Determination of Self-Association of Irinotecan Hydrochloride (CPT-11) in Aqueous Solution

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Received March 6, 1992

Self-association of irinotecan hydrochloride (CTP-11) in an aqueous solution was studied using UV, circular dichroism (CD), ^{1}H-NMR and the quasi-elastic light scattering (QLS) method. The UV spectra showed a hypochromic effect in the aqueous solution. In the CD spectra, typically positive Davydov splitting was observed and the $\Delta\varepsilon$ value was reduced sigmoidally when the concentration of CTP-11 was decreased. In the ^{1}H-NMR, the aromatic signals of higher concentration shifted to a diamagnetic direction compared with those of lower concentration. These observations suggested that CPT-11 molecules are present as monomer in the lower concentration, and the self-association with positive helicity occurs by vertical stacking more than 10 μM of concentration. Its molecules form complete aggregates at more than 2 mM of the concentration. Results of QLS which coincided in the prediction of partition coefficient experiments suggested that CPT-11 molecules formed dimer under the condition. By the regression analysis of CD spectral data, the equilibrium constant for the self-association was calculated to be 2.41×10^{-4} M^{-1}.

Keywords self-association; CPT-11; irinotecan hydrochloride trihydrate; UV spectrum; CD spectrum; ^{1}H-NMR; quasi-elastic light scattering; camptothecin; partition coefficient

Introduction

CPT-11,[1] irinotecan hydrochloride trihydrate, is a novel antitumor agent synthesized from camptothecin which was isolated from Chinese tree *Camptotheca acuminata* DECAISNE (Nyssaceae) by Wall and co-workers.[2] During the synthetic studies of camptothecin derivatives, CPT-11 was obtained as a stable and water-soluble derivative, and it has a weaker toxicity than camptothecin. The structure of CPT-11 is shown in Fig. 1.

On the development studies of CPT-11 as a new antitumor drug, we found that CPT-11 formed aggregates in an aqueous solution by determining the partition coefficient in the chloroform/water system (Fig. 1). In the present paper, we wish to describe the determination of the self-association mode of CPT-11.

Experimental

Material CPT-11, irinotecan hydrochloride trihydrate, (+)-(4*S*)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]-1*H*-pyrano-[3′,4′:6,7]-indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione hydrochloride trihydrate had been synthesized by Yakult Honsha Co., Ltd.[1]

Measurement of Partition Coefficients in the Chloroform/Water System Each 200, 150, 100 and 50 mg of CPT-11 was exactly weighed and was dissolved in 25 ml of water. Each solution and the same volume of chloroform were mixed. The mixture was shaken for 6 h at room temperature, and was then allowed to stand for 18 h. The chloroform layer and water layer were separated, and the solute contents of these solutions were measured by high performance liquid chromatography. Instrument: LC-6A liquid chromatography system (Shimadzu), column: AM-312 (ODS, 5 μm, YMC), 150 mm × 6 mm i.d. Mobile phase: methanol–water (10 : 9) containing 5 mM sodium *n*-heptanesulfonate. Flow rate: 1.4 ml/min. Column temperature: 40 °C. Detect: UV 254 nm. Internal standard: *n*-propyl *p*-hydroxybenzoate.

Ultraviolet and Visible (UV) Spectrophotometry UV spectra were recorded on a Shimadzu UV-VIS recording spectrophotometer UV-240 using 1, 2, 5 or 10 mm of quartz cells. CPT-11 (203 mg) was dissolved in an aqueous solvent or organic solvents to make exactly 300 ml, and this solution was used as an initial solution (1 mM). Other solutions having several concentrations were prepared by gradually diluting the initiation. We used weakly acidic 0.01 M potassium dihydrogenphosphate (KH_2PO_4) solution as an aqueous solvent to suppress opening a 6-membered lactone ring in CPT-11 molecule.

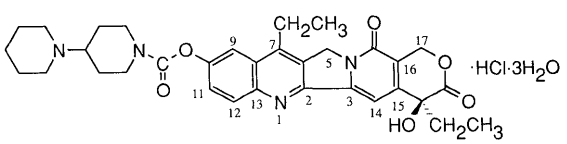

Fig. 1. Structure of CPT-11 (Irinotecan Hydrochloride Trihydrate)

Circular Dichroism (CD) Spectrometry CD spectra were measured on a JASCO CD Spectrometer CD-500 equipped with a JASCO Data Processor. CPT-11 (2.032 g) was dissolved in 0.01 M KH_2PO_4 solution to make exactly 300 ml, and this solution was used as an initial solution (10 mM). Other solutions with several concentrations were prepared by gradually diluting the initial solution.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy The ^{1}H-NMR spectra of CPT-11 solutions (20 μM and 2 mM) in D_2O were measured on a JEOL GX-400 NMR spectrometer by the homo-gated decoupling method using 3-(trimethylsilyl)propionic acid-d_4 (TSP) as an internal standard. Chemical shifts of aromatic region (δ in D_2O): (20 μM) 8.23 (1H, d, J=8 Hz, H-12), 8.02 (1H, d, J=2 Hz, H-9), 7.79 (1H, s, H-14), 7.68 (1H, dd, J=8, 2 Hz, H-11); (2 mM) 7.94 (1H, d, J=8 Hz, H-12), 7.76 (1H, d, J=2 Hz, H-9), 7.48 (1H, s, H-14), 7.39 (1H, dd, J=8, 2 Hz, H-11).

Quasi-Elastic Light Scattering (QLS) The refractive index was measured on a Otsuka RM-102 refractometer. The refractive index of CPT-11 was 1.1312, and the refractive index increment (dn/dC) was calculated to be 0.2016. QLC was done at 90° of the incident light angle on a Dynamic light scattering spectrophotometer model DLS-700 by Otsuka Electronics Co., Ltd. A light source is Ne–He laser 5 mW, and the wavelength of incident light is 632.8 nm. The temperature of sample solutions was maintained at 25 °C during measurements of refractive index and QLS.

Results and Discussion

The result of the partition coefficient in the $CHCl_3/H_2O$ system is shown in Fig. 2. The slope of the regression curve was calculated to be 2.05778, which estimated that CPT-11 molecules formed dimer.

The UV spectra of CPT-11 were measured in aqueous or organic solutions (MeOH, $CHCl_3$ and dimethyl sulfoxide (DMSO)) in the concentration range of 1 μM to 1 mM. The spectra of an aqueous solution showed two characteristic absorption maxima due to strong π–π^* transitions at 355 and 368 nm (shown in Figs. 3 and 4). The molecular extinction coefficient (ε) at 368 nm exhibited the conspicuous hypochromic effect when the concentration of the solute increased, although that at the 355 nm did not change. The hypochromic effect shows a regular arrangement of aromatic chromophores such as self-association. It is also suggested that the aggregates of oligomeric units may be

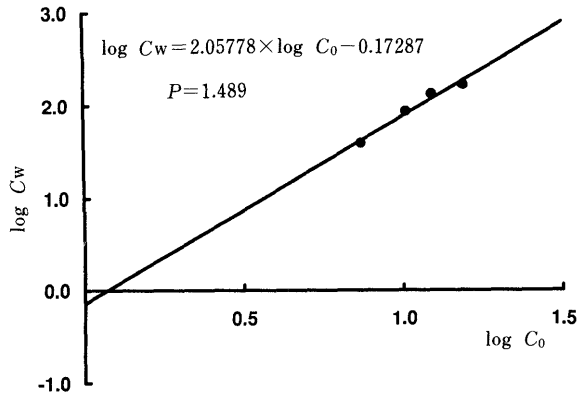

Fig. 2. Partition Coefficient in the Chloroform/Water System

C_0 is the concentration of CPT-11 in the chloroform layer, and C_w is the concentration in the water layer. Each point is the average value of 3 measurements.

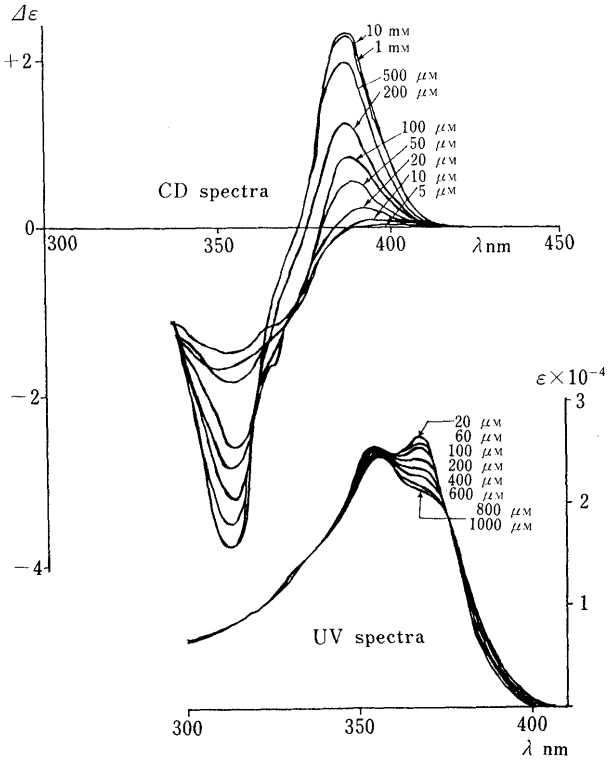

Fig. 3. CD and UV Spectra of CPT-11 in an Aqueous Solvent

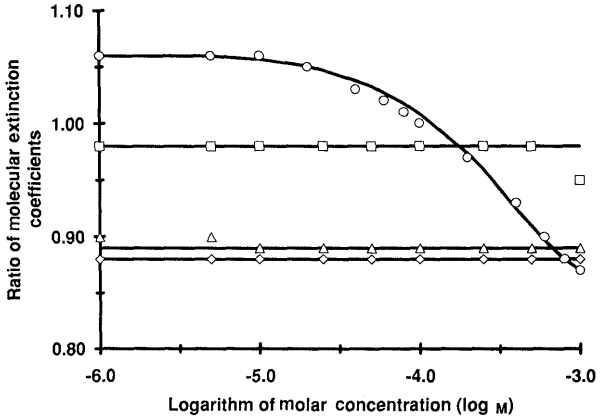

Fig. 4. UV Spectral Changes in an Aqueous Solvent and Other Organic Solvents

Absorption maxima in each solvent were observed at 355 and 366 nm in aq. KH_2PO_4 (○), at 358 and 370 nm in MeOH (□), at 362 and 378 nm in $CHCl_3$ (△), or at 357 and 383 nm in DMSO (◇). Ratio of molecular extinction coefficient on the longer wavelength side to that on the shorter wavelength side was plotted as an ordinate with a logarithm of molar concentration as an abscissa.

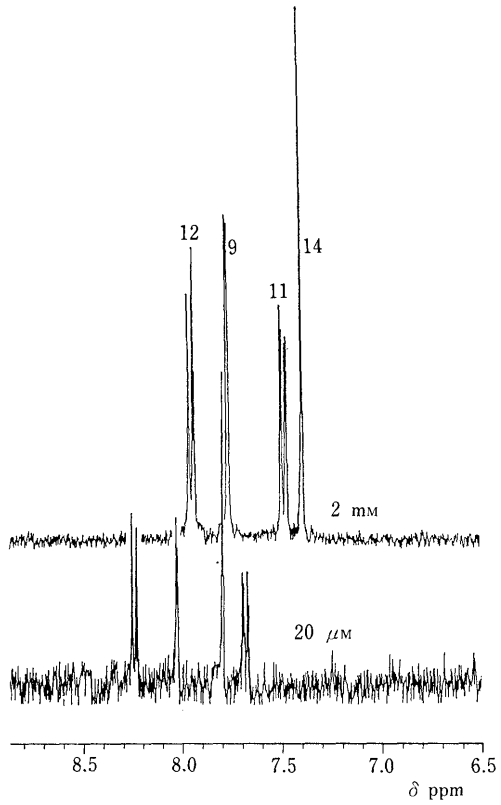

Fig. 5. ^{1}H-NMR Spectra of CPT-11 at 2 mM and 20 μM of the Solute Concentrations in Deuterium Oxide

TSP was used as internal standard.

formed, because the bathochromic shifts were not observed. The self-association of CPT-11 does not occur in any organic solvent because no change of the molecular extinction coefficients was observed (shown in Fig. 4).

The exciton chirality method of CD spectrum was established in the 1970s by Harada and Nakanishi.[3] By this method, two identical chromophores undergoing strong π–π^* transitions electrostatically interact with each other to exhibit two strong CD Cotton effects of opposite sign and same rotational strength. This Cotton effect amplitude is inversely proportional to the square of the interchromophoric distance. If the distance is short enough, this method is applicable to the non-bonding system such as the self-association. Hoshino and co-workers succeeded in determining the self-association of anthocyanin pigments of flowers.[4]

In the CD spectra (shown in Fig. 3) over 2 mM of concentration, the typically positive Davydov splitting curve was observed in the UV absorption bands region (300—400 nm), and the molar CD amplitude of the first Cotton ($\Delta\varepsilon_1$) was approximately +2.3. The $\Delta\varepsilon_1$ was reduced sigmoidally when the concentration was decreased, and then it converged to zero less than 10 μM. This converging indicates that the chromophores are non-interactive with each other. It is suggested that all CPT-11 molecules are present as monomers under 10 μM. The molecules are gradually arranged depending on the increase of concentra-

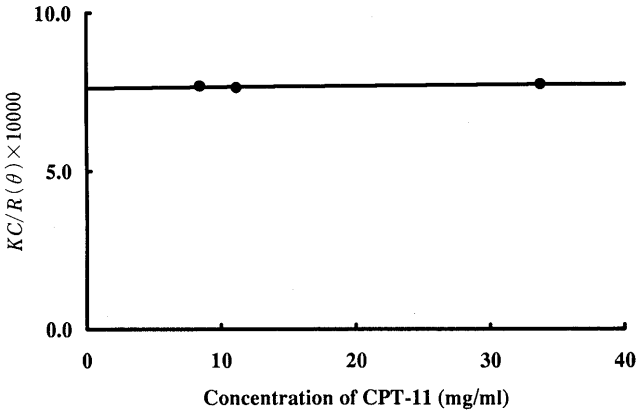

Fig. 6. Debye's Plot for CPT-11 in Water

For the associating system, the molecular weight of the aggregates was determined by extrapolation $KC/R(\theta)$ to infinite dilution. Preliminary test showed that the accuracy of $R(\theta)\times1000000$ was 2.9231 ± 0.0218 (10 runs, at 9.5 mg/ml). It was, however, difficult to determine the QLS under the conditions of low concentrations (less than 2 mM = 1.35 mg/ml) because the reproducibility of the $R(\theta)$ was poor.

tion from 10 μM to 2 mM, and all the molecules completely form aggregates more than 2 mM. The helicity of the aggregated molecules is clockwise, since the sign of the exciton chirality of the CD curve is positive.

The structure of the aggregates was estimated to be of two types. One is the vertically stacking type by hydrophobic and/or π-electron interactions, and the other is the horizontally extending type by mainly hydrogen bonds. In order to determine either the vertical type or the horizontal one, we measured ^{1}H-NMR spectra at 2 mM and 20 μM in deuterium oxide by the homo-gated decoupling method. The aromatic proton signals at 2 mM shifted to higher field compared with those at 20 μM (Fig. 5). Especially, 0.4 ppm of diamagnetic shift at 14-position was observed. Accordingly, CPT-11 molecules stack vertically, and the driving force is suggested to be hydrophobic and/or π-electron interactions.

We measured the molecular weight of CPT-11 aggregates in the aqueous solution by the QLS method, which is used to determine diffusion coefficients, size, shape, molecular weight and internal motion of biopolymers.[5] If the particle size of aggregates such as CPT-11 is much smaller than the wavelength of the incident light, the interference factor of the scattering light can be neglected, and the Debye's plotting method expressed as follows is applicable in this aggregates system of CPT-11.

$$\frac{K\cdot C}{R(\theta)}=\frac{1}{\mathrm{Mw}}+2\cdot A_2\cdot C+\cdots$$

C, concentration of CPT-11; Mw, molecular weight of CPT-11; A_2, the second virial coefficient.

where K is calculated using the increment (dn/dC) of the refractive index of the CPT-11 solution at constant temperature. The dn/dC of aq. CPT-11 solution was given as 0.2016 by measurement of the refractive index at several concentrations at 25 °C. The Rayleigh ratio, $R(\theta)$, corrected by the volume, was obtained from the experiments using vertically polarized incident light at 25 °C. The molecular weight of the aggregates was calculated to be approximately 1310 by extrapolation of $KC/R(\theta)$ to infinite dilution (Fig. 6) together with the second virial coefficient (1.78×10^{-4}).

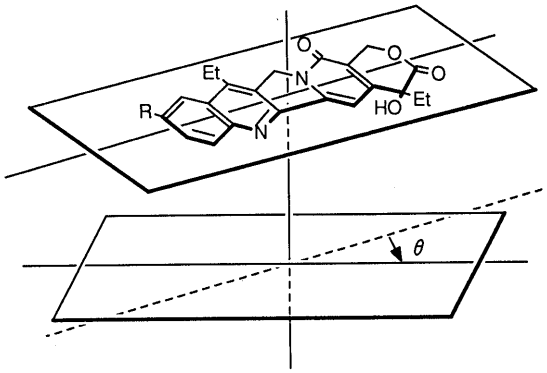

Fig. 7. Proposal Structure of a Dimer in an Aqueous Solution

It therefore suggests that CPT-11 molecules form dimers in higher concentration of the solution.

The equilibrium constant at room temperature in this self-association system was calculated from CD spectral data. When two molecules aggregate to form a dimer, the curve should be linear on the double reciprocal plot with concentration *versus* ratio of amplitudes ($\Delta\varepsilon/\Delta\varepsilon_{max}$). The equilibrium constant was calculated to be 2.41×10^{-4} M^{-1} ($R=0.9977$) by the regression analysis.

Conclusion

CPT-11 molecules are present as monomers of less than 10 μM concentration in the aqueous solution because the CD amplitude of Davydov splitting is zero. The molecules are gradually arranged depending on the increase of solute concentration in the range of 10 μM to 2 mM, and form complete aggregates more than 2 mM. In the self-association system, the molecules stack vertically because the diamagnetic shifts were observed in the ^{1}H-NMR. The positive CD splitting shows clockwise helicity of aggregated chromophores. By measuring QLS, the molecular weight of the aggregate was calculated to be 1310, and the aggregates are present as dimers. It agreed with the estimation from the partition coefficient experiment. However, CPT-11 did not aggregate in any organic solvents. The proposal structure of this aggregate is shown in Fig. 7.

Acknowledgments The authors are grateful to Dr. Tsutomu Hoshino of Niigata Univeristy for his valuable advice on this work. Also we would like to thank Ms. Ritsuko Fukushige, Mr. Katsushi Sasa and Dr. Shoichi Nakamura of Otsuka Electronics Co., Ltd. for measurement of QLS.

References

1) *a*) S. Sawada, S. Okajima, R. Aiyama, K. Nokata, T. Furuta, T. Yokokura, E. Sugino, K. Yamaguchi, and T. Miyasaka, *Chem. Pharm. Bull.*, **39**, 1446 (1991); *b*) K. Nitta, T. Yokokura, S. Sawada, T. Kunimoto, T. Tanaka, N. Uehara, H. Baba, M. Takeuchi, T. Miyasaka, and M. Mutai, "Recent Advances in Chemotherapy, Antitumore Section I. The Proceedings of the 14th International Congress of Chemotherapy," ed., by J. Ishigami, Tokyo Univ. Press, Tokyo, 1985, p. 29; *c*) T. Taguchi, A. Wakui, H. Niitani, H. Furue, K. Ohta, and T. Hattori, *Jpn. J. Cancer Chemother.*, **17**, 115 (1990); *d*) M. Fukuoka, S. Negoro, H. Niitani, and T. Taguchi, Annual Meeting of American Society of Clinical Oncology, Washington D.C., May 1990.

2) *a*) J.-C. Cai and C. R. Hutchington, "The Alkaloids," Vol. 21, ed. by A. Brossi, Academic Press, New York, (1983), p. 101; *b*) M. E. Wall, M. C. Wani, C. E. Cook, K. H. Palmer, and G. A. Sim, *J. Am. Chem. Soc.*, **88**, 3888 (1966).

3) *a*) N. Harada and K. Nakanishi, "Circular Dichroic Spectroscopy,

Exciton Coupling in Organic Chemistry," University Science Books (in Japanese), Tokyo Kagaku-Dojin, Tokyo, 1982; *b*) N. Harada, S.-M. L. Chen, and K. Nakanishi, *J. Am. Chem. Soc.*, **97**, 5343 (1975); *c*) N. Harada, S. Suzuki, H. Ueda, and K. Nakanishi, *ibid.*, **93**, 5577 (1971).

4) *a*) T. Goto, H. Tamura, T. Kawai, T. Hoshino, N. Harada, and T. Kondo, *Ann. N.Y. Acad. Sci.*, **471**, 155 (1986); *b*) T. Hoshino, U. Matsumoto, T. Goto, and N. Harada, *Tetrahedron Lett.*, **23**, 433 (1982); *c*) T. Hoshino, U. Matsumoto, N. Harada, and T. Goto, *ibid.*, **22**, 3621 (1981); *d*) T. Hoshino, U. Matsumoto, and T. Goto, *ibid.*, **21**, 1751 (1980).

5) *a*) T. Kamata, *Bunseki*, **1987** 364; *b*) K. Tomita, *Kagaku To Kogyo*, **60**, 487 (1986); *c*) V. A. Bloomfield, *Ann. Rev. Biophys. Bioeng.*, **10**, 421 (1981).