

**Supplementary Information**  
**For**  
**Dual Hypoxia-responsive Supramolecular Complex for Cancer**  
**Target Therapy**

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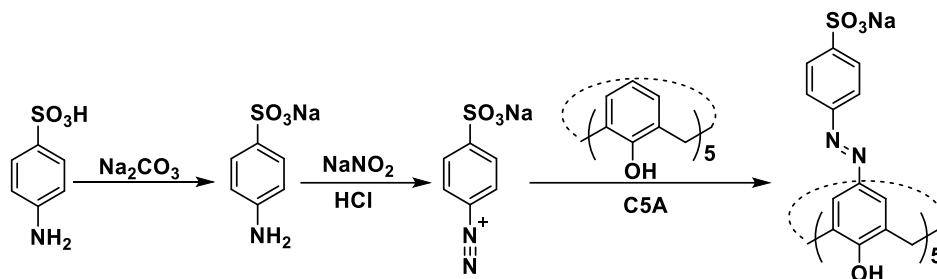
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## 1 Supplementary Methods

### 1.1 Synthesis of SAC5A.



Supplementary Figure 1. The synthetic route of SAC5A.<sup>1</sup>

**SAC5A:** Sulfanilic acid (1.732 g, 10 mmol) was dissolved in water (10 mL) containing sodium carbonate (0.518 g, 5.0 mmol) at 50–55 °C. A solution of  $\text{NaNO}_2$  (0.702 g, 10 mmol in 10 mL of water) was added to sulfanilic acid solution and the resulting mixture was added slowly to the concentrated  $\text{HCl}$  (4.0 mL) at 0–5 °C for 30 min and further stirred at this temperature for 1.5 h. The resultant 4-sulfobenzenediazonium chloride salt solution was slowly added into a solution of 25,26,27,28-tetrahydroxycalix[5]arene (C5A, 1.1 g, 2.0 mmol) and sodium acetate trihydrate (4.08 g, 30 mmol) in MeOH-DMF (26 mL, 5:8, v/v) to obtain a red suspension. The red mixture was allowed to stir for 2 h more in an ice bath, then acidified by 150 mL of aqueous  $\text{HCl}$  (0.25%) and warmed at 60 °C for 30 min to produce reagent as a reddish viscous solid. The residue was recrystallized with water/methanol (60 mL, 1:1, v/v), then the solution was cooled, filtered, and dried to provide a reddish solid (SAC5A) in a quantitative yield.

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  7.81 (s, 10H, calix-Ar-H), 7.77 – 7.71 (m, 20H, Ar-H), 3.96 (s, 10H, Ar- $\text{CH}_2$ -Ar) ppm;



40 °C for 2 h. TLC (DCM/MeOH = 10:1) showed most of BE was consumed. The resulting mixture was evaporated under reduced pressure to remove excess *N*-Methyl-piperazine. Then 5 mL of toluene was added to the residue and the mixture was evaporated under reduced pressure. The crude product was purified by silica gel chromatography (4% to 12% MeOH in DCM) to obtain a light-yellow NMP-BE (80 mg, 68%).

It was found that BE can transform into an insoluble solid during the concentration process after chromatography. To avoid/minimize product loss, we used BE-OH to generate NMP-BE as illustrated in the Supplementary Figure 3. To a solution of BE-OH (200 mg, 0.344 mmol, in 4 mL of anhydrous THF) stirred at 0 °C was added with Et<sub>3</sub>N (96 µL, 0.692 mmol) and EtSO<sub>2</sub>Cl (48 µL, 0.507 mmol). The mixture was stirred for around 30 min until TLC showed BE-OH was consumed. Then the reaction mixture was added with 100 µL of water to quench the excess EtSO<sub>2</sub>Cl and followed by addition of anhydrous Na<sub>2</sub>SO<sub>4</sub>. The mixture was filtrated, washed with EtOAc and the filtrate was concentrated under reduced pressure to obtain a residue. The residue was dissolved in 3 mL of anhydrous THF and added with 306 µL of *N*-Methyl-piperazine. The resulting mixture was heated at 40 °C for 2 h (note: Extending the reaction time to 48 h resulted the mess of the reaction according to <sup>1</sup>H-NMR and we speculated a product with C6-C7 double bond was formed via double bond migration) until most of the penultimate ethyl sulfonyl ester was consumed according to TLC analysis. Then the mixture was directly subjected to preparative TLC (DCM/MeOH (5:1)). The product attached in silica-gel powder was eluted with (DCM/MeOH (12:1)). The eluent was evaporated under reduced pressure to obtain ≈ 5.0 mL of solution. 2 mL of *t*-BuOH and 2.0 mL of H<sub>2</sub>O was added to the solution and the mixture was evaporated carefully. The resultant solution was lyophilized at –70 °C to obtain NMP-BE (105 mg, 46% for 2 steps) as a freeze-dried white powder.

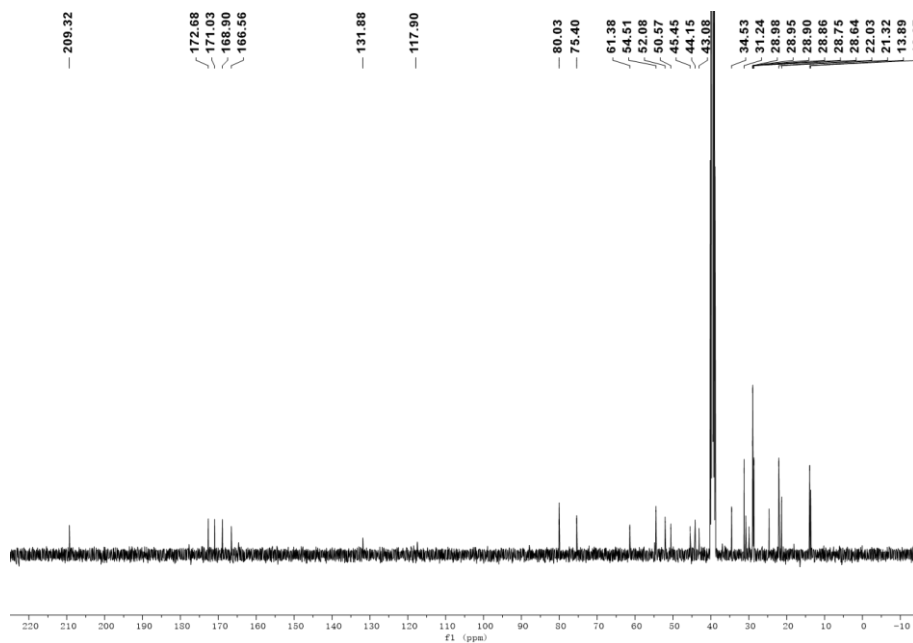
$[\alpha]^{14}_{\text{D}} = + 70.3$  ( $c = 0.5$ , CHCl<sub>3</sub> : MeOH = 1:1);

$\nu_{\text{max}}$  (KBr): 3430, 2959, 2924, 2854, 1750, 1659, 1520, 1460, 1402, 1261, 1194, 1054, 1033, 800, 772 cm<sup>-1</sup>;

<sup>1</sup>H NMR (400 MHz, DMSO) δ 8.48 (t, *J* = 6.0 Hz, 1H), 8.37 (s, 1H), 6.57 (s, 1H), 5.50 (t, *J* = 6.0 Hz, 1H), 5.16 (ddd, *J* = 9.9, 7.2, 2.8 Hz, 1H), 4.27 (d, *J* = 18.9 Hz, 1H), 4.03 (d, *J* = 18.9 Hz, 1H), 3.88 (dd, *J* = 15.9, 6.8 Hz, 1H), 3.58 (dd, *J* = 15.9, 5.2 Hz, 1H), 3.39 (m, 1H), 3.11 (d, *J* = 13.7 Hz, 1H), 2.97 – 2.86 (m, 2H), 2.80 (s, 3H), 2.74 (dd, *J* = 18.9, 5.7 Hz, 1H), 2.35 (s, 8H), 2.18 (s, 3H), 1.57 (s, 2H), 1.49 (s, 3H), 1.23 (br s, 22H), 1.02 (d, *J* = 6.8 Hz, 3H), 0.85 (t, *J* = 6.7 Hz, 3H) ppm;

**a**

**b**



Supplementary Figure 4. (a)  $^1\text{H}$  NMR spectrum of NMP-BE in  $\text{DMSO-}d_6$ , 400 MHz, 25 °C. (b)  $^{13}\text{C}$  NMR spectrum of NMP-BE in  $\text{DMSO-}d_6$ , 101 MHz, 25 °C.

The product obtained by method 2 was further subjected to HPLC analysis, the result indicated  $\approx 92.9\%$  purity. The instrument and condition for the HPLC analysis is listed below:

Instrument: SHIMADZU 20AT

Column: Sciences Inertsil ODS-SP 3  $\mu\text{m}$ , 4.6\*250 mm

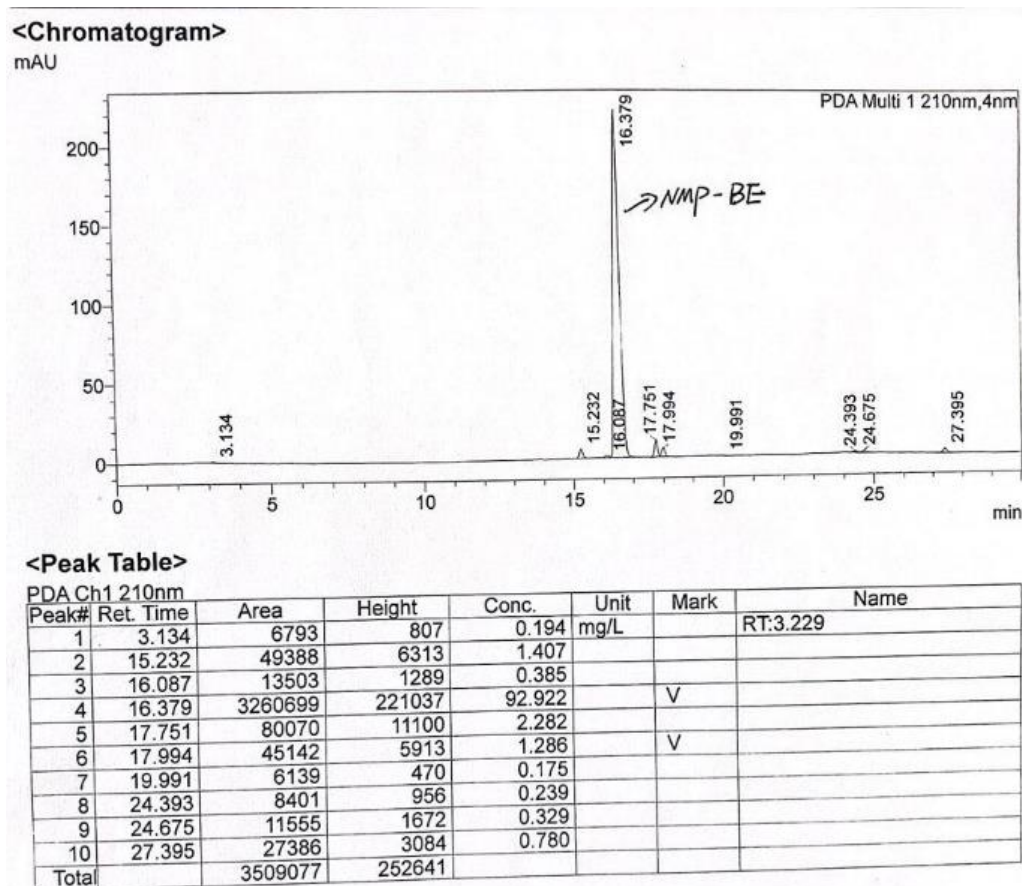
Detection wavelength: 210 nm

Velocity of flow: 1 mL/min

Diluent: 70%  $\text{CH}_3\text{CN}$

Mobile phase A:  $\text{CH}_3\text{CN}$ :  $\text{H}_2\text{O}$ :  $\text{H}_3\text{PO}_4$  = 10: 90: 0.1.

Mobile phase B:  $\text{CH}_3\text{CN}$ :  $\text{H}_2\text{O}$ :  $\text{H}_3\text{PO}_4$  = 90: 10: 0.1.



Supplementary Figure 5. The HPLC trace of NMP-BE obtained via method 2. NMP-BE could be further purified to 98.9% purity employing preparative liquid chromatography involving 2 steps. Firstly, the sample was subjected to preparative liquid chromatography. The instrument and condition are listed below:

Instrument: Agela FL-H050G

Column: GEMINI C18 250 x 21.2 mm, 5  $\mu$ m 110A

Mobile phase A: CH<sub>3</sub>CN: H<sub>2</sub>O: TFA = 10: 90: 0.05.

Mobile phase B: CH<sub>3</sub>CN: H<sub>2</sub>O = 90: 10.

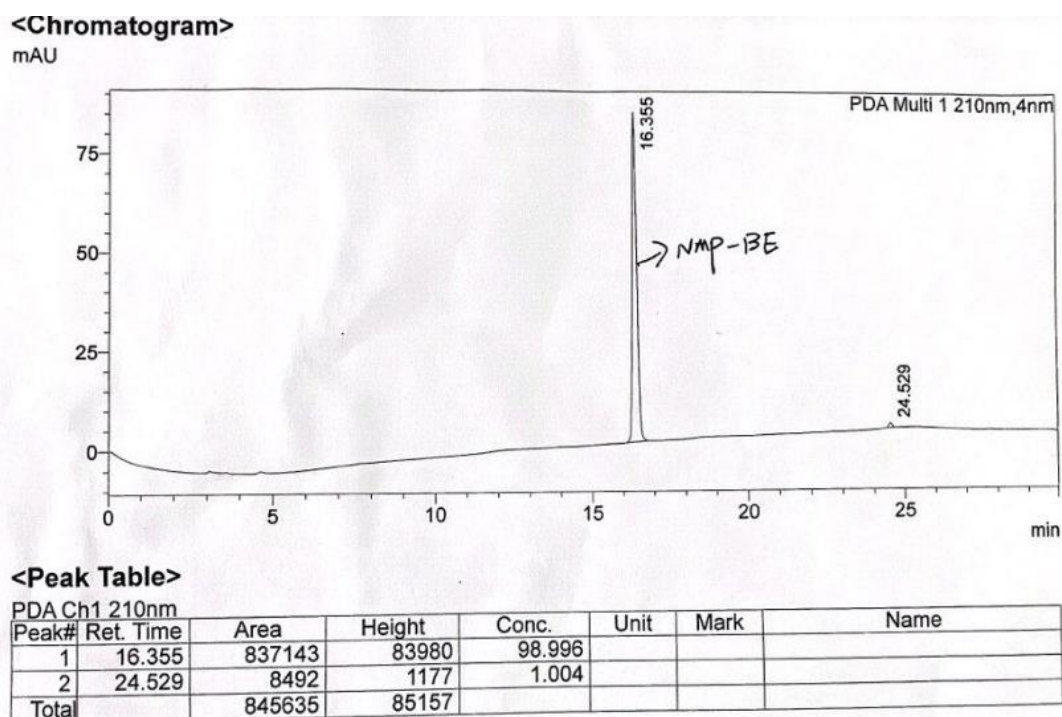
Then the eluent was concentrated and lyophilized to obtain a white powder, which was subjected to the second time preparative liquid chromatography.

Instrument: Agela FL-H050G

Column: GEMINI C18 250 x 21.2 mm, 5  $\mu$ m 110A

Mobile phase A: 10 mM NH<sub>4</sub>HCO<sub>3</sub> (with ammonia to adjust pH to 9).

Mobile phase B: CH<sub>3</sub>CN: H<sub>2</sub>O = 90: 10.



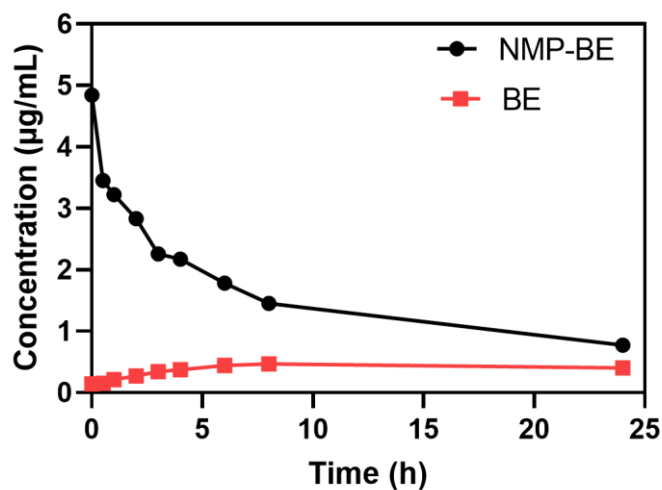
Supplementary Figure 6. The HPLC trace of NMP-BE after purification with preparative liquid chromatography.

Then the eluent was concentrated and lyophilized to obtain a white powder to store at  $-20^{\circ}\text{C}$ .



## 2 Supplementary Notes

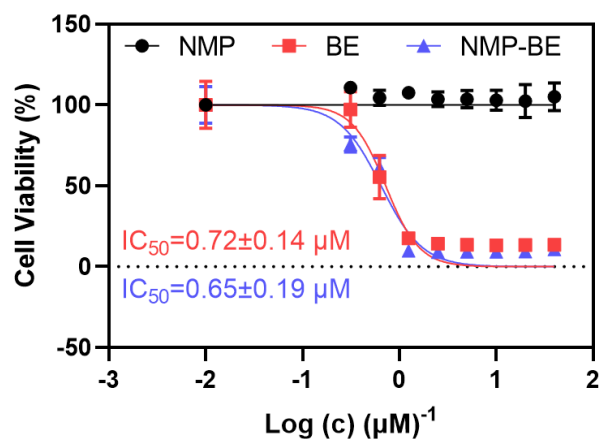
### 2.1 *In vitro* release rate of BE from prodrug NMP-BE



Supplementary Figure 7. *In vitro* release rate of BE. NMP-BE was prepared into a solution of HEPES (10 mM, pH = 7.4) at a concentration of 5 µg/mL. The buffer was extracted at different time points, and the peak area of NMP-BE or BE was detected by HPLC, and the concentration of NMP-BE or BE was calculated by external standard method.

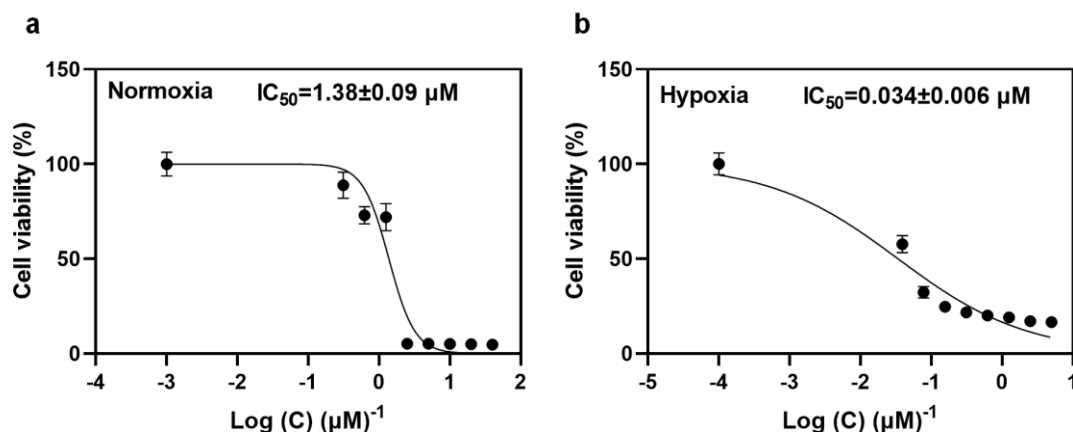
We speculate that the possible reason for the low concentration of released BE is mainly the low water solubility of BE, and BE was precipitated in HEPES buffer.

### 2.2 Cell viability and $IC_{50}$ of NMP, BE or NMP-BE against PANC1 cells via MTT assays



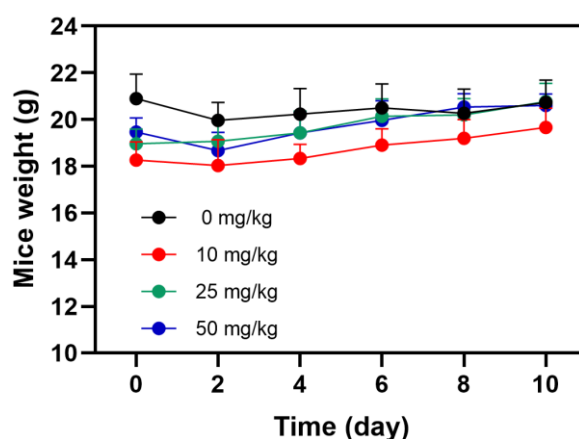
Supplementary Figure 8. Cell viabilities of PANC1 cells treated with various concentrations of NMP, BE or NMP-BE under normoxic conditions for 72 h ( $n = 6$  biologically independent samples).  $IC_{50}$  for BE =  $0.72 \pm 0.14 \mu M$ ;  $IC_{50}$  for NMP-BE =  $0.65 \pm 0.19 \mu M$ . Data are presented as mean  $\pm$  SD.

### 2.3 Hypoxia-selective cytotoxicity of NMP-BE in PANC1 cells



Supplementary Figure 9. Cell viabilities of PANC1 cells treated with various concentrations of NMP-BE under normoxic (a) or hypoxic (b) conditions for 24 h ( $n = 6$  biologically independent samples).  $IC_{50}$  for NMP-BE (Normoxia) =  $1.38 \pm 0.09 \mu M$ ;  $IC_{50}$  for NMP-BE (Hypoxia) =  $0.034 \pm 0.006 \mu M$ .

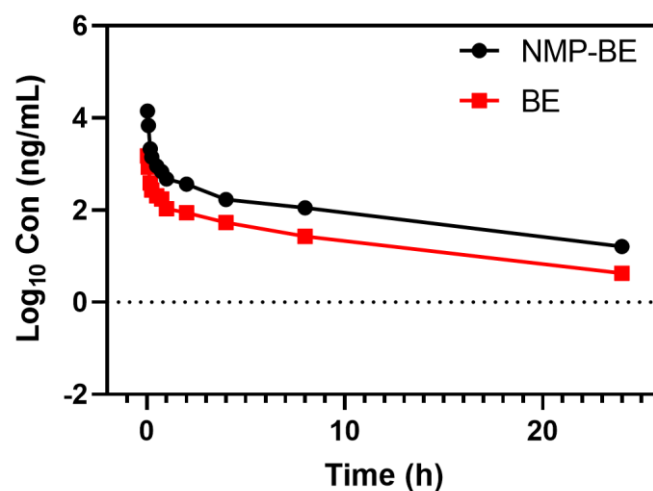
### 2.4 Acute toxicity of NMP-BE derivative in mice



Supplementary Figure 10. Weight curves of mice administrated with different concentrations (0, 10, 25, 50 mg/kg) of NMP-BE fumaric acid on the tail vein for 10

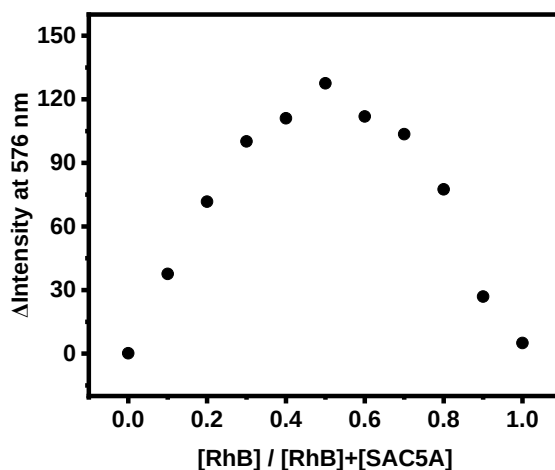
days. Data are presented as the mean  $\pm$  SD ( $n = 3$  mice per group).

## 2.5 Pharmacokinetic analysis of NMP-BE



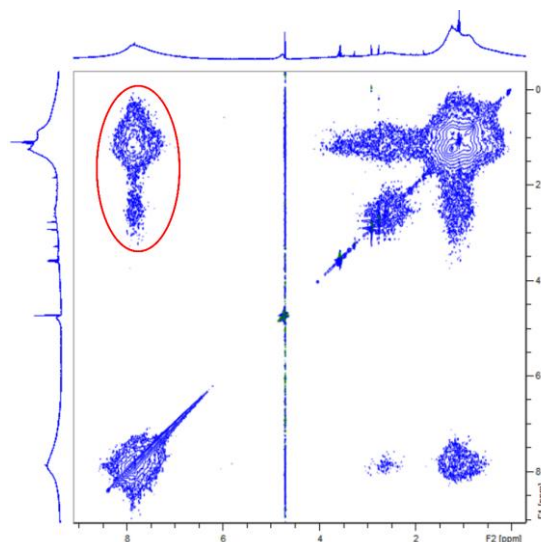
Supplementary Figure 11. PK study of NMP-BE (iv administration). BE and NMP-BE plasma concentration-time curve in mice after a single iv administration of NMP-BE (10 mg/kg). Data are presented as the mean  $\pm$  SD ( $n = 3$  mice per time point).

## 2.6 Job's plot for the complexation of RhB with SAC5A



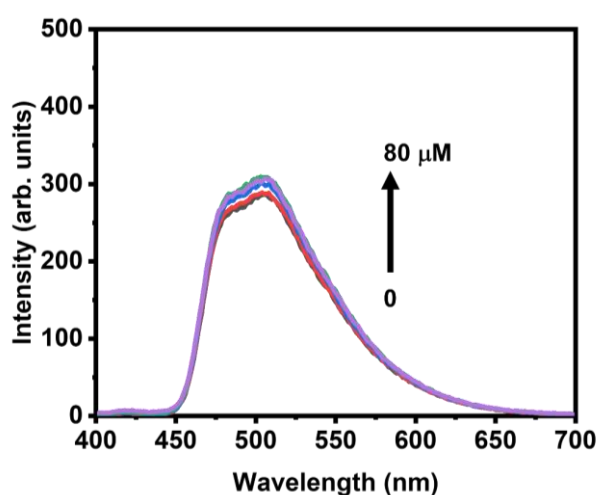
Supplementary Figure 12. Job's plot for solutions of RhB and SAC5A,  $\lambda_{\text{ex}} = 497$  nm,  $\lambda_{\text{em}} = 594$  nm,  $[\text{RhB}] + [\text{SAC5A}] = 1.0 \mu\text{M}$ .

## 2.7 The NOESY of NMP-BE@SAC5A



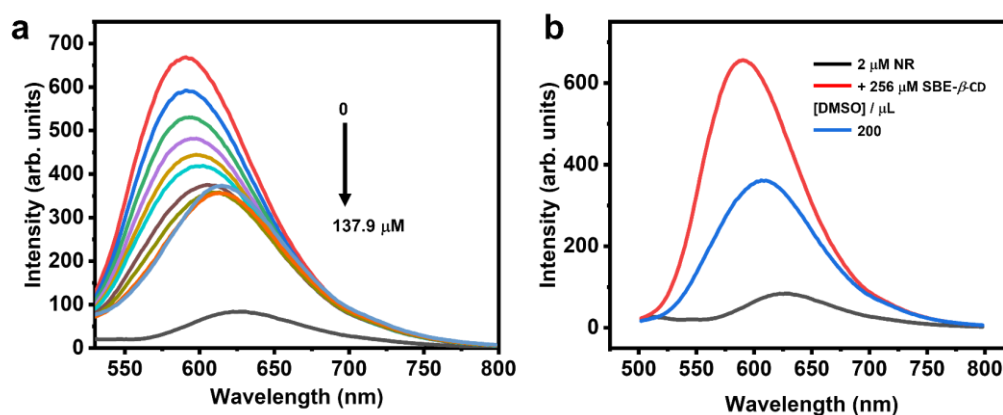
Supplementary Figure 13. The NOESY spectrum of NMP-BE@SAC5A in D<sub>2</sub>O, 400 MHz, 25 °C. It shows a cross-peak between the alkyl chain hydrogens on NMP-BE and the phenyl hydrogens on SAC5A, thus clearly indicating the host-guest interactions between NMP-BE and SAC5A (red circle). D<sub>2</sub>O passivates the associated hydrogen and broadens the peaks, so that the hydrogen correlation between molecules is a little scattered.

## 2.8 Fluorescence titrations of SC5A and SBE- $\beta$ -CD with NMP-BE



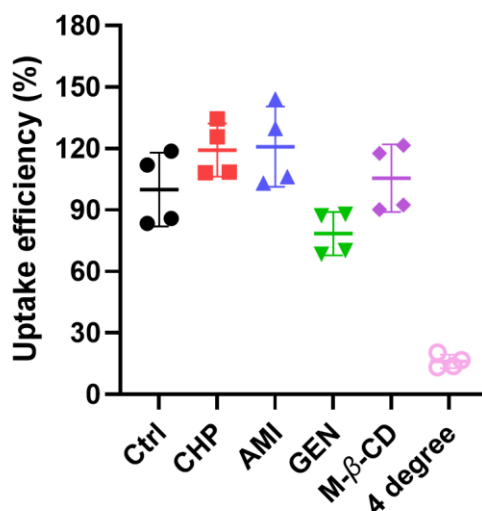
Supplementary Figure 14. Competitive fluorescence titration of LCG@SC5A (1.0/1.0  $\mu$ M) with NMP-BE (up to 80  $\mu$ M) in HEPES buffer (10 mM, pH = 7.4) at 25 °C,  $\lambda_{\text{ex}}$  =

368 nm. The 1:1 binding affinity between SC5A and LCG has been reported as  $1.48 \times 10^6 \text{ M}^{-1}$  in the previous work.<sup>2</sup>



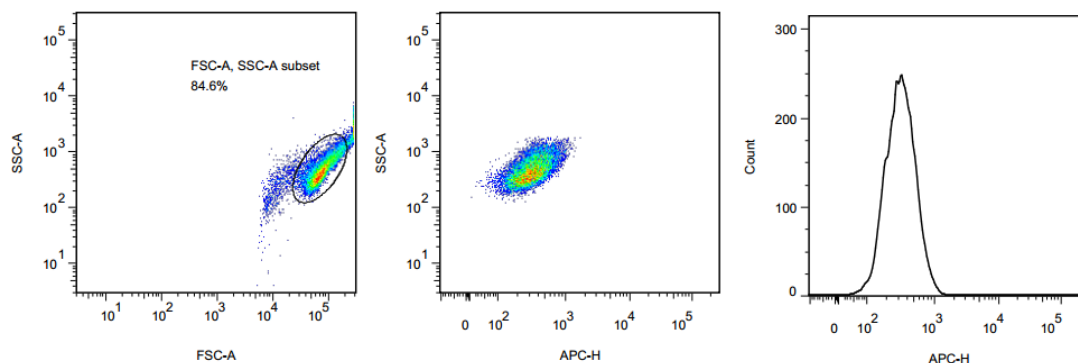
Supplementary Figure 15. (a) Competitive fluorescence titration of NR@SBE- $\beta$ -CD (2.0/256  $\mu\text{M}$ ) with NMP-BE (up to 137.9  $\mu\text{M}$ ) in HEPES buffer (10 mM, pH = 7.4) at 25  $^{\circ}\text{C}$ ,  $\lambda_{\text{ex}} = 500 \text{ nm}$ . (b) The effect of DMSO (the solvent for NMP-BE) on the host-guest pair.

## 2.9 The cellular uptake of CY5-DM@SAC5A

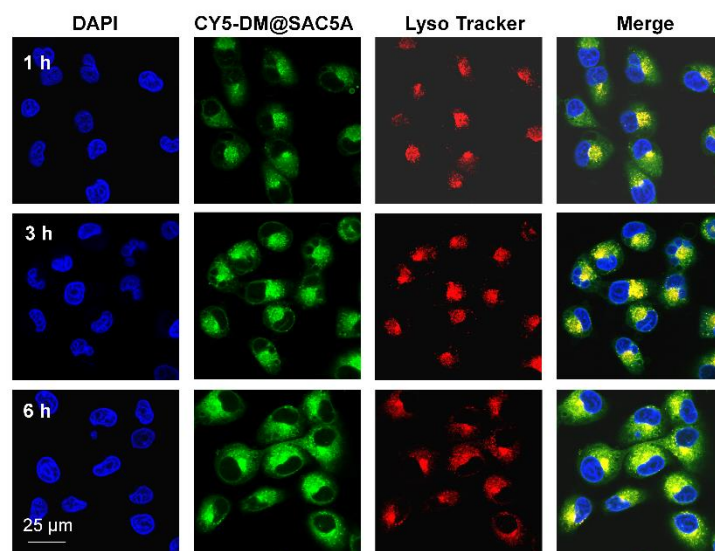


Supplementary Figure 16. Inhibition of CY5-DM@SAC5A uptake was studied using specific endocytosis inhibitors. Chlorpromazine (CHP): inhibitor of clathrin-mediated endocytosis; amiloride (AMI): inhibitor of macropinocytosis; Genistein (GEN): inhibitor of caveolae-mediated endocytosis; Methyl- $\beta$ -cyclodextrin (M- $\beta$ -CD): lipid rafts-mediated endocytosis; 4  $^{\circ}\text{C}$ : energy-mediated endocytosis. Data are presented as

mean  $\pm$  SD ( $n = 4$  biologically independent samples).

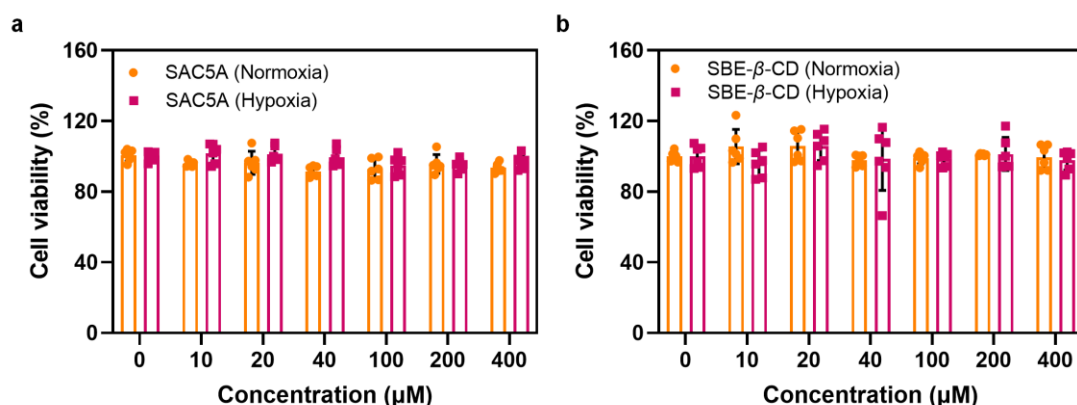


Supplementary Figure 17. Gating strategy for identification of the fluorescence of PANC1 cells treated with CY5-DM@SAC5A.



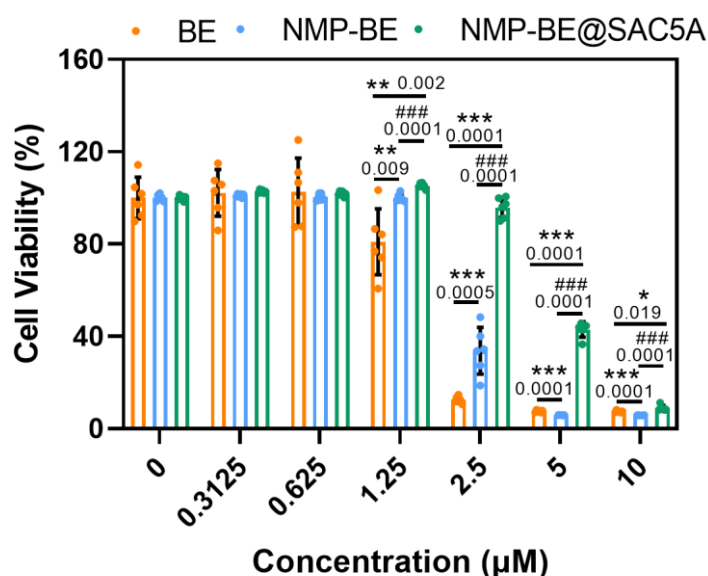
Supplementary Figure 18. Time-dependent cellular uptake of CY5-DM@SAC5A. PANC1 cells were treated with CY5-DM@SAC5A for 1, 3, and 6 h. and then *in vivo* co-localized with Lyso Tracker Red and imaged by CLSM. Blue: DAPI; Green: CY5-DM; Red: Lyso Tracker. Scale bar, 25  $\mu$ m. This was repeated independently for 3 times with similar results.

## 2.10 *In vitro* hypoxia imaging and cytotoxicity of SBE- $\beta$ -CD



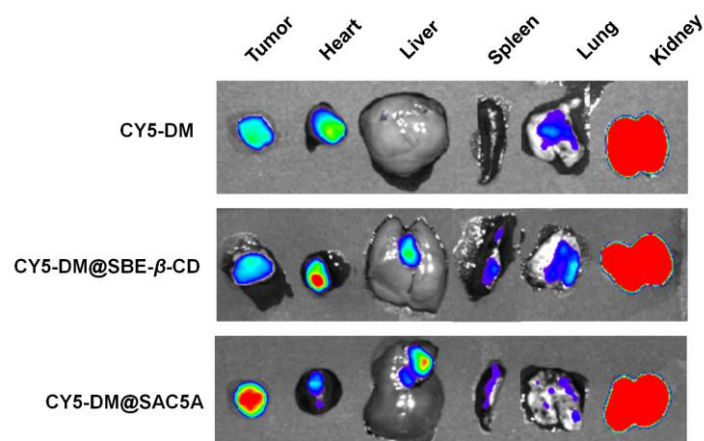
Supplementary Figure 19. The cytotoxicity of SAC5A and SBE- $\beta$ -CD. (a) Cell viability of PANC1 cells treated with various concentrations of SAC5A for 24 h. (b) Cell viability of PANC1 cells treated with various concentrations of SBE- $\beta$ -CD for 24 h. Data are presented as mean  $\pm$  SD ( $n = 6$  biologically independent samples).

## 2.11 *In vitro* cytotoxicity of BE, NMP-BE or NMP-BE@SAC5A in HPNE cells



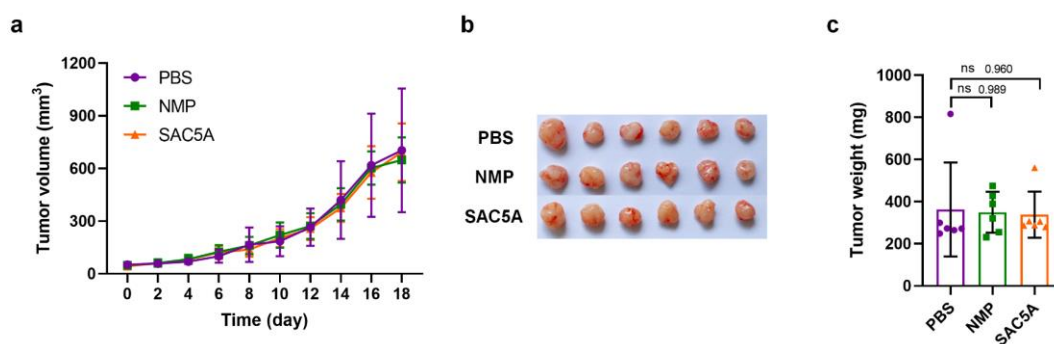
Supplementary Figure 20. Cell viability of HPNE cells treated with various concentrations of BE, NMP-BE or NMP-BE@SAC5A for 24 h. Data are presented as mean  $\pm$  SD ( $n = 6$  biologically independent samples).  $P$  values are calculated by paired  $t$  test: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus BE group; ### $P < 0.001$  versus NMP-BE group.

## 2.12 *Ex vivo* fluorescence imaging of CY5-DM@SAC5A



Supplementary Figure 21. The fluorescence imaging of the major organs *in ex vivo* at 24 h post injection of CY5-DM, CY5-DM@SBE- $\beta$ -CD and CY5-DM@SAC5A.

## 2.13 *In vivo* anticancer effects of NMP and SAC5A



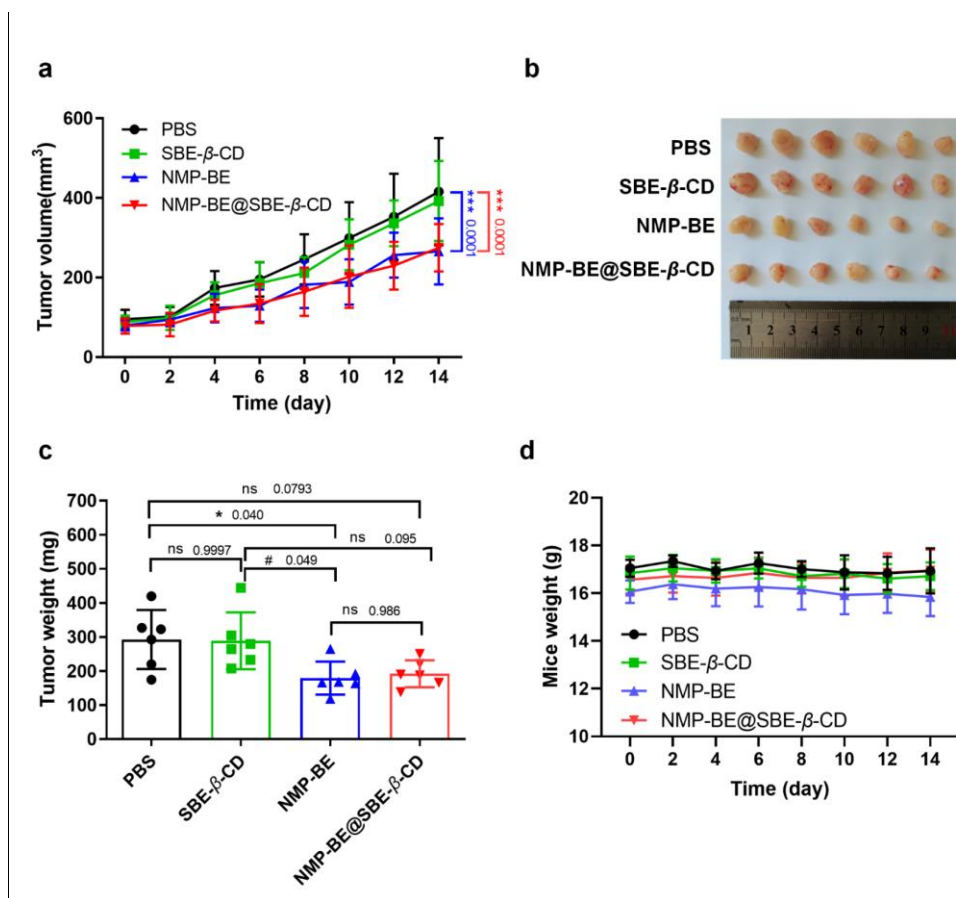
Supplementary Figure 22. *In vivo* anticancer effects of NMP and SAC5A. (a) Average tumor volume growth curves of mice treated with PBS, NMP, and SAC5A. Data are presented as mean  $\pm$  SD ( $n = 6$  mice per group).  $P$  values are calculated by two-way ANOVA with Turkey's multiple comparisons test: ns: no significance; (b) Images of dissected tumors in various groups. (c) Weights of tumors from the mice after treatment. Data are presented as mean  $\pm$  SD ( $n = 6$  biologically independent samples).  $P$  values are calculated by one-way ANOVA with Turkey's multiple comparisons test: ns: no significance.

Note: (b) During the experiment, the tumor images were taken of PBS vs NMP, PBS vs SAC5A groups respectively. As the PBS group consisted of the same tumors, the tumor



images of all three groups were ultimately combined.

## 2.14 *In vivo* anticancer effects of NMP-BE@SBE- $\beta$ -CD



Supplementary Figure 23. *In vivo* anticancer effects of NMP-BE@SBE- $\beta$ -CD. (a) Average tumor volume growth curves of mice treated with PBS, SBE- $\beta$ -CD, NMP-BE, and NMP-BE@SBE- $\beta$ -CD. Data are presented as mean  $\pm$  SD ( $n = 6$  mice per group).  $P$  values are calculated by two-way ANOVA Turkey's multiple comparisons test: \*\*\* $P < 0.001$  versus PBS group; (b) Images of dissected tumors in various groups. (c) Weights of tumors from the mice after treatment. Data are presented as mean  $\pm$  SD ( $n = 6$  biologically independent samples).  $P$  values are calculated by one-way ANOVA Turkey's multiple comparisons test: \* $P < 0.05$  versus PBS group; # $P < 0.05$  versus SBE- $\beta$ -CD group; ns: no significance. (d) Weight changes of the mice in different groups (PBS, SBE- $\beta$ -CD, NMP-BE and NMP-BE@SBE- $\beta$ -CD). Data are presented as mean  $\pm$  SD ( $n = 6$  mice per group).

## Supplementary References

1. Yue, Y. X., et al. Promoting tumor accumulation of anticancer drugs by hierarchical carrying of exogenous and endogenous vehicles. *Small Struct.* 2200067 (2022).
2. Yu, H., Geng, W. C., Zheng, Z., Gao J. Guo, D. S. & Wang, Y. Facile fluorescence monitoring of gut microbial metabolite trimethylamine *N*-oxide via molecular recognition of guanidinium-modified calixarene. *Theranostics* **9**, 4624–4632 (2019).