On the Encapsulation and Assembly of Anticancer Drugs in a Cooperative Fashion

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General Information

All solvents were dried before use. All materials were obtained from commercial suppliers and used without further purification. Analytical thin-layer chromatography (TLC) was performed on silica-gel plates w/UV254. ¹H NMR and ¹³C NMR spectra were recorded on 400, 600 or 700 MHz spectrophotometers. UV-Vis spectra were measured using Shimadzu UV-2401PC spectrophotometer. Fluorescence spectra were recorded with Shimadzu RF-5301. Dynamic light scattering (DLS) measurements were completed with Zetasizer NanoZS instrument (ZEN3600). Transmission electron microscopy (TEM) images were recorded with FEI Tecnai G2 Spirit TEM microscope working at 80 kV. Specimens for cryo-TEM imaging were prepared using Vitrobot (FEI, Hillsboro, OR). All TEM grids used for cryo-TEM imaging were pretreated with plasma air to render the lacey carbon film hydrophilic. Samples of 1^{6-} , $1^{6-} \subseteq 2^{2^{2+}}$, and $1^{6-} \subseteq 3^+$ were imaged at following concentrations: 0.6 mM solution of 1^{6-} ; for $1^{6-} \simeq 2_{2^{2+}}$, 0.1 mM of 1^{6-} and 0.2 mM of 2^{+} ; for $1^{6} \subset 3^{+}$, 0.2 mM of 1^{6} and 0.2 mM of 3^{+} (all samples in 10 mM phosphate buffer at pH = 7.0 ± 0.1). 5 µL of each sample solution was loaded onto a copper grid coated with lacey carbon film (Electron Microscopy Sciences, Hatfield, PA) in a controlled humidity chamber and subsequently blotted by two pieces of filter paper from both sides of the grid. This process engenders a thin film of solutions (typically ~300nm). The blotted samples were then plunged into liquid ethane that was precooled by liquid nitrogen. The vitrified samples were stored in liquid nitrogen before cryo-TEM imaging. To prevent sublimation, crystallization, and melting of the vitreous ice film, the cryo-holder temperature was maintained below -170 °C during the entire imaging process. Cryo-TEM imaging was conducted on a FEI Tecnai 12 TWIN electron microscope operating at a voltage of 100 kV. Crvo-TEM micrographs were acquired using a 16-bit 2KÅ~2K FEI Eagle bottom mount camera.

Synthetic Procedures and Characterizations



Scheme S1. Synthesis of dual-cavity basket 1.

Synthetic procedure: The synthetic route for obtaining **1** is shown in Scheme S1. Compound **5** was obtained by following literature procedure (*RSC Advances*, *5*, 66339-66354) while compound **4** by using our recently reported methodology (*Chem. Eur. J.* **2019**, *25*, 1115).

Compound 6: Compound **4** (100 mg, 0.18 mmol) was suspended in 12 mL of anhydrous toluene. Compound **5** (203 mg, 0.56 mmol) was added, followed by stirring for 10 min and the addition of 1.2 mL of pyridine. The mixture was refluxed for 18 h with the Dean-Stark apparatus. Following, the solvent was removed under vacuum to give a solid residue. The residue was dissolved in dichloromethane, which was successively washed with diluted HCl, water and brine. The organic layer was dried with anhydrous Na₂SO₄ and the solvent removed under reduced pressure. The crude product was purified by column chromatography (SiO₂, dichloromethane/methanol = 80:1) to yield 115 mg (73%) of **6** as a yellow solid. ¹H NMR (600 MHz, CDCl₃) δ (ppm) = 7.80 (s, 4H), 7.36-7.28 (m, 10H), 5.52 (s, 2H), 5.07 (s, 4H), 3.71 (t, *J* = 6.8 Hz, 4H), 2.38 (t, *J* = 7.4 Hz, 4H), 2.03 – 1.94 (m, 4H). ¹³C NMR (151 MHz, CDCl₃) δ (ppm) = 172.30, 167.55, 148.51, 135.82, 131.14, 128.97, 128.54, 128.23, 128.20, 118.55, 66.38, 61.25, 37.36, 31.49, 23.80. HRMS (ESI): calculated (M+Na)⁺= 875.0402, found 875.0343.

Compound 7: Under an atmosphere of nitrogen, compound 6 (115 mg, 0.13 mmol) was dissolved in 14 mL of anhydrous dioxane, followed by the addition of: Bu₄NBr (174 mg, 0.54 mmol), 4A molecular sieves (174 mg), K₂CO₃ (374 mg, 2.7 mmol), PPh₃ (14 mg, 0.054 mmol) and Pd(OAc)₂ (7.3 mg, 0.027 mmol). The solution was kept stirring at 90° overnight. The solvent was evaporated under vacuum and the residue dissolved in dichloromethane. Following, dichloromethane was washed with water, brine and dried with anhydrous Na_2SO_4 . The evaporation of the solvent gave crude product, which purified by the column chromatography was $(SiO_2,$

dichloromethane/methanol = 80:1) to give 40 mg (43%) of compound 7 as a yellow solid. ¹H NMR (400 MHz, CDCl₃) δ (ppm) = 7.88 (s, 2H), 7.31 – 7.26 (m, 4H), 6.18 (s, 1H), 5.02 (s, 2H), 3.63 (t, J = 6.7 Hz, 2H), 2.30 (t, J = 7.4 Hz, 2H), 2.03 – 1.76 (m, 2H). ¹³C NMR (151 MHz, CDCl₃) δ (ppm) = 172.24, 167.30, 149.04, 135.79, 134.75, 131.22, 128.51, 128.20, 128.15, 118.98, 66.34, 50.65, 37.33, 31.47, 23.75. HRMS (ESI): calculated (M+Na)⁺= 2100.6401, found 2100.6390.

Compound 1: Compound 7 (20 mg, 0.01 mmol) was dissolved in 5 mL of glacial acetic acid containing HBr (33%) and stirred at 60 ° for 6 h. The solvent was evaporated under vacuum and the resulting solid washed with water and dichloromethane to yield 12 mg (80%) of compound **1** as a pale yellow solid. ¹H NMR (700 MHz, DMSO-d₆) δ (ppm) = 11.92 (s, 1H), 8.21 (s, 2H), 6.84 (s, 1H), 3.50 (t, J = 5.8 Hz, 2H), 2.15 (t, J = 7.1 Hz, 2H), 1.78 – 1.57 (m, 2H). ¹³C NMR (176 MHz, DMSO-d₆) δ (ppm) = 173.81, 167.72, 150.10, 135.10, 130.30, 119.12, 48.69, 36.89, 30.78, 23.13. HR-MS (ESI): calculated (M+Na)⁺= 1559.3551, found 1559.3511.



Figure S1. ¹H NMR spectrum (600 MHz, CDCl₃) of compound 6 at 298 K.



Figure S2. ¹³C NMR spectrum (151 MHz, CDCl₃) of compound 6 at 298 K.



Figure S3. ¹H NMR spectrum (400 MHz, CDCl₃) of compound 7 at 298 K.



Figure S4. ¹³C NMR spectrum (151 MHz, CDCl₃) of compound 7 at 298 K.



Figure S5. ¹H NMR spectrum (700 MHz, DMSO-d₆) of compound 1 at 298 K.



Figure S6. ¹³C NMR spectrum (176 MHz, DMSO-d₆) of compound 1 at 298 K.



Figure S7. Fluorescence spectra of doxorubicin (2.0 μ M, $\lambda_{ex} = 500$ nm) without and with basket 1⁶⁻ (2.4 to 24 μ M) in CH₃OH at 298 K.



Figure S8. A change in the emission intensity of 1.0 μ M doxorubicin **2**⁺ (10 mM PBS at pH = 7.0) as a function of the increasing concentrations of **1**⁶⁻ was subjected to global (550–590 nm) nonlinear regression analysis using 2:1 stoichiometric model (see supramolecular.org, *Chem. Soc. Rev.*, **2011**, *40*, 1305-1323); a random distribution of residuals is shown in the bottom plot. For the titration experiment in Figure 3B, $K_1 = 370070.81 \ (\pm 6.8\%)$ and $K_2 = 7998094.41 \ (\pm 4.03\%)$. The reported $K_1 = 3.2 \pm 0.8 \cdot 10^5 \ M^{-1}$ and $K_2 = 9 \pm 1 \cdot 10^6 \ M^{-1}$ represent the arithmetic mean and standard deviation from two independent measurements.

Zeta Potential Distribution



Figure S9. Zeta ζ potential (the red curve peaks at -33.6 mV) corresponding to the mixture of 0.2 mM basket 1⁶⁻ and 0.4 mM doxorubicin 2⁺ in 10 mM PBS buffer at pH=7.0 was measured using DLS (Zetasizer NanoZS instrument, ZEN3600).



Figure S10. A change in the emission intensity of 3^+ (13.3 µM in 10 mM PBS at pH = 7.0) as a function of the increasing concentrations of 1^{6-} was subjected to global (520 540 nm) nonlinear regression analysis using 1:1 stoichiometric model to give $K_1 = 21327.18$ (±0.61%) M⁻¹ (see supramolecular.org, *Chem. Soc. Rev.*, **2011**, 40, 1305-1323); a random distribution of residuals is shown in the bottom plot. For the titration experiment in Figure 4C, $K_1 = 21258.74$ (±0.8%). The reported $K_1 = 2.12 \pm 0.01 \cdot 10^4$ M⁻¹ represents the arithmetic mean and standard deviation from two independent measurements.



Figure S11. The size distribution of particles (DLS) for 10 mM PBS buffer at pH=7.0 containing 0.6 mM basket 1⁶⁻ and topotecan **3**⁺ obtained from DLS measurement at 298 K.



Figure S12. TEM image of a mixture of 0.2 mM solution of basket 1^{6-} and topotecan 3^+ in 10 mM PBS buffer at pH=7.0. The solution was deposited on a copper grid and stained with uranyl acetate.



Figure S13. Cryo-TEM image of 0.2 mM solution of basket 1⁶⁻ and topotecan **3**⁺ in 10 mM PBS buffer at pH=7.0.



Figure S14. (Left) Fluorescence spectra of 10 mM PBS solution of 20 μ M doxorubicin **2**⁺ and 10 μ M basket **1**⁶⁻ ($\lambda_{ex} = 500$ nm) were obtained by lowering pH of the solution (shown in the inset). (Right) A plot showing fluorescence intensity of **1**⁶⁻ at 585 nm as a function of the solution's pH. As a note, the fluorescence of DOX **2**⁺ is quenched when residing inside basket **1**⁶⁻. The release of the encapsulated drug leads to increase in its fluorescence; for a control experiment, see Figure S15.



Figure S15. (Left) Fluorescence spectra of 10 mM PBS solution of 20μ M doxorubicin 2^+ at different pH values of the solution. (Right) Fluorescence intensity of 1^{6-} at 585 nm as a function of the solution's pH. The experiment demonstrates a rather small change in the fluorescence intensity of DOX 2^+ within the examined pH range.



Figure S16. (Left) Fluorescence spectra of 10 mM PBS solution (pH = 7.0) of 10 μ M doxorubicin 2⁺ and 5 μ M basket 1⁶⁻ in the presence of increasing quantity of spermine (inset). (Right) Fluorescence intensity of 1⁶⁻ at 585 nm as a function of the increasing quantity of spermine.

Computational Studies

The Monte-Carlo (MC) conformational searches of 1^{6-} , $1^{6-} \subset 2^+$, $1^{6-} \subset 2_2^{2^+}$ and $1^{6-} \subset 3^+$ were completed with the Maestro suite (Schrodinger) using OPLS3e molecular mechanics (MM) force field in implicit water solvent. For each search, we used systematic torsional sampling method with 300 steps per rotatable bond and 50,000 steps overall. The energy window for saving structures was set to 12 kJ/mol.

BASKET 16-

For 1⁶⁻, the MC/MM search gave 1489 conformers (<6.5 kJ/mol) with the global minimum found 2 times.

Final report:

3271 unique conformations found 3271 minimized with good convergence Found 620 confs within 1.00 kcal/mol (4.18 kJ/mol) of glob. min. Found 2323 confs within 2.00 kcal/mol (8.37 kJ/mol) of glob. min. Found 3231 confs within 3.00 kcal/mol (12.55 kJ/mol) of glob. min. Found 3271 confs within 5.00 kcal/mol (20.92 kJ/mol) of glob. min. Global minimum E = -1641.79 kJ/mol found 2 times.

DOXORUBICIN 2⁺

For $1^{6} \subset 2^{+}$, four MC/MM conformational searches were completed, each with a different starting pose in of doxorubicin 2^{+} (A–D below) inside the cavity of 1^{6} .

The starting pose **A** (below on the left) consisted of doxorubicin forming no particular noncovalent interactions with three carboxylate arms from 1^{6-} but holding its aromatic part deep inside the cavity. MC/MM search gave 610 conformers (<6.5 kJ/mol) with the global minimum (below on the right) found 7 times; note that DOX 2^+ holds onto two carboxylates from the basket.





Final report:

1238 unique conformations found
1238 minimized with good convergence
Found 153 confs within 1.00 kcal/mol (4.18 kJ/mol) of glob. min.
Found 677 confs within 2.00 kcal/mol (8.37 kJ/mol) of glob. min.
Found 1238 confs within 3.00 kcal/mol (12.55 kJ/mol) of glob. min.
Global minimum E = -1872.17 kJ/mol found 7 times.

The starting pose **B** consisted of doxorubicin forming two hydrogen bonds and a salt bridge with three carboxylate arms and placing its aromatic part deep inside the cavity. MC/MM search gave 1755 conformers (<6.5 kJ/mol) with the global minimum found 17 times. Additional results are shown in Figure 5A.





Final report:

1755 unique conformations found		
1755 minimized with good convergence		
Found	74 confs within	1.00 kcal/mol (4.18 kJ/mol) of glob. min.
Found	198 confs within	2.00 kcal/mol (8.37 kJ/mol) of glob. min.
Found	391 confs within	3.00 kcal/mol (12.55 kJ/mol) of glob. min.
Found	1742 confs within	5.00 kcal/mol (20.92 kJ/mol) of glob. min.
Found	1755 confs within	10.00 kcal/mol (41.84 kJ/mol) of glob. min.
Global minimum $E = -1886.64 \text{ kJ/mol}$ found 17 times.		

The starting pose C (below on the left) consisted of doxorubicin forming one hydrogen bond and a salt bridge with two carboxylate arms and placing its aromatic part deep inside the cavity. The most stable conformer is shown on the right. MC/MM search gave 295 conformers (<6.5 kJ/mol) with the global minimum (below on the right) found 1 time.





Final report:

1095 unique conformations found		
1095 minimized with good convergence		
Found	102 confs within	1.00 kcal/mol (4.18 kJ/mol) of glob. min.
Found	473 confs within	2.00 kcal/mol (8.37 kJ/mol) of glob. min.
Found	955 confs within	3.00 kcal/mol (12.55 kJ/mol) of glob. min.
Found	1095 confs within	5.00 kcal/mol (20.92 kJ/mol) of glob. min.
Global minimum $E = -1877.10 \text{ kJ/mol found 1 times.}$		

The starting pose **D** (below on the left) consisted of doxorubicin forming no interactions with the carboxylate arms and placing its aliphatic part deep inside the cavity. The most stable conformer is shown on the right. MC/MM search gave 281 conformers (<6.5 kJ/mol) with the global minimum (below on the right) found 1 time.





Final report:

1219 unique conformations found

1219 minimized with good convergence

Found105 confs within1.00 kcal/mol (4.18 kJ/mol) of glob. min.Found419 confs within2.00 kcal/mol (8.37 kJ/mol) of glob. min.

Found 823 confs within 3.00 kcal/mol (12.55 kJ/mol) of glob. min. Found 1219 confs within 5.00 kcal/mol (20.92 kJ/mol) of glob. min. Global minimum E = -1855.17 kJ/mol found 1 time.

For $1^{6} \subset 2_{2}^{2^{+}}$, five MC/MM conformational searches were completed, each with the starting conformation in which doxorubicin 2^{+} adopted distinct poses (A–C below) in cavities of 1^{6} .

The starting pose A (below on the left) consisted of doxorubicin in the top cavity forming no favorable noncovalent contacts with three carboxylate arms and placing its aromatic part deep inside the cavity. Doxorubicin in the bottom cavity formed a salt bridge and a hydrogen bond with two carboxylate arms. MC/MM search gave 27 conformers (<6.5 kJ/mol) with the global minimum found 7 times (below on the right).





Final report:

931 unique conformations found

931 minimized with good convergence

Found	13 confs within	1.00 kcal/mol (4.18 kJ/mol) of glob. min.
Found	38 confs within	2.00 kcal/mol (8.37 kJ/mol) of glob. min.
Found	142 confs within	3.00 kcal/mol (12.55 kJ/mol) of glob. min.
Found	927 confs within	5.00 kcal/mol (20.92 kJ/mol) of glob. min.
Found	931 confs within 1	0.00 kcal/mol (41.84 kJ/mol) of glob. min.
Global minimum $E = -2101.12 \text{ kJ/mol found 7 times.}$		

The starting pose **B** (below on the left) consisted of doxorubicin in both cavities forming two hydrogen bonds and a salt bridge with three carboxylate arms and placing its aromatic part deep inside the cavity. MC/MM search gave 33 conformers (<6.5 kJ/mol) with the global minimum found 15 times (shown on the right); also depicted in Figure 5B. Note that a change in the orientation of the drug in the bottom cavity for 120° gives two additional diastereomeric complexes (**B1** and **B2** poses). The examination of **B1** and **B2** with MC/MM yielded comparable structures with steric energies E = -2124.52 kJ/mol and -2123.01 kJ/mol.





Final report:

353 unique conformations found

353 minimized with good convergence

Found	30 confs within	1.00 kcal/mol (4.18	kJ/mol) of glob. min.
Found	52 confs within	2.00 kcal/mol (8.37	kJ/mol) of glob. min.
Found	194 confs within	3.00 kcal/mol (12.55	5 kJ/mol) of glob. min.
Found	353 confs within	5.00 kcal/mol (20.92	2 kJ/mol) of glob. min.
Global m	inimum $E = -212$	4.00 kJ/mol found	15 times.

The starting pose C (below on the left) consisted of doxorubicin in the top cavity forming no favorable noncovalent contacts with three carboxylate arms and placing its aliphatic part deep inside the cavity. Doxorubicin in the bottom cavity forms a salt bridge and two hydrogen bonds with three carboxylate arms. MC/MM search gave 43 conformers (<6.5 kJ/mol) with the global minimum (below on the right) found 3 times.





Final report:

585 unique conformations found
585 minimized with good convergence
Found 15 confs within 1.00 kcal/mol (4.18 kJ/mol) of glob. min.
Found 86 confs within 2.00 kcal/mol (8.37 kJ/mol) of glob. min.
Found 323 confs within 3.00 kcal/mol (12.55 kJ/mol) of glob. min.
Found 585 confs within 5.00 kcal/mol (20.92 kJ/mol) of glob. min.

Global minimum E = -2103.44 kJ/mol found 3 times.

TOPOTECAN 3⁺

For $1^{6} \subset 3^{+}$, two MC/MM conformational searches were completed, each with the starting conformation in which topotecan 3^{+} adopted a distinct pose (A–B below) in the cavity of 1^{6} .

The starting pose **A** (below on the left) consisted of topotecan placing its lactone part deep inside the cavity without having any particular noncovalent contacts with carboxylate arms. MC/MM search gave 544 conformers (<6.5 kJ/mol) with the global minimum (below on the right) found 5 times.





Final report:

2908 unique conformations found		
2907 minimized with good convergence		
Found	255 confs within	1.00 kcal/mol (4.18 kJ/mol) of glob. min.
Found	995 confs within	2.00 kcal/mol (8.37 kJ/mol) of glob. min.
Found	2341 confs within	3.00 kcal/mol (12.55 kJ/mol) of glob. min.
Found	2908 confs within	5.00 kcal/mol (20.92 kJ/mol) of glob. min.
Global minimum $E = -1532.24 \text{ kJ/mol}$ found 5 times.		

The starting pose **B** (below on the left) consisted of topotecan placing its aromatic part deep inside the cavity without having any particular noncovalent contacts with carboxylate arms. MC/MM search gave 272 conformers (<6.5 kJ/mol) with the global minimum (below on the right) found 1 time.





Final report:

438 unique conformations found

438 minimized with good convergence

Found	116 confs within	1.00 kcal/mol (4.18 kJ/mol) of glob. min.
Found	347 confs within	2.00 kcal/mol (8.37 kJ/mol) of glob. min.
Found	412 confs within	3.00 kcal/mol (12.55 kJ/mol) of glob. min.
Found	438 confs within	5.00 kcal/mol (20.92 kJ/mol) of glob. min.
Global m	inimum $E = -156$	3.70 kJ/mol found 1 times.