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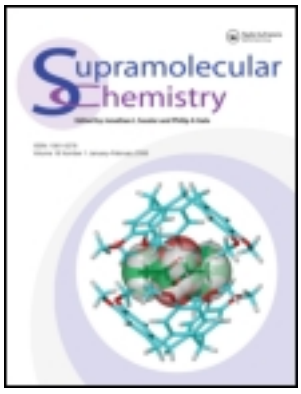

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Characterisation and antiproliferative activity of irinotecan and sulphonatocalixarene inclusion complex

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The interaction between water-soluble sulphonatocalix[4]arene (SC4A) and irinotecan (CPT-11) was investigated by using UV spectrophotometry. Inclusion complex of SC4A with CPT-11 was confirmed by ^{1}H NMR and DSC analysis. Water solubility study showed that SC4A has remarkable solubilisation on CPT-11 and the complex has good water solubility. The antiproliferative activity of the complex was evaluated. The results showed that the complexation of CPT-11 with SC4A increases the antiproliferative activity of CPT-11.

Keywords: sulphonatocalixarene; inclusion complex; irinotecan; antiproliferative activity; supramolecular chemistry

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1. Introduction

Irinotecan (CPT-11, Scheme 1) is the semi-synthetic derivative of the anticancer alkaloid camptothecin (*1*), and is used clinically to treat advanced colorectal cancer (*2*, *3*), lung cancer (*4*, *5*) and malignant lymphoma (*6*, *7*). CPT-11 has a low solubility, it needs to be prepared with CPT-11 hydrochloride for improving its solubility.

During the last several decades, a combination of supramolecules for biological and pharmaceutical applications has acquired great interest in supramolecular chemistry (*8*, *9*). Calixarenes, which contain a repeating phenolic unit formed into a macrocycle via methylene bridges (*10*), are noted for their ability to form host–guest complexes by trapping organic compounds and ions in their toruslike cavities. Above all, water-soluble para-sulphonatocalixarene were widely used in medicinal chemistry fields (*11–13*) due to their innocuousness (*14*) and good solubility (*15*) (up to 0.1 M) in aqueous media. These sulphonatocalixarenes provide not only a hydrophobic environment (benzene rings), but also hydrophilic heads (SO_3^-), so they can encapsulate some drug molecules into their cavity leading to an increase in the solubility and the stability (*8*, *11*), or improvement in the bioavailability (*12*) of the drug.

In this work, we investigated the binding mechanics and the binding mode of sulphonatocalix[4]arene (SC4A) with CPT-11 by UV, NMR and DSC (Differential Scanning Calorimetry) analysis. Finally, the solubility of the SC4A·CPT-11 complex was studied, and its antiproliferative activity was evaluated.

2. Experimental

2.1 General

CPT-11 was purchased from Knowshine (Shanghai) Pharmachemicals Inc. (Shanghai, China). The HT-29 human colon cancer line was purchased from Shanghai Men-Die-Ta Biotech. Co. Ltd (Shanghai, China). Para-SC4A was synthesised and purified according to the literature procedures, and verified by ^{1}H NMR (*16*,*17*). All other chemicals, reagents and solvents (analytical or purer grade) were purchased from commercial suppliers. NMR experiments were performed on a Varian Mercury VX300 spectrometer (300 MHz) at 298 K in a deuterium oxide solution. Two-dimensional Rotating Frame Overhauser Effect Spectroscopy (ROESY) was performed in D_2O (300 MHz) with a mixing time of 400 ms. DSC analysis was performed with a NETZSCH DSC 204 instrument from 25 to 400°C with a heating rate of 10 K/min.

2.2 Prediction of the interaction between CPT-11 and SC4A by UV spectrophotometry

The same amounts of CPT-11 were added into a series of 10-ml comparison tubes. After different amounts of SC4A were gently added into these tubes, respectively, these solutions were diluted to graduation with MeOH–H_2O, shook up and were allowed to settle for about 15 min. Then by using the same concentration of SC4A in MeOH–H_2O as blanks, the difference-UV absorption spectra of these solutions were determined, and the absorbance was measured at 232 nm.

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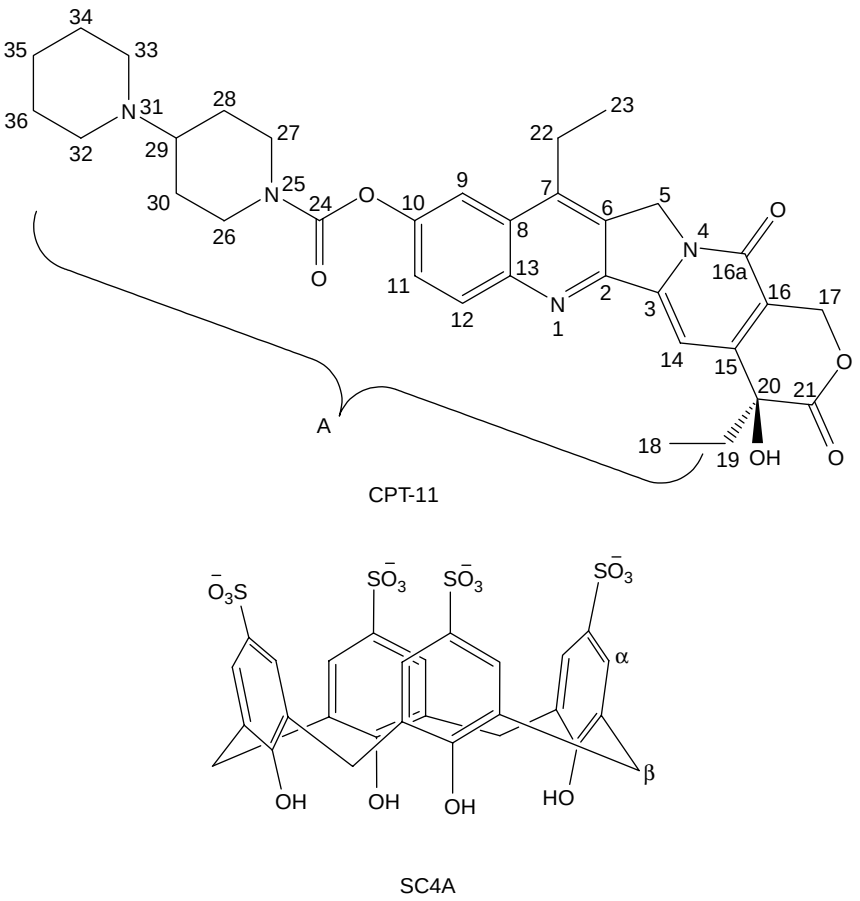

Scheme 1. Chemical structures of CPT-11 and SC4A. Protons of CPT-11 are shown for NMR purposes.

For the titration of host into a solution of guest, the relationship between the change of guest absorbance and the host concentration is given by Equation (1) (*18*):

$$\frac{(A - A_0)}{[H]} = K_S(A_\infty - A_0) - K_S(A - A_0), \qquad (1)$$

where A_0 is the absorbance of the guest in the absence of host, $[H]$ is the concentration of host SC4A at each titration point, A_∞ is the absorbance when all the guest molecules are complexed with host (i.e. guest with large excess of host), A is the observed absorbance at each titration point and K_S is the binding constant (M^{-1}).

2.3 Preparation and characteristics of SC4A·CPT-11 complex

To generate SC4A·CPT-11 complex, CPT-11 and SC4A were completely dissolved in a mixed solution of methanol and water (v:v = 1:9) and stirred for about 48 h at room temperature. The mixed solution was evaporated to remove methanol and water, and dried in vacuum to give SC4A·CPT-11 complex.

^{1}H NMR and 2D ROESY were carried out on Varian Mercury VX300 spectrometer with a 5-mm sample tube, and deuterated water was typically used as solvent in NMR experiments. No internal reference was used to avoid possible interference with the complexation between CPT-11 and SC4A, so the solvent signal (D_2O, 4.694 ppm) was used as an internal reference.

DSC analysis was carried out for pure CPT-11, pure SC4A, physical mixtures of CPT-11 with SC4A at a 1:1 molar ratio and complex of CPT-11 with SC4A.

2.4 Aqueous solubility

Excess amount of complex was put into 5 ml of water and then was stirred for 1 h. After removing the insoluble substance by filtration, the filtrate was evaporated under reduced pressure to dryness and the residue was dosed by weighing method (*19*).

2.5 Cell culture and growth inhibition assays

The human colon carcinoma HT-29 cell lines were grown in Roswell Park Memorial Institute 1640 medium

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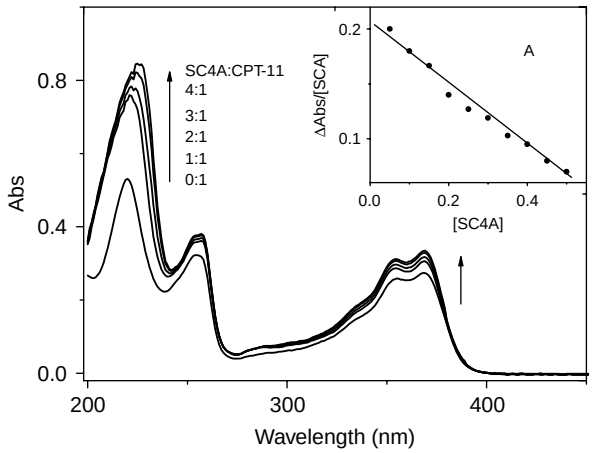

Figure 1. UV spectra of CPT-11 in the absence and presence of SC4A in solution. Concentration of CPT-11: 0.1 mM; concentration of SC4A: 0, 0.1, 0.2, 0.3 and 0.4 mM; A is the curve of absorbance difference at various concentrations of SC4A at 232 nm.

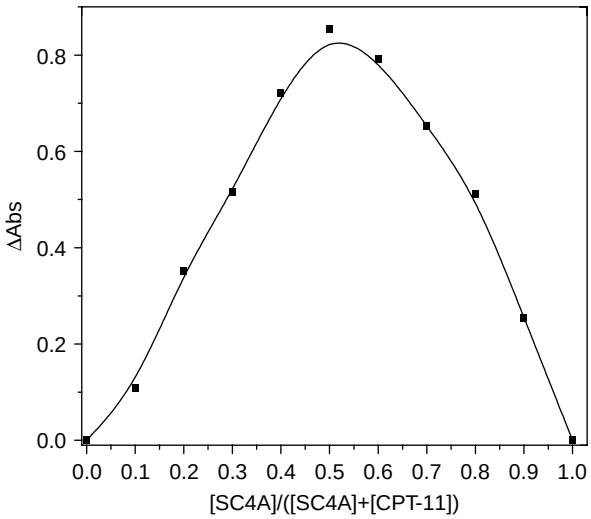

Figure 2. Job plot for SC4A binding of CPT-11. The total concentration was maintained at 0.5 mM and the changes of absorbance were measured at 232 nm.

containing 10% heat-inactivated foetal calf serum and 2 mM glutamine, and then maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The cell culture media and supplements originated from Hyclone.

Antiproliferative activity of the complex and CPT-11 was evaluated as cell survival after treatment for 48 h. Cell viability was evaluated by a microculture tetrazolium reduction assay using Cell Proliferation and Cytotoxicity Assay Kit from Roche (Indianapolis, IN, USA). Briefly, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was added to cell cultures in 96-microwell flat-bottomed plates for 4 h incubation at 37°C. Plates were then centrifuged and MTT-containing culture medium was removed. Precipitated formazan was dissolved in 150 ml dimethyl sulphoxide. Results were read with 15 min in a spectrometer at 562 nm, and the means of triplicates were calculated.

Table 1. The chemical shifts (δ_H) of protons for free CPT-11 and complex.

Proton	δ_H[a] (Free CPT-11)	δ_H (Complex)	$\Delta\delta$
5-1	4.344	4.317	−0.027
5-2	4.135	4.053	−0.082
9	6.916	7.146	0.229
11	7.368	7.628	0.260
12	7.043	7.275	0.232
14	7.393	7.589	0.196
17-1	5.292	5.391	0.099
17-2	5.175	5.260	0.085
18	0.871	0.861	0.010
19	1.799	1.854	0.055
22	2.718	2.847	0.129
23	1.056	2.847	0.129
26-1&27-1[b]	3.475	3.908	0.433
26-2	3.425	3.040	−0.385
27-2	3.063	3.040	−0.023
28-1&30-1	1.924	2.085	0.161
28-2&30-2	1.410	1.536	0.126
29	2.916	3.477	0.561
32-1&33-2	2.142	2.854	0.712
32-2&33-1	2.999	2.608	−0.391
34, 35&36	1.727	1.863	0.136

[a] δ_H is proton chemical shift in the centre position, ppm.
[b] & represents that protons in here can be exchanged with each other.

3. Results and discussion

3.1 UV spectrum and binding constant

A series of solutions containing the same amounts of CPT-11 and different amounts of SC4A were determined by UV spectrophotometry for examining whether an interaction exists between CPT-11 and SC4A. As can be seen from Figure 1, upon the addition of SC4A, one maximum wavelength of CPT-11 is slightly red shifted from 232 to 238 nm, whereas all maximal absorbance of CPT-11 at 232, 255 and 370 nm increase markedly. The results indicate that there exists an interaction between CPT-11 and SC4A.

To determine the stoichiometry of the complexation, the Job plots were obtained from the UV titration data. The formation of 1:1 complex was clearly confirmed (Figure 2). The K_S value of the complex was calculated by using Scatchard method. It is $7.2 \times 10^3\ M^{-1}$, indicating that the complex of SC4A with CPT-11 is remarkably stable.

3.2 Characteristics of the SC4A·CPT-11 complex

^{1}H NMR experiments of free CPT-11, SC4A and SC4A·CPT-11 complex were performed to clarify the

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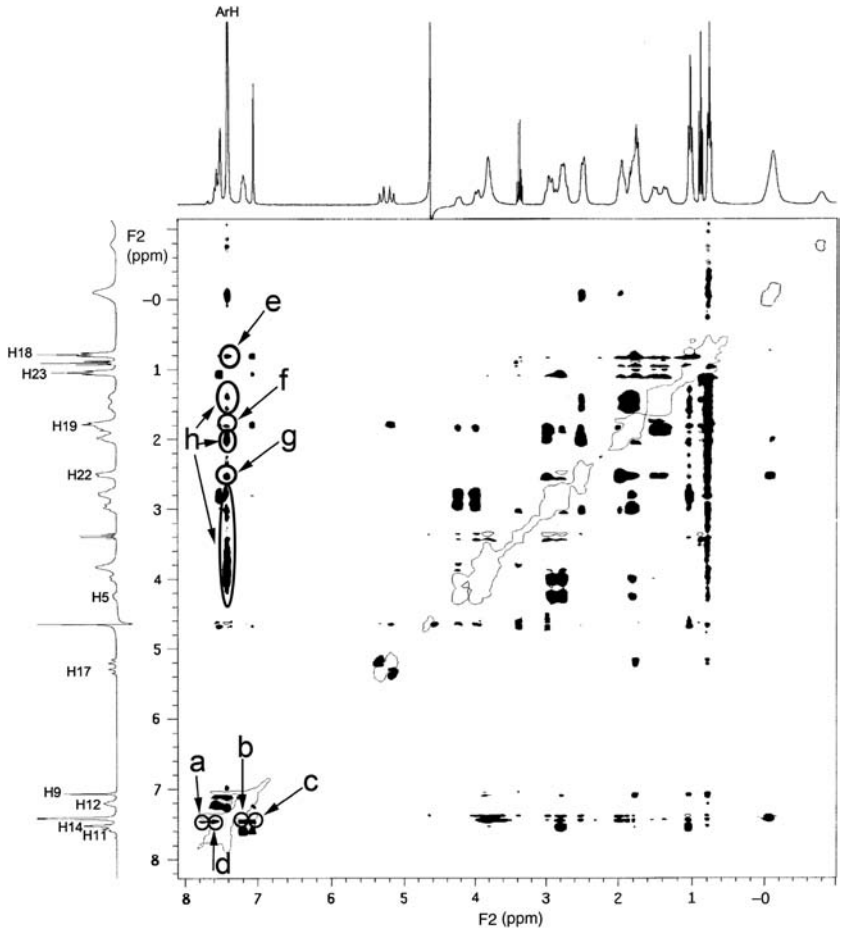

Figure 3. 2D ROESY spectra of SC4A·CPT-11 complex in D_2O. Annotated cross peaks indicate intermolecular interactions between CPT-11 and SC4A.

interaction of CPT-11 with SC4A. SC4A only presents two peaks, one for protons of benzene ring (α-H), another for protons of CH_2 (β-H). On the one hand, chemical shift values of aromatic ring protons of SC4A increase from 7.333 to 7.444 ppm, and protons chemical shifts of CH_2 (β-H) on SC4A have no change. On the other hand, chemical shift values of some protons in CPT-11 also markedly change due to the complexation of SC4A with CPT-11 (Table 1). As can be seen from Table 1, chemical shift values of protons in quinoline ring (H9, H11, H12 and H22) and in piperidinopiperidine ring (H26–30, H32–36) and that of proton H14 change markedly due to the complexation of CPT-11 with SC4A, but the change of chemical shift values of protons H17, H18 and H19 in α-hydroxyl δ-lactone ring, as well as protons H5 and H23 is small. These observations suggest that a strong interaction exists between quinoline ring and piperidinopiperidine ring of CPT-11 and aromatic ring of SC4A, and SC4A cavity might bind with the two parts of quinoline ring and piperidinopiperidine ring. In addition, comparing the peak area of protons H18, H19 and all protons of quinoline ring in CPT-11 with those of the aromatic ring of SC4A also proves that the 1:1 molar ratio exists for SC4A to CPT-11 in the complex.

To further confirm the binding mode of SC4A with CPT-11, 2D ROESY experiment was performed for SC4A·CPT-11 complex (Figure 3). There are three clear cross peaks (circled **a**, **b** and **c**) between protons of quinoline ring of CPT-11 and those of the aromatic ring of SC4A. The peak **a** represents the correlation involving proton H11 in quinoline ring of CPT-11, peaks **b** and **c** represent the correlation involving protons H12 and H9, respectively. The results suggest that the cavity of SC4A includes the quinoline ring of CPT-11. Moreover, cross peak **d** suggests that an interaction exists between aromatic ring protons (Ar-H, i.e. α-H) of SC4A and proton H14 of CPT-11, cross peak **e** represents an interaction between Ar-H and proton H18 of CPT-11, cross peak **f** represents an interaction between Ar-H and proton H19 of CPT-11 and cross peak **g** represents an interaction between Ar-H and proton H22 of CPT-11. At the same time, several cross peaks **h** appear due to an interaction of aromatic ring

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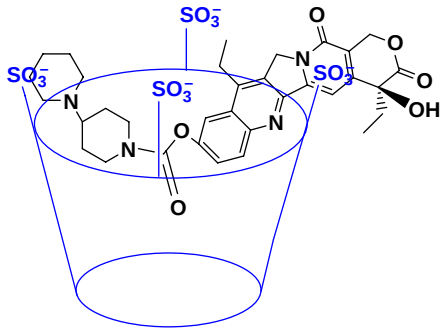

Figure 4. Possible binding mode of SC4A·CPT-11 complex.

protons of SC4A with piperidinopiperidine ring protons of CPT-11. There are no cross peaks for protons of CH_2 (β-H) with all protons of CPT-11. On the basis of the above observations, we can deduce reasonably the binding mode of SC4A with CPT-11 as shown in Figure 4. In this mode, the cavity of SC4A includes mainly the quinoline ring and the piperidinopiperidine ring of CPT-11 alongside face **A** through hydrogen-bonding interaction, electrostatic interaction, π−π interaction, etc.

DSC analysis is widely used to study the interaction between drugs and macrocyclic compounds in the solid state (*20*). DSC thermograms for each pure component, physical mixture and SC4A·CPT-11 complex are shown in Figure 5. The DSC isotherm for free CPT-11 is characterised by a sharp endothermic peak at 276°C. No endothermic peaks are observed for SC4A due to their amorphous nature. DSC thermogram of physical mixture is only the simple superposition of CPT-11 with SC4A. No characteristic CPT-11 endothermic curve is observed in DSC thermograms for SC4A·CPT-11 complex due to the formation of the SC4A·CPT-11 complex in the solid state. A broad peak at 100–118°C is dehydration of water molecules combining in CPT-11, SC4A and complex.

3.3 *Aqueous solubility*

Water solubility of the complex is assessed by the preparation of its saturated solution. Compared with water solubility of free CPT-11 (0.9 mg/ml) and CPT-11 hydrochloride (7 mg/ml), that of SC4A·CPT-11 complex is dramatically increased to approximately 25 mg/ml (enhancing about 27.8-fold for free CPT-11 and 3.6-fold for CPT-11 hydrochloride, respectively). In the control experiment, a clear solution was obtained after dissolving 58 mg of SC4A·CPT-11 complex, which is equivalent to 25 mg of CPT-11 in 1 ml water at room temperature. This result subsequently confirms the reliability of the obtained satisfactory water solubility of SC4A·CPT-11 complex.

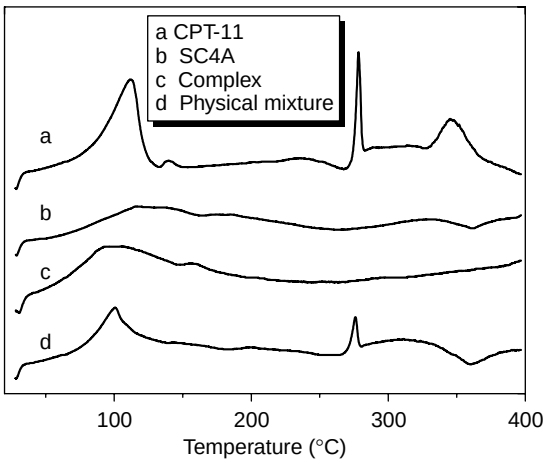

Figure 5. DSC thermograms for (a) CPT-11, (b) SC4A, (c) SC4A·CPT-11 complex and (d) physical mixture (SC4A: CPT-11, 1:1, molar ratio).

3.4 *Antiproliferative activity*

Cell growth inhibition tests for SC4A·CPT-11 complex were evaluated *in vitro* for antiproliferative activity against the human colon carcinoma HT-29 cell lines by the MTT cytotoxicity assay using parent SC4A and free CPT-11 as reference compounds (Figure 6). As expected, no antiproliferative activity of SC4A can be observed in the concentration range of tests. It can be seen from Figure 6 that both CPT-11 and SC4A·CPT-11 complex exert highly antiproliferative activities, and the latter exhibits a higher antiproliferative activity on the human colon carcinoma HT-29 cell than free drug CPT-11.

IC_{50} (the half maximal inhibitory concentration) values that represent the concentration of a drug required for 50% reduction of cellular growth were calculated by using origin 6.0 software to process the data of cell viability rate at various concentrations of the drug. IC_{50}

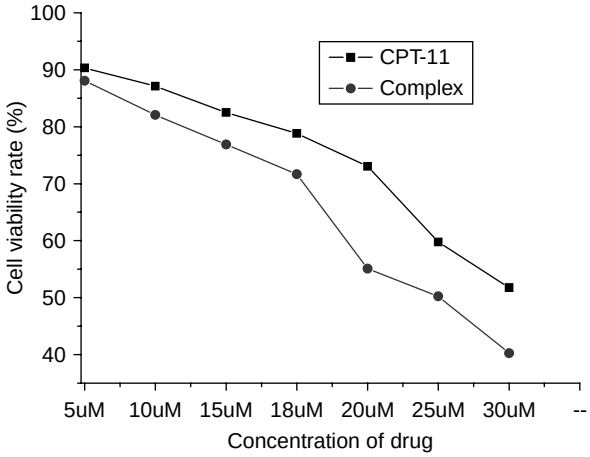

Figure 6. Inhibition of human HT-29 colon carcinoma cell adhesion by SC4A·CPT-11 complex and CPT-11.

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values of SC4A·CPT-11 complex and CPT-11 are 18.9 and 26.4 μM, respectively, which indicates that the complex has higher antiproliferative activities than free CPT-11. Statistical significance is indicated with $P < 0.01$. Structurally, the hydroxyl lactone ring is a prerequisite for the antiproliferative activity of CPT-11 (*21*,*22*). SC4A binding of CPT-11 can increase the stability of the hydroxyl lactone ring, which should be the main factor of the antiproliferative activities of CPT-11.

4. Conclusions

In conclusion, UV spectroscopic studies were carried out to gain insight into interactions between CPT-11 and SC4A. We actualised inclusion of an anticancer drug CPT-11 by using water-soluble SC4A. The SC4A·CPT-11 complex was characterised by using NMR and DSC analyses. Water solubility experiments show that SC4A are able to solubilise CPT-11 to high levels. Finally, we evaluated the antiproliferative activity of the complex. The obtained results indicate that the complexation of CPT-11 and SC4A can increase the antiproliferative activity of CPT-11, which has potential application in the medical fields for the complex.

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