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Liposomal Stabilization of Camptothecin's Lactone Ring

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Camptothecin displays unprecedented antitumor activities against human colon cancer.[1] To date its full therapeutic utility has been limited by poor water-solubility and the aqueous instability of the lactone ring moiety. Ring opening is rapid, resulting in a complete loss of biological activity.[2] In this communication, we demonstrate that liposome-bound camptothecin is stable, thus suggesting that liposomes may serve as useful drug delivery systems for solubilizing camptothecin and conserving both its lactone ring and antitumor activity.

In order to study the equilibrium associations of camptothecin with lipid bilayers, we exploited the drug's intense intrinsic fluorescence. Fluorescence is associated with the extended conjugation of the quinoline ring system. Upon association with small unilamellar vesicles (SUVs) composed of L-α-dimyristoyl phosphatidylglycerol (DMPG), the λ_{max} value of camptothecin's emission spectrum shifts to lower wavelength, or blue shifts, some 16 nm (Figure 1).

While these spectral changes provide qualitative evidence that camptothecin binds membranes, fluorescence anisotropy titration offers the most sensitive means for quantitatively assessing the extent of drug binding. For example, Figure 2 shows a 19-fold enhancement in camptothecin's steady-state anisotropy (a) value upon drug association with DMPG vesicles. Similar results are shown for drug binding to electroneutral L-α-dimyristoyl phosphatidylcholine (DMPC). Analysis of the data using double-reciprocal plots[3] gave identical overall association constants of 100 M^{-1}.

Having determined the concentrations of DMPC and DMPG required to assure a bound drug fraction in excess of 97%, we then used an HPLC assay[4] to evaluate the stability of free and liposome-bound drug (Figure 2, inset). We observed rapid hydrolysis of free camptothecin in PBS at 37 °C ($t_{1/2}$ = 16.6 min). In

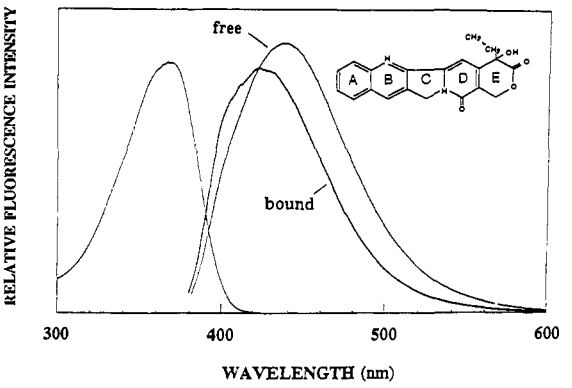

Figure 1. Fluorescence excitation and emission spectra of camptothecin (1 μM, PBS buffer, 37 °C). Also shown is the emission spectra for DMPG-bound drug (0.29 M lipid).

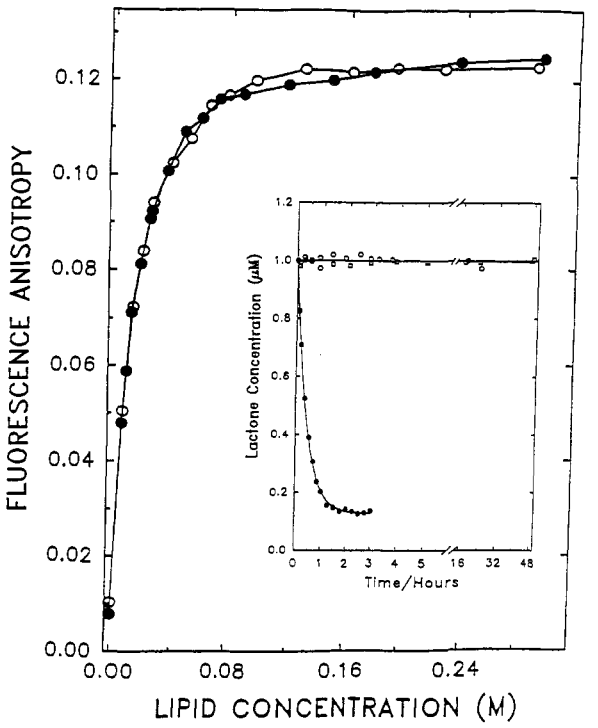

Figure 2. Equilibrium binding of camptothecin to SUVs composed of DMPG (○) and DMPC (●). The inset shows the change in lactone concentration as a function of time for free drug (●), DMPC-bound drug (○), and DMPG-bound drug (□). Drug and lipid concentrations of 1 μM and 0.29 M, respectively, were used (PBS buffer, 37 °C).

marked contrast, however, camptothecin was found to be stable in both DMPC and DMPG bilayers, with no evidence of ring opening even at time points as long as 48 h. Stabilization was also achieved in liposomal formulations containing drug concentrations of 2 mM, where the lipid:drug ratio was reduced to 150.

The fact that liposome-associated camptothecin is stable suggests that the drug's lactone ring penetrates into the bilayer. Two types of spectroscopic data are available which support this notion. The first type of evidence comes from Figure 1, where blue shifting of the drug's emission spectrum is observed upon association with membrane. Such a spectral shift is indicative of a change in the dielectric constant of the medium surrounding the fluorophore, as when a compound leaves an aqueous environment and intercalates in between the lipid acyl chains.[5]

Additional evidence that camptothecin's fluorochrome penetrates into the lipid bilayer comes from iodide quenching data.[6]

(1) Giovanella, B. C.; Stehlin, J. S.; Wall, M. C.; Nicholas, A. W.; Liu, L. F.; Silber, R.; Potmesil, M. *Science (Washington, D.C.)* **1989**, *246*, 1046-1048.

(2) Wani, M. C.; Nicholas, A. W.; Manikumar, G.; Wall, M. E. *J. Med. Chem.* **1987**, *30*, 1774-1779.

(3) Burke, T. G.; Tritton, T. R. *Biochemistry* **1985**, *24*, 1768-1776.

(4) The kinetics of lactone ring opening was monitored by HPLC with fluorescence detection. Separation of parent drug from carboxylate form was achieved on an Ultrasphere C-18 column using an isocratic mobile phase consisting of 32% acetonitrile, 67% 0.1 M acetate buffer (pH 5.5), and 1% 0.1 M sodium dodecyl sulfate. The detergent sodium dodecyl sulfate was added to samples prior to injection.

(5) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, 1983.

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Iodide has an immeasurably small permeation of the bilayer[7] and is able to discriminate between molecules free in solution from those located in the interior of the membrane. While camptothecin free in solution was quenched readily by iodide (V = 1.8 M^{-1}; K_{SV} = 44.3 M^{-1}), drug bound to DMPC membranes was much more difficult to quench (V = 0 M^{-1}; K_{SV} = 6.2 M^{-1}). Membrane-bound drug is thus much less accessible to quenching by iodide, presumably because the fluorochrome locates deep with the bilayer.

In summary, the important anticancer drug camptothecin has been shown here to be stable when harbored within liposomal bilayers of DMPC and DMPG. Because liposomes have been shown to be useful clinically in the delivery of other anticancer drugs such as doxorubicin, daunorubicin, and *cis*-platinum complexes (see refs 8–10 and references therein), and because liposome suspensions stabilize the pharmacologically active form of camptothecin, liposomal drug delivery systems may be of potential utility for introducing camptothecin (or related lipophilic analogues) to cancer victims. Studies of such formulations in experimental models are presently in progress.

(6) The static (V) and dynamic (K_{SV}) quenching constants for free and membrane-bound drug were determined at 37 °C as described previously (Burke, T. G.; Tritton, T. R. *Biochemistry* **1985**, *24*, 5972–5980). Experiments were conducted at a constant halide concentration of 0.5 M. A lipid concentration of 100 mg/mL was used. Camptothecin's excited-state lifetime (τ) value for drug free in solution was determined, using a 200-MHz frequency-domain fluorometer, to be 4.2 ns, similar to the average τ value for membrane-bound drug (3.7 ns). Since $K_{SV} = \tau k_q$, where k_q is the collisional rate constant, the observed differences in K_{SV} between free and membrane-bound species are dominated by differences in the collision rate constant (k_q).

(7) Jendrasiak, G. L. *Chem. Phys. Lipids* **1972**, *9*, 133–146.

(8) Forssen, E. A.; Coulter, D. M.; Proffitt, R. T. *Cancer Res.* **1992**, *52*, 3255–3261.

(9) Perez-Soler, R.; Priebe, W. *Cancer Res.* **1990**, *50*, 4260–4266.

(10) Khokhar, A. R.; Al-Baker, S.; Brown, T.; Perez-Soler, R. *J. Med. Chem.* **1991**, *34*, 325–329.

Geometry and Dynamics of Benzene Chemisorbed on a Pt/η-Al_2O_3 Catalyst: A ^{13}C Dipolar NMR Study

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It has been suggested from infrared investigations of benzene chemisorbed on alumina-supported platinum catalysts that the symmetry of the molecule is C_{3v}.[1,2] This result was found to be consistent with earlier infrared studies of benzene adsorbed on a Pt(111) single-crystal surface,[3] in which a C_{3v} symmetry was also assigned.[4] Since molecular distortion may be a precursor to catalytic activity, and since highly disperse metal-supported particles may result in an increase of reactivity in structure-sensitive processes involving carbon–carbon bonds,[5] the measurements reported in this communication were performed using 95%-disperse Pt/η-Al_2O_3. Given that infrared studies provide only indirect information about a possible alternation of short and long C–C bond lengths, a direct NMR determination of the C–C distances would constitute a more stringent test of such a distortion. We report here the results of a dipolar NMR study which indicates the absence of significant distortion of benzene chemisorbed on highly disperse Pt/η-Al_2O_3 as well as rapid ring reorientation at temperatures as low as 6 K.

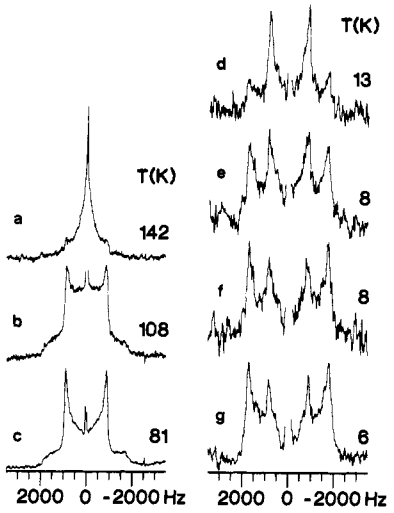

Figure 1. ^{13}C spectra of doubly-labeled benzene (1,2-$^{13}C_2$) chemisorbed on Pt/η-Al_2O_3 obtained at 15 MHz (1.4 T) using a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with π pulses of width 9.6 μs (B_{1C} = 4.86 mT) and 15.4% rf duty factor. The narrow central peak cropped for clarity in the spectra shown in Figure 1b–g is partially due to spin-locking by the average rf field.

Nutation NMR spectroscopy[5] is especially suited for measuring interatomic distances in orientationally disordered solids and has been used to confirm bond alternation in *trans*-polyacetylene.[6] In that case, a superposition of two Pake doublet patterns permitted unambiguous confirmation of two C–C bond lengths. The Pake doublet splitting is proportional to the inverse third power of the ^{13}C–^{13}C internuclear distance and is the parameter used to measure bond lengths in this experiment.[5] In this work, we used the Carr–Purcell–Meiboom–Gill (CPMG) sequence,[7] which in principle offers twice the spectral resolution of a nutation experiment and thus a better chance of detecting two nearly equal bond lengths. We recently showed theoretically as well as experimentally that the Pake splitting measured using the CPMG pulse sequence scales linearly with the radio frequency (rf) duty factor and that appropriate extrapolation of a plot of doublet splitting versus duty factor yields C–C distances accurate to ~1%.[8]

The ^{13}C CPMG spectra of benzene-$^{13}C_2$ chemisorbed on Pt/η-Al_2O_3 obtained with cross polarization and proton decoupling[5] are shown in Figure 1.[9] The spectra change significantly with temperature, indicating dynamical behavior which is very different from what is observed in bulk solid benzene. In the latter at temperatures >90 K, the molecule is known to reorient rapidly about the hexad axis, and the doublet splitting should be reduced to one-half the rigid-lattice value.[10] However, as Figure 1 shows,

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(1) Haaland, D. M. *Surf. Sci.* **1981**, *102*, 405.

(2) A much smaller distortion has recently been reported in bulk benzene: Jeffrey, G. A.; Ruble, J. R.; McMullan, R. K.; Pople, J. A. *Proc. R. Soc. (London)* **1987**, *A414*, 47.

(3) Lehwald, S.; Ibadi, H.; Dehmuth, J. E. *Surf. Sci.* **1979**, *78*, 577.

(4) A NEXAFS study of a similar sample, however, indicated a change of less than 0.02 Å; see: Horsley, J. A.; Stöhr, J.; Hitchard, A. P.; Newbury, D. C.; John, A. L.; Settle, F. J. *J. Chem. Phys.* **1985**, *83*, 6099.

(5) Yannoni, C. S.; Kendrick, R. D. *J. Chem. Phys.* **1981**, *74*, 747. Horne, D.; Kendrick, R. D.; Yannoni, C. S. *J. Magn. Reson.* **1983**, *38*, 493.

(6) Yannoni, C. S.; Clarke, T. C. *Phys. Rev. Lett.* **1983**, *51*, 1191.

(7) Carr, H. Y.; Purcell, E. M. *Phys. Rev.* **1954**, *94*, 630. Meiboom, S.; Gill, D. *Rev. Sci. Instrum.* **1958**, *29*, 688.

(8) Engelsberg, M.; Yannoni, C. S. *J. Magn. Reson.* **1990**, *88*, 393.

(9) The 0.2-g sample contained 5 wt % platinum, as determined by X-ray fluorescence spectroscopy. The catalyst was prepared from chloroplatinic acid by reduction in H_2 at 593 K for 2 h, followed by outgassing for 3 h at the same temperature. Subsequently it was cooled and outgassed at 298 K for 2 h. The catalyst thus activated was then equilibrated with doubly-^{13}C-labeled benzene (benzene-1,2-$^{13}C_2$ from Isotech) at ambient temperature. The sample was then outgassed at 298 K for 6 h. This procedure leaves benzene molecules chemisorbed on the Pt surface, removing most of the physisorbed material, see: ref 1 and Tirendi, C. F.; Mills, G. A.; Dybowski, C. R. *J. Phys. Chem.* **1984**, *88*, 5765. The N_2 BET surface area of the catalyst before reduction was 179 m^2/g. The amount of benzene chemisorbed under these conditions, as determined by uptake measurements, was estimated to be 0.11 mmol of benezene per gram of catalyst. The dispersion of the Pt as measured by hydrogen uptake experiments is high, ~95%.

(10) Andrew, E. R.; Eades, R. G. *Proc. R. Soc. (London)* **1953**, *A218*, 537.

0002-7863/92/1514-8319$03.00/0