Supporting Information

Glutamine-β-Cyclodextrin for Targeted Doxorubicin Delivery to Triple-Negative Breast Cancer Tumors via the Transporter ASCT2

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Supplemental Methods

Materials and reagents for Synthesis.

Trifluoroacetic acid (TFA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), tert-Butyl bromoacetate (tBu) and sodium hydride (NaH, 60% dispersion in mineral oil) were purchased from J&K Chemical (Beijing, China). *N*,*N*-dimethylformamide (DMF, superdry) was obtained from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China).

Conjugation of tBu and β-CD

β-CD (11.3 g, 0.01 mol) was dissolved in 150 mL of DMF and dropwise added NaH (4 g, 0.1 mol) and tBu (19.5 g, 0.1 mol) at room temperature (RT) with stirring. The system was then heated to 80 °C for overnight reaction in nitrogen atmosphere. Subsequently, 50 mL of deionized water was added to the system to remove remaining NaH in ice bath. Then the system was precipitated with 2 L ethanol and washed twice with methanol. The precipitation was collected via vacuum suction filtration and dried in vacuum at 45 °C to give CD-tBu. (yield, 87.2%) The ^{1}H-NMR spectrum of CD-tBu was shown in Figure S1.

Synthesis of CD-COOH

CD-tBu (10 g, 8 mmol) was dissolved in 200 mL of deionized water at 30 °C and 30 mL of TFA was dropwise added with stirring. The system was then stirred at 30 °C overnight. The reaction mixture was dried via reduced pressure distillation with oil pump and washed twice with ethanol to give CD-COOH. (yield, 93.2%) The ^{1}H-NMR spectrum of CD-COOH was shown in Figure S2.

Synthesis of GLN-CD

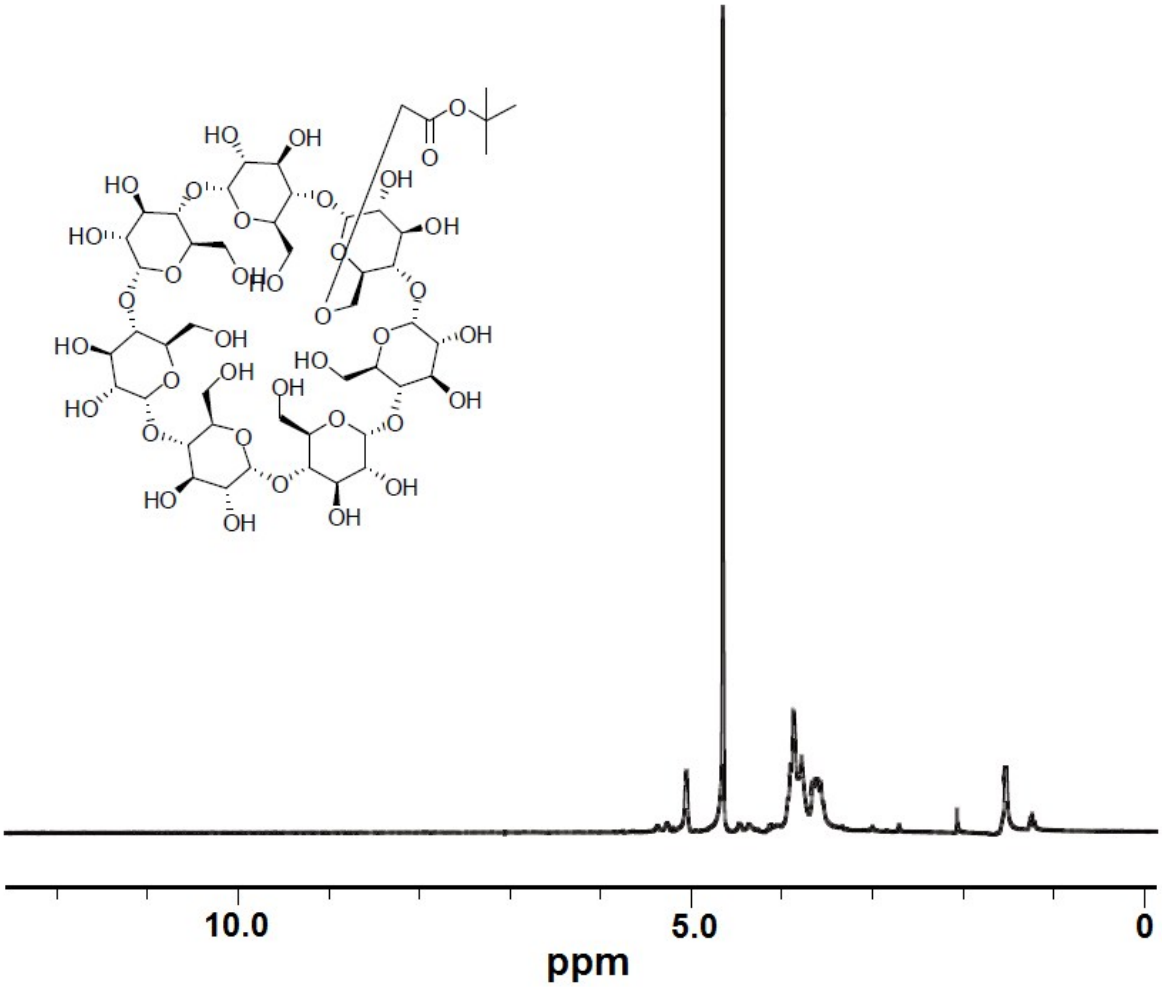
CD-COOH (2.4 g，2 mmol) was dissolved in 30 mL of deionized water. EDC·HCl (1.2 g, 6 mmol) and NHS (0.7 g, 6 mmol) were added and stirred for 30 min at RT to pre-activate the carboxyl of CD-COOH. The mixture was then added to GLN solution (1.2 g, 8 mmol) for

further 48 h reaction. The system was dried via reduced pressure distillation with oil pump and washed twice with methanol to remove remaining EDC and NHS. Subsequently, raw product was purified by cation exchange resin with deionized water as mobile phase. The mobile phase was collected and vacuum freeze dried to give GLN-CD. (yield, 61.6%)

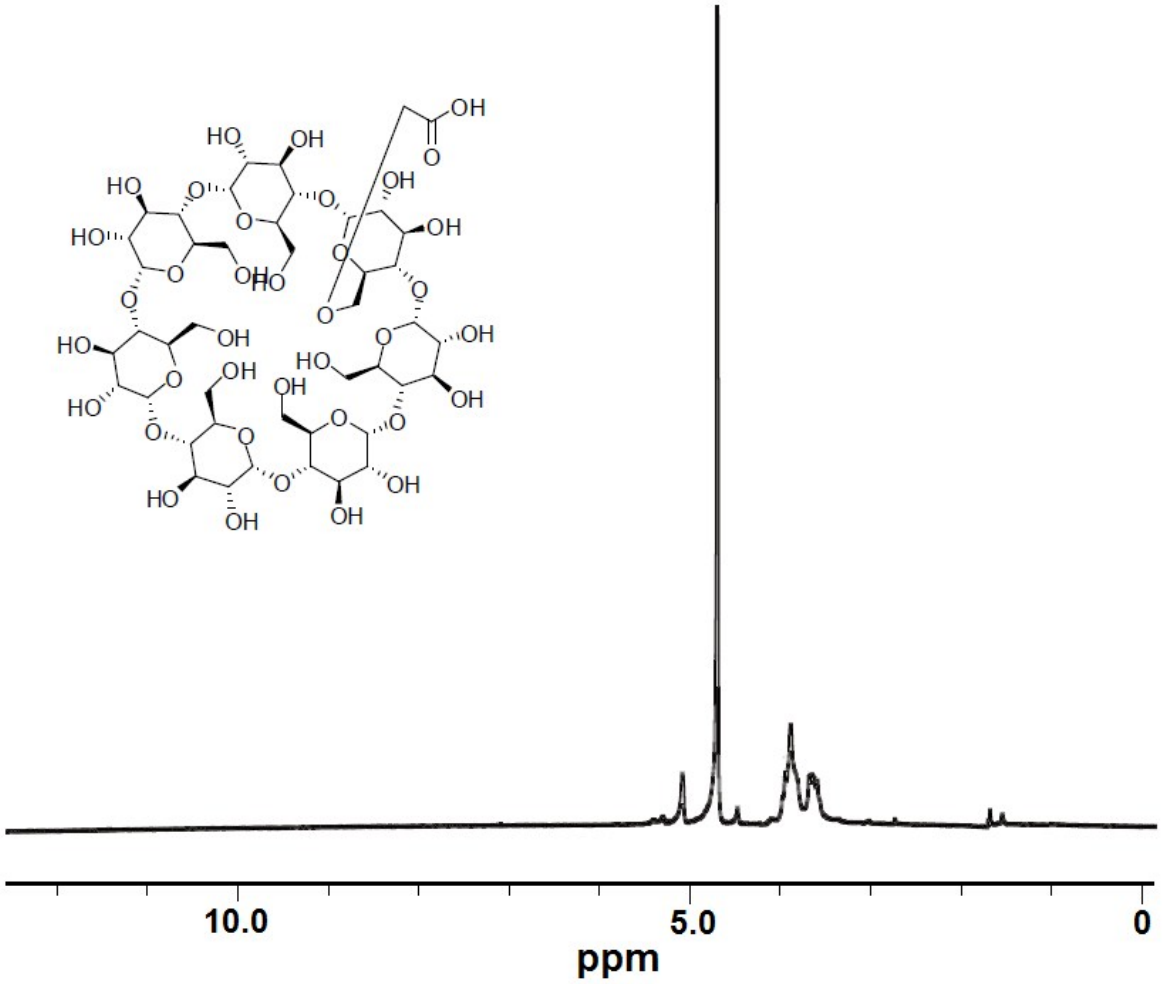
DOX analysis by HPLC assay

DOX was extracted from plasma (0.5 mL) and tissue homogenate (1 mL) by adding 2 and 4 mL of chloroform-methanol (4:1, v/v) mixture, respectively, with continuous stirring for 10 min. After centrifugation at 3000 rpm for 10 min, the organic phases were collected and evaporated to dryness at 30°C under a stream of nitrogen gas. The dry residues were then dissolved in 100 μL of methanol and 50 μL of the sample was injected into the HPLC column for analysis. The HPLC assay was performed with a Nova-Pak C18 column (4 μm, 150 mm × 3.9 mm) at a controlled temperature of 30°C. The mobile phase consisted of methanol, acetonitrile, 0.01 mol/L NH4H2PO4 and acetic acid (50:20:28:0.6, v/v) and the flow rate was 1.0 mL/min.

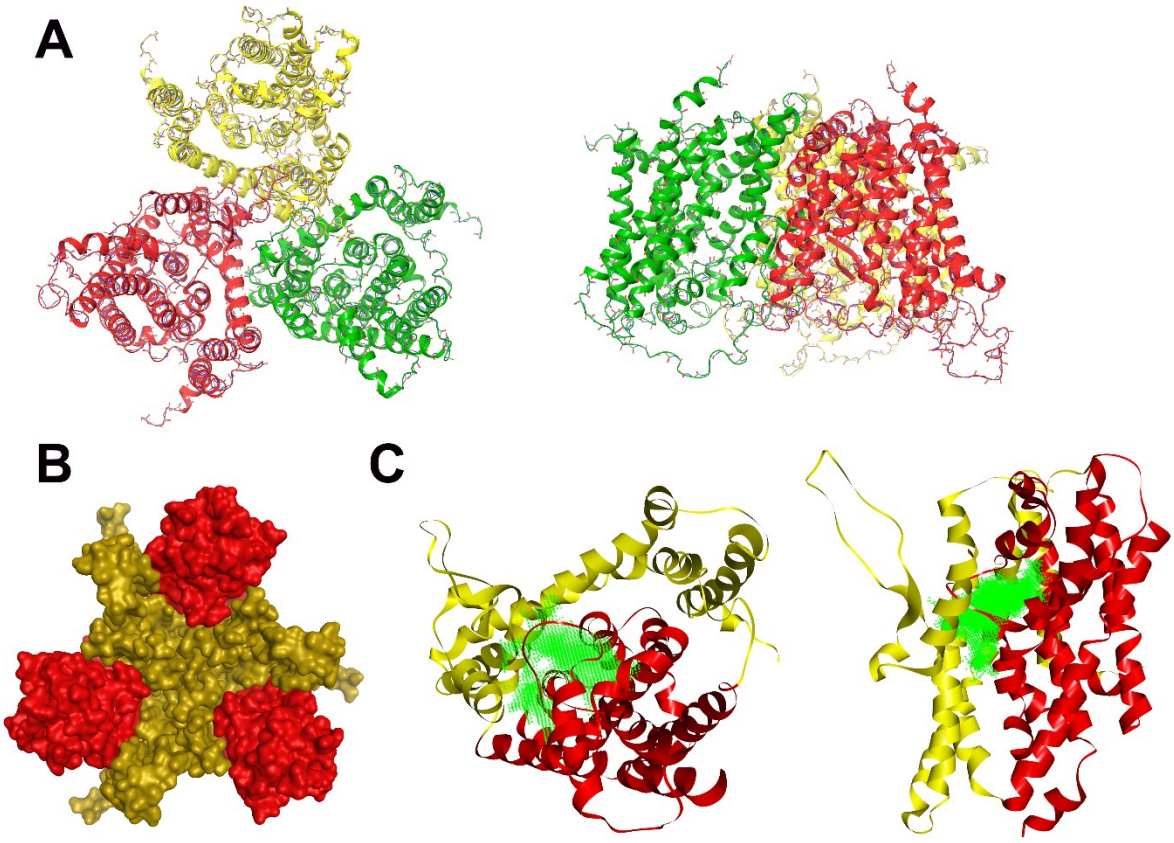
Supplemental Figures



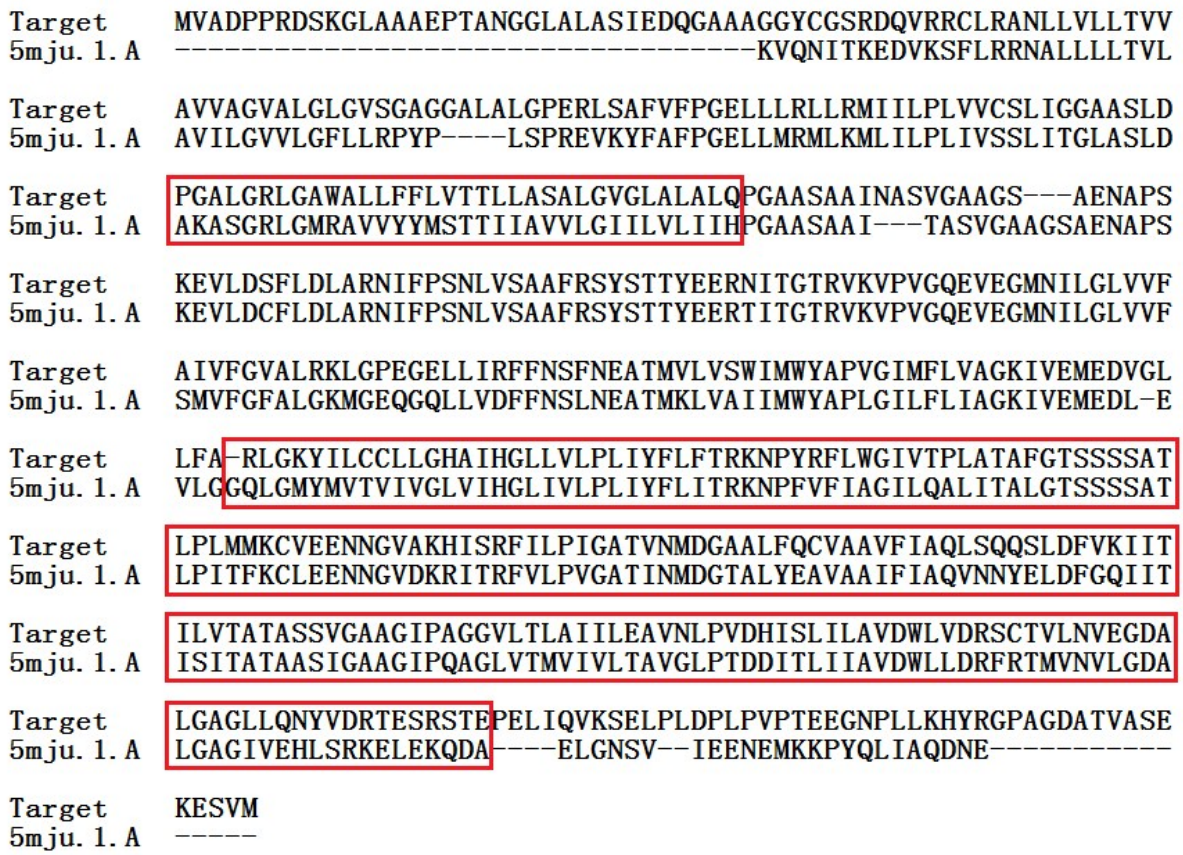
Supplementary Fig. 1. ^{1}H-NMR of CD-tBu.



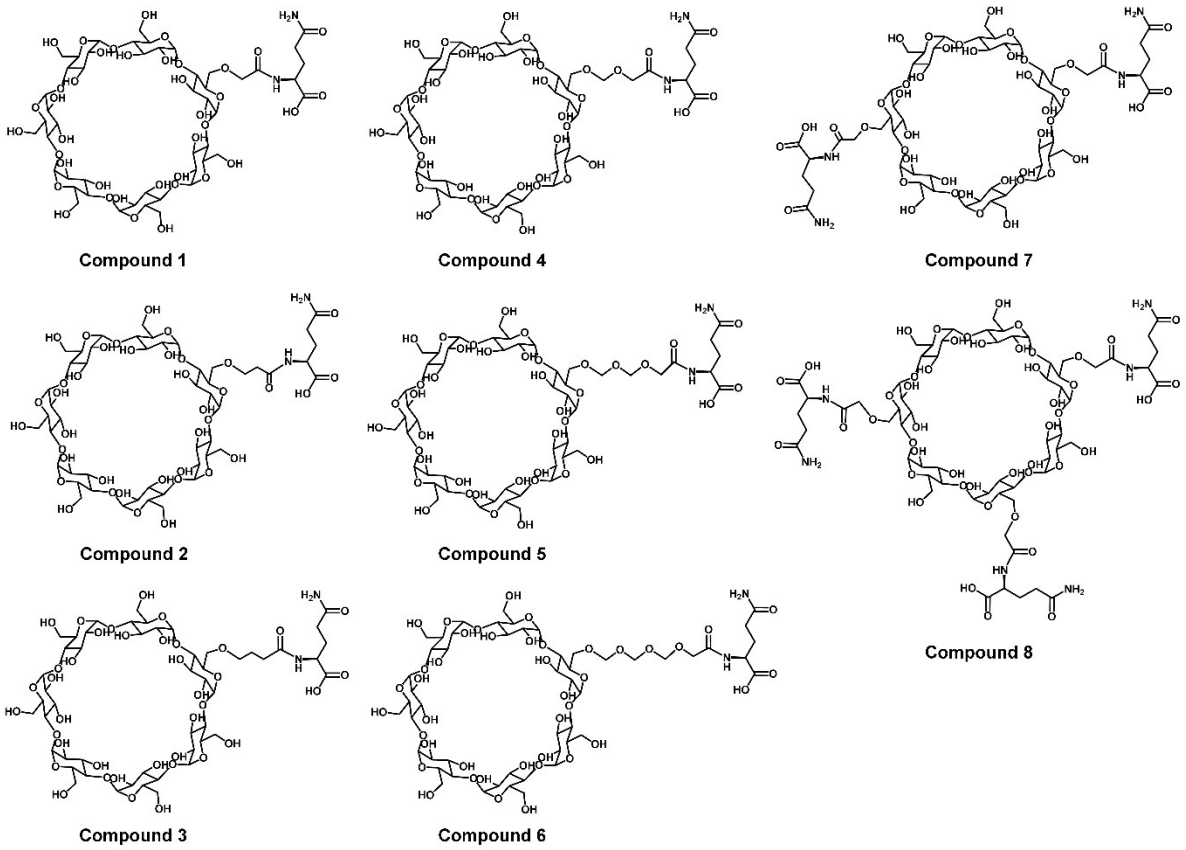
Supplementary Fig. 2. ^{1}H-NMR of CD-COOH.



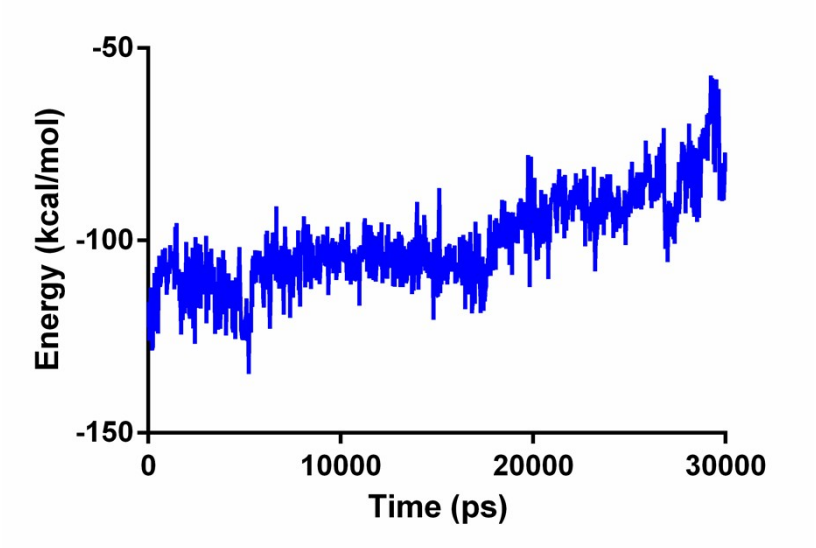
Supplementary Fig. 3. The model of ASCT2 was built based on the crystal structure of the homologous EAAT1 (5mju). (**A**) The structure of ASCT2 trimer viewed from the extracellular solution and from the membrane. Yellow, red and green indicate three respective chains. (**B**) Surface of the ASCT2 model. Red and yellow were used to highlight transport domain (TranD) and scaffold domain (ScaD), respectively. (**C**) ASCT2 monomer viewed from the extracellular solution and parallel to the membrane. Red, TranD; yellow, ScaD; green points, predicted GLN binding pocket by *Discovery Studio*.



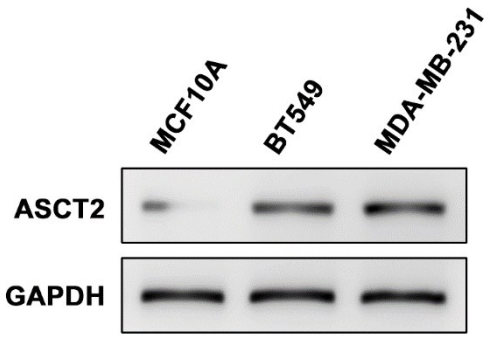
Supplementary Fig. 4. Sequence alignment between ASCT2 (NP_005619.1) and EAAT1 (5mju.A) by *Clustal*. Red boxes indicate TranD.



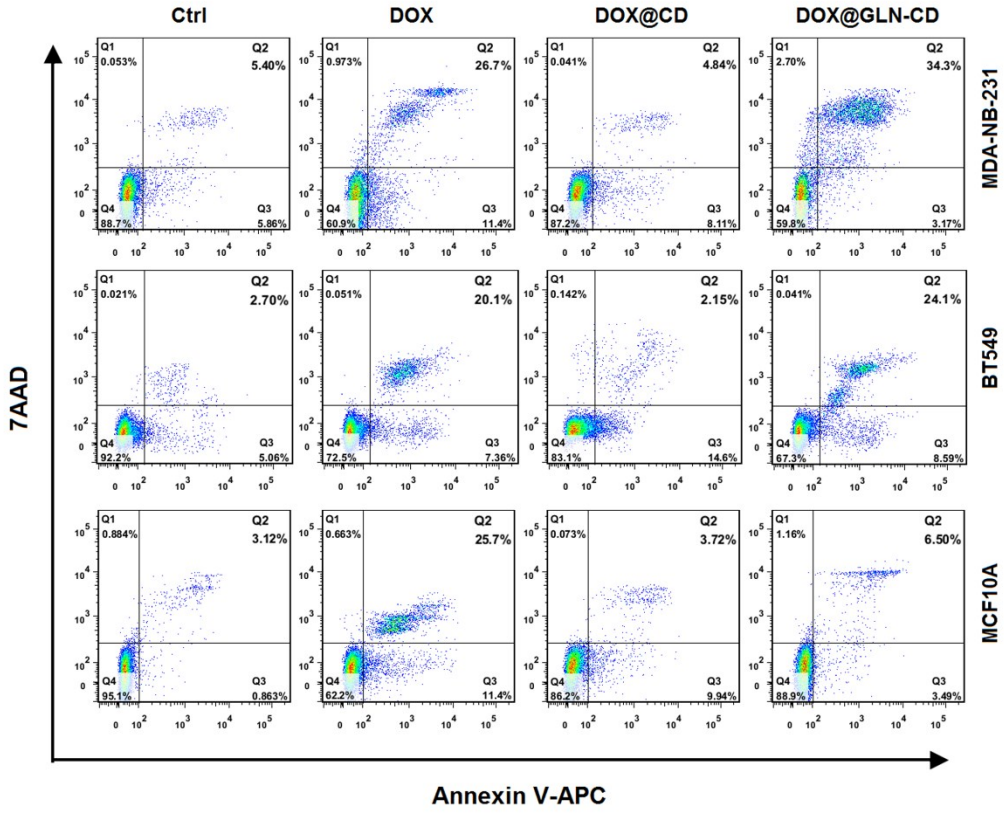
Supplementary Fig. 5. Structural formula of GLN-CD derivatives.



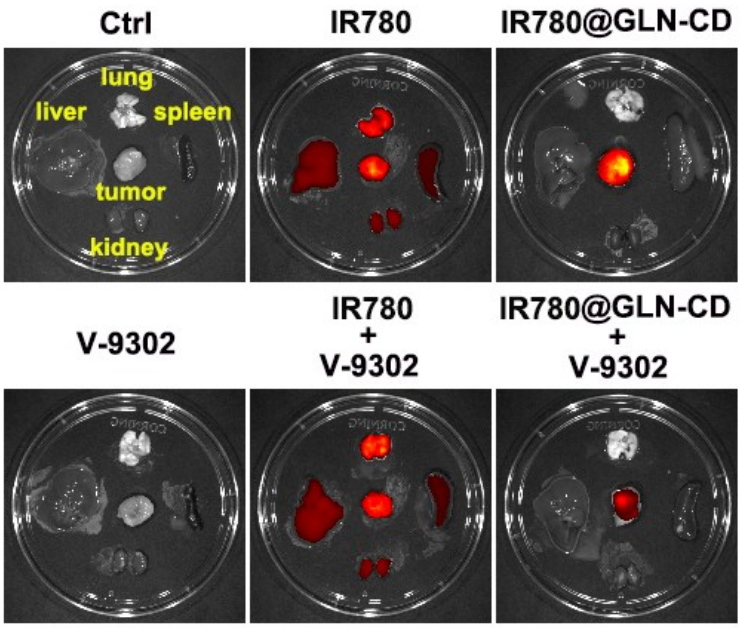
Supplementary Fig. 6. The detailed docking energy change of GLN interactions with ASCT2 residues during the entire simulation period.



Supplementary Fig. 7. The expression of ASCT2 in MCF10A, BT549 and MDA-MB-231 cells. Antibodies: anti-ASCT2 (1:2000; #8057 , Cell Signaling Technology) and anti-GADPH (1:1000; sc-47724, Santa Cruz Biotechnology)



Supplementary Fig. 8. Apoptosis induced by free DOX or inclusion complexes (3.68 μM) was detected by FCM via double staining of Annexin V and 7AAD.



Supplementary Fig. 9. The accumulation of IR780@GLN-CD in tumors was antagonistically decreased by ASCT2 inhibitor, V-9302 (S8818; Selleck, Houston, TX, USA). IR780@GLN-CD inclusion complexes were prepared using a saturated solution-based method with some modifications. After administered intraperitoneally with V-9302 (75 mg/kg) for 4 h, the tumor-bearing mice were administered intravenously with IR780@GLN-CD inclusion complexes (10 μg IR780 each mouse). Equivalent amount of IR780 was used as control. The mice were then sacrificed and their tissues (liver, spleen, lung, kidney, and tumor) were collected, washed with normal saline and subsequently imaged with the IVIS Spectrum (λex, 745 nm; λem, 800 nm) 6 h after injection.

Supplemental Tables

Table S1. The loading and encapsulation efficiency of DOX at various DOX/GLN-CD ratios.

DOX:GLN-CD	1:0.5	1:1	1:1.5	1:2
LE%	26.47±3.2	26.74±1.8	21.12±4.2	16.984±3.7
EE%	43.21±2.6	87.68±3.1	96.46±5.4	98.24±4.8

The loading efficiency (LE) and encapsulation efficiency (EE) of DOX were measured using the UV-based method. Each sample was detected at least thrice.

Table S2. IC50 of DOX and inclusion complexes in MDA-MB-231, BT549 and MCF10A cells.

Cell lines	MDA-MB-231	BT549	MCF10A
DOX	0.967±0.043	2.226±0.312	1.446±0.279
DOX@CD	NA	NA	NA
DOX@GLN-CD	1.681±0.151	3.410±0.422	NA

NA, not available. The IC50 was calculated with *GraphPad Prism 6*. Each sample was detected at least thrice.