for 48 hours. The mixture was evaporated and re-dissolved in methanol, excessive NaBH\textsubscript{4} was then added. Thereafter, the product α-CDE\textsubscript{1} was extracted with water from ethyl acetate, and purified by preparative TLC (2.4 mg, 10.8%, \(R_f\)\textsubscript{α-CDE1/α-CDNH2} = 2.21).

Chemical synthesis of β-CDPg: A mixture of β-CDNH\textsubscript{2} (200 mg, 0.177 mmol) and 4 equivalents of Pg (200 mg, 0.740 mmol) in anhydrous pyridine (or DMF) (3 mL) was flushed with N\textsubscript{2} and stirred vigorously at room temperature for 48 hours. The mixture was evaporated and re-dissolved in methanol, excessive NaBH\textsubscript{4} was then added, and the mixture was stirred overnight. Thereafter, the product was extracted with water from ethyl acetate, purified by cation-exchange column chromatography with water, preparative TLC, and re-crystallization, and the final product β-CDPg (30.4 mg, 12.41%, \(R_f\)\textsubscript{β-CDPg/CDNH2} = 2.27; Solvents used: 1-propanol/ethyl acetate/water/Ammonium hydroxide=3:1:2:1) was given.

Synthesis of α-CDPg: A mixture of α-CDNH\textsubscript{2} (25.8 mg, 0.027 mmol) and excessive progesterone (39.2 mg, 0.124 mmol) in anhydrous pyridine (1.5 mL) was flushed with N\textsubscript{2} and stirred vigorously at room temperature for 48 hours. The mixture was evaporated and re-dissolved in methanol, excessive NaBH\textsubscript{4} was then added and incubated overnight. the product α-CDPg was then extracted with water from ethyl acetate, and purified by preparative TLC (0.8 mg, 2.3%, \(R_f\)\textsubscript{α-CDPg/α-CDNH2}=1.44; Solvents used: 1-propanol/ethyl acetate/water/Ammonium hydroxide=3:1:2:1).

Synthesis of Ada-DOX: Ada-COCl was dissolved in anhydrate dichloromethane (DCM) mixed with Et\textsubscript{3}N, and then stirred at room temperature for 3 hours under N\textsubscript{2}. Doxorubicin hydrochloride was then added, stirred overnight and separated by flash chromatography
(CH₂Cl₂:CH₃OH = 100:1, v/v). Ada-DOX was obtained and purified by flash column with a yield of 80.5%.

Generation of the CDE₁-Ada-DOX inclusion complex supramolecule: The 1:1 stoichiometric inclusion complex of CDE₁ with Ada-DOX (CDE₁-Ada-DOX) was readily prepared by the co-precipitation method through host-guest interactions. The CDE₁-Ada-DOX supramolecules were subject to further chemical and biological analysis in comparison with the non-ER-targeted CD-Ada-DOX inclusion complex in which no estrogen moiety was conjugated.

The 1H-NMR spectra of β-CDOT in D₂O detected using a Bruker NMR spectrometer at a frequency of 400 MHz showed two doublet signals at 7.37 and 7.69 ppm for the protons in the benzene rings of the tosylate (not shown). The signals at 5.61–5.80 ppm were assigned to the tertiary carbons of CD in the glucose unit connection. Tetratertiary protons connected to the hydroxyl groups in α and β positions were assigned to the signals at 3–4 ppm. In addition, the active protons in hydroxyl groups showed at around 2 ppm.

The 1H-NMR spectra of β-CDNH₂ in D₂O detected using a Bruker NMR spectrometer at a frequency of 400 MHz showed the signals at 4.90 ppm which were assigned to the tertiary carbons for CD in the glucose unit connection (not shown). The tetratertiary protons connected to the hydroxyl groups were assigned to the signals at 3–4 ppm. In addition, the active protons in hydroxyl groups as well as the protons in –NH₂ showed at around 2 ppm.

The FTIR spectra of β-CDNH₂ exhibited similar peaks as β-CD, and the frequencies observed at the wavenumbers of 3,319.26 cm⁻¹, 2,928.53 cm⁻¹, 1,153.04 cm⁻¹, and 1,029.24 cm⁻¹ corresponded to the symmetric and antisymmetric stretching of O-H, C-H, C-C, and bending vibration of O-H, respectively (not shown). For β-CDNH₂, the frequencies for N-H stretching were between wavenumbers of 3,274.10 to 3,492.83 cm⁻¹ and C-N stretching was at 1,022.95 cm⁻¹.

The structure of (β) CDE₁ was confirmed by several spectral methods including UV-vis, HR-MS, 1H- and 13C-NMR, circular dichroism, and FTIR spectroscopy. The UV-vis absorption spectra of CDE₁ and E₁ (insert) in saturated aqueous solution showed a characteristic peak as E₁ at 285 nm without obvious shifting.

The positive-ion mode ([M+H] +) HR-MALDI-TOF-MS spectral study of β-CDE₁ showed a major ion peak of C₆₀H₃₃NO₃₅ for CDE₁ at m/z of 1,388.268 with an intensity of 100.0%. The exact m/z of CDE₁ was 1,387.56. The mass of β-CDE₁ is close to the perceived limit of facile, passive membrane transport (>1,500 Da).

1H NMR spectral data for β-CDE₁ in DMSO-d₆ were gained in a Bruker spectrometer at a frequency of 800 MHz: 1.21, 1.86, 2.48, 3.28, 3.54, 3.59, 3.69, 4.45, 4.80, 5.66, 5.73, 6.40, 6.46, 7.01, and 8.98 ppm. The 1H-NMR spectra of CDE₁ showed characteristic peaks for the polysaccharide ring of CD from 3-6 ppm, protons in E₁ residue were assigned to 6-7 ppm, and the signal at 9 ppm downfield was assigned to the active proton of the benzene ring in E₁.

13C NMR spectral data of β-CDE₁ in DMSO-d₆ obtained from a Bruker spectrometer at a frequency of 200 MHz and probe temperature of 298 k: 11.61, 23.17, 26.25, 27.18, 29.09, 39.52, 51.73, 59.98, 72.45, 81.63, 102.09, 112.80, 114.99, 126.10, 130.58, 137.29, and 154.91 ppm. The 13C-NMR spectra of β-CDE₁ also showed characteristic peaks for polysaccharide carbons from 0–110 ppm, and 6 signals in downfield for the sp hybrid carbons of E₁ residue at 112.80, 114.93, 125.90, 130.67, and 155.10 ppm were assigned.

In the circular dichroism spectral measurement of β-CDE₁, the signal of CDE₁ at 1.0 mM in DMF was recorded in the wavelength range of 260 to 450 nm. The signal of DOX, Ada-DOX, CDE₁, and CDE₁-Ada-DOX at 1.0 mM in DMF was recorded with the cutoff of <265 nm. A
significant intensity change was observed with the CDE₁-Ada-DOX inclusion complex at
genegative and positive extrema (290 and 350 nm, respectively) at which the [θ] value changed by
the value of $0.791 \times 10^3$ and $0.555 \times 10^3$ deg cm$^2$·dmol$^{-1}$, respectively, in comparison with Ada-
DOX, and $2.318 \times 10^3$ and $0.576 \times 10^3$ deg cm$^2$·dmol$^{-1}$ in comparison with CDE₁ for both
positive and negative cotton effect.

The FTIR spectra of E₁ and CDE₁ showed both CD (3,319 cm$^{-1}$ for O-H stretching; 1,023 cm$^{-1}$
for C-O vibration) and E₁ characteristic signal (2,944 cm$^{-1}$ for C-H stretching). The FTIR spectra
were recorded for CDE₁ at 705.01, 932.12, 1,022.60, 1,076.93, 1,150.66, 1,368.48, 3,186.18, and
3,274.59 cm$^{-1}$.

β-CDE₁ was found very stable in aqueous solution for 1.5 years with no changes in the $R_f$
value ($R_{cDE1}/R_{cDNH2} = 2.46$) when tracked using TLC with the developing solvent of 1-
propanol/ethyl acetate/water/NH$_3$·H$_2$O in the ratio of 3:1:2:1 (v/v). The metabolism of CDE₁ is
unknown, but we speculate that CDE₁ will be cleaved to release E₁ in solution.

The solubility of β-CDE₁ is 37.7μg/ml. The assay was carried out using the μSol Solubility
assay developed by pION.

α-CDE₁ was also synthesized using a similar synthetic scheme as for β-CDE₁. The positive-
ion mode ESI spectrum of α-CDE₁ acquired using the Agilent 6430 Triple Quad LC/MS system.
The positive-ion mode ([M+H]$^+$) ESI-MS study of α-CDE₁ observed a main ion peak of
C$_{54}$H$_{64}$NO$_{30}$ at an m/z of 1,226.2. The exact m/z of α-CDE₁ was 1,226.5. In the following
experiments, only β-CDE₁ was used and CDE₁ referred to β-CDE₁ unless otherwise indicated.
The synthesized CDE₁ has several roles: to recognize and bind ERs on the tumour cell
membrane and then trigger the internalization of the drug complex through endocytosis; to be
used as a modified estrogen to explore non-genomic events in normal and cancer
cells; to carry the drug cargo to tumour cells through ER-mediated targeting.

In addition, the α/β-CD conjugated withPg was also successfully synthesized and structurally
confirmed by HR-MS for the first time. The m/z of α-CDPg by ESI-MS ([M+H$_2$O+H]$^+$) was
1,288.5 (C$_{57}$H$_{94}$NO$_{31}$, exact m/z = 1,287.57). The m/z of β-CDPg by the positive-ion mode
MALDI-TOF-MS ([M+Na]$^+$) was 1,455.756 (C$_{63}$H$_{101}$NNaO$_{35}$, exact m/z = 1,454.61). In addition
to being used as drug vectors, CDPg can be used as modified Pg to explore the non-genomic and
genomic events elicited by Pg in normal and cancer cells. Both CDE₁ and CDPg are useful tools
differentiating non-classical ligand-activated ER and PgR actions versus the classical effects.

UV/visible spectrometry

The UV/visible spectra of the synthesized moles and supramolecules were recorded using a
Perkin-Elmer Lambda 2 UV/vis double beam spectrophotometer (PerkinElmer Life and
Analytical Sciences Inc., Oak Brook, IL, USA). The UV-Vis spectra were obtained using DMF
as the blank and 2.5-mL standard cuvettes for holding samples. The samples were scanned over a
range of wavelengths of 300–700 nm with scanning step of 10 nm;

High-resolution $^1$H- and $^{13}$C-nuclear magnetic resonance (NMR)

The high-resolution $^1$H NMR experiments were conducted at 25°C on digital NMR Bruker
BioSpin 600 and 800-MHz and spectrometers (Bruker BioSpin Co., The Woodlands, TX, USA)
Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry (MS)

MALDI-TOF mass spectra of β-CDE₁, β-CDPg, and Ada-DOX were obtained on an Applied Biosystems 4700 Proteomics Analyzer coupled with TOF/TOF Optics (Applied Biosystems Inc., Foster City, CA, USA). The instrument was calibrated prior to each analysis using a standard mix of 5 peptides (AB-Sciex, #P2-3143-00). Samples were diluted in 50% acetonitrile and combined 1:1 (v:v) with α-CHCA at 10 mg mL⁻¹ (Sigma-Aldrich Chemicals Inc., St. Louis, MO, USA) as the matrix. 1 µL of this mixture was deposited to a sample plate, air dried and inserted into the mass spectrometer to acquire the spectral data. Spectra were obtained in positive reflective mode and laser power was manually optimized for each compound. Data were analyzed using the Data Explorer software (Applied Biosystems Inc., Foster City, CA, USA).

ESI-MS spectral data for α- and β-CD, α- and β-CDNH₂, α-CDE₁, and α-CDPg were acquired in a positive mode using the Agilent 6430 Triple Quad LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA). Automatic peak detection and mass spectrum deconvolution were performed with Agilent MassHunter workstation Acquisition software B.04.00 (Agilent Technologies Inc., Santa Clara, CA, USA). The MS interface capillary was maintained at 325°C, with a sheath gas flow of 9 L/minute. The spray voltage for positive ion injection was 4.0 kV. The mass analyzer was scanned over a range of 50–3,000 mass-to-charge ratio (m/z).

Fourier transform infrared (FTIR) spectrometry

The instrument used for FTIR analysis of E₁, β-CD, β-CDNH₂, and β-CDE₁ was a Perkin–Elmer 1725 series FTIR spectrometer (Perkin–Elmer Inc., Norwalk, CT, USA) equipped with a room temperature deuterated triglycine sulfate detector and controlled by a Perkin–Elmer 7300 PC. The software used for collecting the FTIR data was the Spectrum version 5.3.1.

Circular dichroism spectrometry

Circular dichroism spectra for DOX, Ada-DOX, CDE₁, and CDE₁-Ada-DOX were recorded at 25°C in an Aviv model 215 circular dichroism spectrometer (Aviv Biomedicals Inc., Lakewood, NJ, USA) using a 0.1-cm cell for three scans with a 0.1 nm bandwidth. The degree of ellipticity (θ) is defined as the tangent of the ratio of the minor to major elliptical axis and the molar ellipticity ([θ]) is reported in deg•cm²•dmol⁻¹.

Cell proliferation assay

The MTT assay was conducted as described previously² to examine the effect of the synthesized CD-based compounds on the viability of MCF-7 and 3T3 cells. Briefly, cells were seeded into a 96-well culture plate at a density of 10⁵ cells/well. After 24 hour incubation, the cells were treated with the drug at 0.01 to 10 µM for 24 hours. Cell viability was determined by reduction of MTT to form insoluble purple formazan that was visualized using a UV spectrometer. The absorbance was measured using a Synergy H4 Hybrid microplate reader (BioTek Inc., Winooski, VT, USA) at a wavelength of 450 nm. The IC₅₀ values were determined using the relative viability over drug concentration curve by Prism 6.03 program (GraphPad Software Inc., La Jolla, CA, USA).
Cell culture
The human breast cancer MCF-7 cell line expressing a high level of ERs and mouse 3T3 fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). MCF-7 and 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. The cells were incubated in 5% CO₂ and 90–100% relative humidity at 37°C. Medium renewal was carried out 2–3 times per week, and cells were subcultured when they achieved 80–90% confluence.

Western blotting
An aliquot of total protein (40 µg) was analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electroblotting of gels onto polyvinylidene difluoride (PVDF) sheets (Millipore, Bedford, MA, USA), the filters were blocked at room temperature with the Tris-buffered saline Tween-20 (TBST) buffer (10 mM Tris-HCl, pH 8.0, 0.1% Tween 20, and 150 mM NaCl) containing 10% non-fat dry milk and then incubated in TBST buffer overnight at 4°C with the antibody to p-p44/42 MAPK (Thr202/Tyr204) (20G11), to ERα, or to β-actin. After washing with TBST buffer, blots were incubated for 1 hour at room temperature with mouse anti-rabbit IgG monoclonal antibody diluted 1:3,000 in TBST buffer and then revealed by ECL approach. The protein content was determined using the bicinchoninic acid (BCA) method after protein extraction from the cells.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)
The TEM images of CDE₁ and CDE₁-Ada-DOX were obtained on the JEM 100CX system (JEOL Ltd, Tokyo, Japan) with an accelerating voltage of 160 kV, and the SEM images were recorded on the JEOL JSM6490 scanning electron microscope (JEOL USA Inc. Peabody, MA, USA). The morphology of the CDE₁ vector and CDE₁-Ada-DOX supramolecules were evaluated by both TEM and SEM with different magnifications from 4 k to 40 k. An aqueous and DMF solution of CDE₁ and CDE₁-Ada-DOX was directly trickled onto a 300-mesh copper grid and dried in a vacuum oven at 35°C for 4 h, and the images were observed using TEM and SEM.

Determination of drug release from CDE₁-Ada-DOX using the dialysis method
The in vitro release profiles of the CDE₁-Ada-DOX inclusion complex and Ada-DOX were determined and compared using a dialysis method as described previously³ using the Synergy H4 hybrid multi-mode microplate reader coupled with Gen5 2.0 data analysis software and Take 3 nano-drop plate (BioTek Instruments Inc., Winooski, VT, USA). Briefly, emission spectra in DMF/H₂O (1:3, v/v) were obtained at different excitation wavelengths to determine the optimal values of λex and λem for spectrophotometry as 485 nm and 600 nm, respectively. Briefly, 2 mL of the drug solution was added to 2 mL PBS at pH 7.4 (0.01 M). The mixture was suspended in a dialysis bag (molecular weight cutoff = 3,000 Da) and dialyzed against 10 mL of PBS containing 50% FBS at 37°C with gentle shaking for 75 h. A 5 µL aliquot of the sample was withdrawn from the medium at designated time points and stored frozen for analysis. The released Ada-DOX was quantified by a microplate reader at λex = 490 nm and λem = 600 nm. A calibration curve was prepared using different concentrations of pure Ada-DOX. To determine the Kd and half-life (t₁/₂ = 0.69/Kd) values for the release of Ada-DOX from the complex in PBS solution...
against 50% FBS solution, the drug release-time curve was fitted using different binding models using Prism 6.03 program (GraphPad Software Inc., La Jolla, CA, USA). The model with the best fit gave the constants. The models we have tried included those with one binding site, two binding site, exponential one-phase association, exponential two-phase accusation, ligand-activator, ligand-inhibitor, sigmoid, and so on. The choice of the best model was confirmed by the F-test, comparison of the relative residuals and the standard error of the parameter estimates from the non-linear regression analysis, and comparison of the Akaike's information criterion (AIC) value.\textsuperscript{4}

**Drug uptake experiments using flow cytometry**

To evaluate drug uptake by cells at pre-determined concentrations of various compounds, CDE\textsubscript{1}, CDE\textsubscript{1}-Ada-DOX, or CD-Ada-DOX at final concentration (1, 2 or 5 \textmu M) were added into 35-mm petri dishes containing 2×10\textsuperscript{5} MCF-7 cells in 3.0 mL culture medium. The cells were incubated for 2–6 h under 5% CO\textsubscript{2} at 37 °C to allow uptake of the drug. Before analysis, the cells were gently washed with phosphate buffered saline (PBS) three times, trypsinized and resuspended in the medium after incubation. The collected cells were redispersed in 500 mL of fresh PBS and stained with 20 \textmu L 4', 6-diamidino-2-phenylindole (DAPI, used as a fluorescent stain for DNA) at 10 ng mL\textsuperscript{-1} for flow cytometric analysis. Flow cytometric analysis was performed on a BD FACScan system (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) by counting 10,000 events per sample. The fluorescence of Ada-DOX was measured with \lambda_{ex} at 490 nm and \lambda_{em} at 600 nm. The untreated cells incubated with DMEM alone (containing 10% FBS, supplemented with 1% of penicillin) were used as the control. Each assay was repeated in triplicate. Next, we conducted competition assay to check whether estrone (E\textsubscript{1}) and tamoxifen changed the drug uptake of CDE\textsubscript{1}-Ada-DOX in MCF-7 cells. Briefly, 5×10\textsuperscript{5} MCF-7 cells/per well were seeded into 35-mm petri dishes and incubated at 37°C with CDE\textsubscript{1}-Ada-DOX at 2 \textmu M for 6 h in the presence of E\textsubscript{1} at 0.1, 1, 5, 10, and 50 \textmu M, or tamoxifen at 0.1, 1, 5, and 10 \textmu M. Afterwards, the cells were washed with PBS three times, trypsinized, and re-suspended in the PBS. The collected cells were then re-dispersed and the fluorescence intensities were determined by a flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

**Statistical analysis**

Data are expressed as the mean ± standard deviation (SD). Data were analyzed using the one-way analysis of variance (ANOVA) followed by \textit{ad hoc} Tukey-Kramer’s test. For multi-group comparison with two factors, two-way ANOVA was used for the data analysis. Differences between two groups were analyzed using unpaired Student’s \textit{t}-test. A \textit{P} < 0.05 was considered statistically significant.
References:


