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Interactions of Daphnodorin B and Daphentin with Calf Thymus DNA

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The interactions of daphnodorin B and daphentin with calf thymus DNA (ctDNA) were investigated. The binding constants of daphnodorin B and daphentin with ctDNA were obtained by UV spectroscopy. Further, the binding mechanisms on the reaction of the two compounds with ctDNA and effect of ionic strength on the fluorescence property of the system have also been investigated. The results of the assay indicate that daphnodorin B intercalates with ctDNA while daphentin interacts with ctDNA by groove binding. And the binding constants of daphnodorin B and daphentin with ctDNA were 3.261×10^5 and 1.063×10^4 L mol^{-1}, respectively.

Key Words: Daphnodorin B, Daphentin, Calf thymus DNA, Binding.

INTRODUCTION

Daphentin and daphnodorin B (Fig. 1) were successfully isolated and purified from the crude extract *Daphne odora var. marginata*. These two compounds are the main bioactive compounds in *Daphne odora var. marginata* and have various biological and medicinal activities, including antibacterial, antiinflammatory, antioxidation, antitumour, anticancer and cardiovascular protection[1]. Daphentin has been clinically used against rheumatoid arthritis, coronary heart disease, angina pectoris and thrombotic vasculitis[2]. It has been reported that daphentin and daphnodorin B demonstrated higher *in vitro* activity against hepatoma SMMC-7721 proliferation compared to breast cancer cell MCF-7, while daphnodorin B exhibited higher activity than daphentin in both celllines[3].

Many chemicals exert their antitumour effects through binding to DNA, inhibiting DNA replication/transcription thus inhibiting growth of tumour cells[4]. DNA is the intracellular target of a wide range of anticancer and antibiotic medicines[5,6]. In recent years, the interaction of nucleic acid and small molecule has been extensively explored with fluorescent dyes, metal complexes and medicines[7-12]. These studies can facilitate understanding the drug action and mechanism, designing new and promising drugs and developing sensitive chemical probes for nucleic acid structure[13]. To our best knowledge, there has been no direct study on the interactions of daphentin and daphnodorin B with DNA. Herein we report the binding mode and binding affinity of daphnodorin B and daphentin with calf thymus DNA (ctDNA) with supporting experimental evidences.

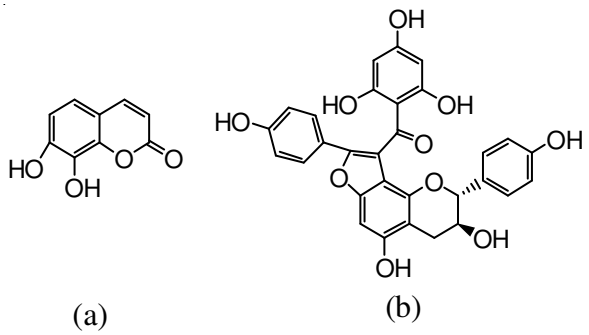

Fig. 1. Chemical structures of daphnetin (a) and daphnodorin B (b)

Many techniques have been employed to investigate the interaction of DNA with small molecules, including fluorescence spectroscopy, UV-spectrophotometry, electrophoresis, nuclear magnetic resonance, electrochemical methods[14-17]. UV-VIS absorption and fluorescence spectroscopy are regarded as effective methods among these techniques because they are sensitive, rapid and simple[18].

In this work the interactions of daphnodorin B and daphentin with calf thymus DNA (ctDNA) in *tris*-HCl buffer were explored by UV spectrum, fluorescence spectrum, DNA melting point, iodide anion effect, ionic strength effect and. The elucidation of the binding modes will be useful for further understanding the pharmacological actions and effects of the two compounds. This work will also be beneficial for future design and development of novel DNA probes and small molecule medicines.

EXPERIMENTAL

The stock solutions (1×10^{-3} mol L^{-1}) of daphnetin and daphnodorin B (≥ 90 %) were prepared by dissolving in anhydrous methanol. Calf thymus DNA (Sigma Chem. Co, USA) was dissolved in 0.05 mol L^{-1} *tris*-HCl buffer solutions (pH 7.4) as stock solution and stored at 4 ºC. The concentration of DNA was determined by UV absorption at 260 nm using a molar absorbption coefficient ε = 6600 L mol^{-1} cm^{-1}. Purity of the DNA was confirmed by monitoring the ratio of the absorbance at 260 nm to that at 280 nm. The solution gave a ratio of > 1.8 at A_{260}/A_{280}, indicating that DNA was sufficiently free from protenin[12]. *Tris*-HCl buffer solution (0.05 mol L^{-1}, pH 7.4) was used to control the pH of the working solution and NaCl was used to adjust the ionic strength of the solution. Other chemicals were analytical-reagent grade and double distilled water was used throughout. All solution were shaken thoroughly and allowed to equilibrate for 0.5 h before spectral measurement were made at room temperature. The primary data were transferred to the ORIGIN graphic program for plotting and analysis. All experiments, unless specified otherwise, were carried out at room temperature.

UV-VIS absorption spectrum: All UV-VIS absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer (Japan) with a matched set of 1 cm path length quartz cuvettes. The slit width was 1.5 nm. The absorption titration was performed at 1.0×10^{-5} mol L^{-1} of the daphentin and daphnodorin B and by varying the concentration of double stranded ctDNA. In order to prevent interference from DNA absorption, the data were obtained by keeping the same concentration of ctDNA in the reference cuvette.

Fluorescence spectrum: All fluorescence measurements were performed on a Hitachi F-4600 fluorescence spectrophotometer (Japan) using a quartz cuvette of 1 cm path length. The fluorescence spectra were measured by fixing the concentration of daphnodorin B and daphentin 5.0×10^{-6} mol L^{-1} while varying the DNA concentrations at 298, 303 and 308 K.

Fluorescence polarization: 1.00×10^{-5} mol L^{-1} daphnodorin B and daphentin solutions (2 mL each) were titrated with increasing concentrations of ctDNA in a 1 cm cuvette. The added volume of ctDNA was no more than 20 μL to avoid complications due to dilution effect. Excitation was set at 295 and 310 nm with a slit width of 5 nm for daphentin and daphnodorin B, respectively. The mixture was shaken thoroughly and then equilibrated for 0.5 h at room temperature.

The measurement of polarization follows equation[15]:

$$P = \frac{(F_{VV} - F_{VH}G)}{(F_{VV} + F_{VH}G)} \quad (1)$$

$$G = \frac{F_{HV}}{F_{HH}} \quad (2)$$

where F_{VV} is the vertical polarization intensity vertical with excitation; F_{VH} is the horizontal polarization intensity vertical with excitation. F_{HV} is the vertical polarization intensity parallel with excitation; F_{HH} is the horizontal polarization intensity parallel with excitation.

DNA melting points: Melting point curves were recorded using an UV-VIS double beam spectrophotometer equipped with a thermostatic cell compartment. The measurements were carried out in *tris*-HCl buffer (0.05 mol L^{-1}, pH 7.4) using continuous heating from 40 to 95 ºC at 260 nm.

KI quenching experiment: A series of assay solutions containing various amount of KI and a fixed amount of sample-DNA were prepared to measure the fluorescence intensity. The quenching constants of the sample-DNA complex at different KI concentrations were determined.

Ionic strength effect: The fluorescence of a fixed amount of sample-DNA was measured with various concentrations of NaCl.

RESULTS AND DISCUSSION

UV-VIS spectral studies: In general, the binding of small molecule to DNA helix is often characterized through absorption spectral titrations, followed by the changes in the absorbance and shift in the wavelength[19]. This phenomenon indicates that the small molecule has intercalated into DNA base pairs and is involved in a strong interaction in the molecular stack between the aromatic chromophore and the base pairs. The spectral changes have been rationalized as follows[20,21]: the empty π*-orbital of the small molecule couples with the π*-orbital of the DNA base pairs, which causes an energy decrease and a decrease of the π-π* transition energy. Therefore, the absorption of the small molecule should exhibit red-shift. At the same time, the empty π*-orbital is partially filled with electrons to reduce the transition probability, which leads to hypochromism.

The absorption spectra of daphnodorin B and daphentin are shown in Fig. 2. In the absence of DNA, the absorption peak of daphnodorin B at 307 nm, with the increase of DNA concentration, the absorption peak from 307 nm red shift to 312 nm and have a notable hypochromicities. However, no obvious red shift (325 nm) and no notable hypochromicities were observed for daphentin. The electronic absorption spectra indicate that the binding mode of daphnodorin B with ctDNA is to be intercalation, while daphentin not. To quantify the binding strength of daphentin/daphnodorin B with DNA, the intrinsic binding constant K_b was determined by equation[22]:

$$\frac{[DNA]}{(\varepsilon_a - \varepsilon_f)} = \frac{[DNA]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_b - \varepsilon_f)} \quad (3)$$

where [DNA] is the concentration of DNA, ε_a, ε_f and ε_b correspond to the extinction coefficients for the free sample, for each addition of DNA to the sample and for the sample in the fully bound form, respectively. The values of K_b that daphnodorin B and daphentin to DNA were calculated to be 3.261×10^5 L mol^{-1} and 1.063×10^4 L mol^{-1}, respectively. The value of K_b described in the literature for classical intercalators (ethidium-DNA, 4.94×10^5 L mol^{-1} [23]; proflavin-DNA, 4.1×10^5 L mol^{-1} [24]). The values of K_b obtained here were consistent with the same magnitude of the fluorescence quenching constant K reported in the fluorescence spectral characteristic. These data demonstrated that daphnodorin B can strongly intercalate into DNA base while daphentin not, agreeing with the hypothesis.

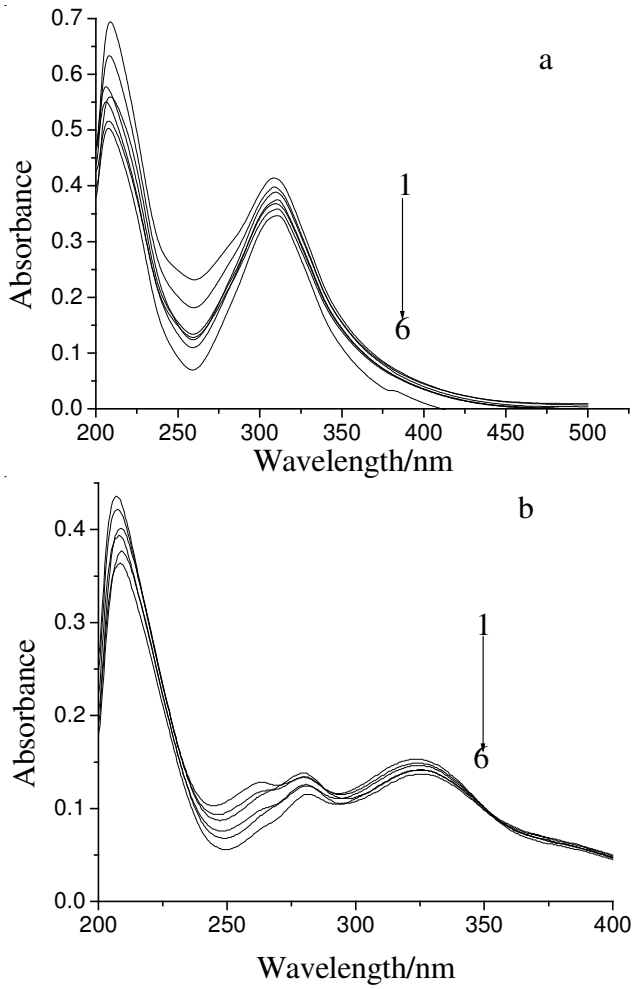

Fig. 2. UV-VIS spectra of daphnodorin B (a) and daphentin (b) with increasing concentration of ctDNA $C_{(Daphnodorin\ B)} = C_{(Daphentin)} = 1 \times 10^{-5}$ mol L^{-1}; [DNA]: 0, 0.21×10^{-5}, 0.42×10^{-5}, 0.63×10^{-5}, 0.84×10^{-5}, 1.05×10^{-5} mol L^{-1}, respectively from 1 to 6

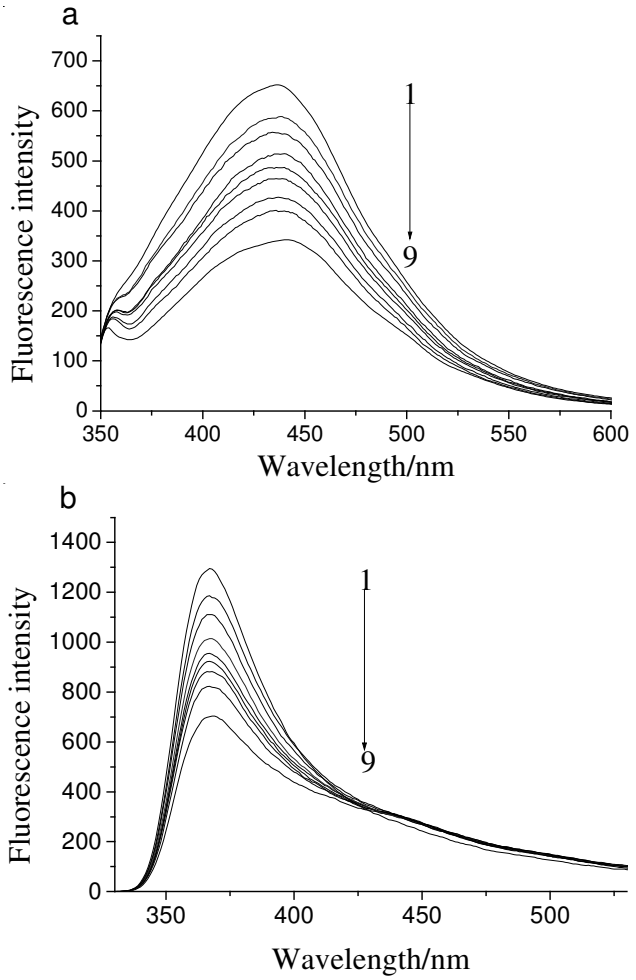

Fig. 3. Fluorescence quenching spectra of daphnodorin B (a) and daphentin (b) as ctDNA were added. Conditions: pH 7.4; $C_{Daphnodorin\ B} = 5.0 \times 10^{-6}$ mol L^{-1} from 1 to 9: C_{ctDNA} = 0, 1.05, 2.1, 3.15, 4.2, 5.25, 6.3, 7.35 and 8.4×10^{-6} mol L^{-1}

Fluorescence spectroscopic studies

Fluorescence quenching spectra: The fluorescence spectra of daphnodorin B and daphentin are shown in Fig. 3. As can be known from Fig. 3 the fluorescence intensity of daphnodorin B and daphentin were dramatically quenched when the ctDNA was added at room temperature, which indicated both of daphnodorin B and daphentin could interact with ctDNA.

Fluorescence quenching constant: Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching, such as excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional process[25]. The different mechanism of quenching are classified as either dynamic quenchin or static quenching. Dynamic and static quenching result from collision between fluorophores and aquencher. Dynamic quenching is due to diffusion. Since higher temperatures lead to larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature. In contrast, increased temperature is likely to result in decreased stability of complexes and thus lower values of the static quenching constants[26].

In order to confirm the quenching mechanism, the fluorescence quenching was analyzed according to the equation of Stern-Volmere[27]:

$$F_0/F = 1 + Kq\ t[Q] = 1 + K_{SV} \quad (4)$$

where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of quencher, respectively. Kq is the quenching rate constant of biomolecule. τ_0 the average lifetime of the fluorescence molecule in the absence of quenching reagent and its value is about 10^{-8} s, [Q] is the concentration of quencher. K_{SV} is the Stern-Volmer quenching constant and also is static quenching constant when it is static quenching reaction.

The quantitative analysis of the binding ctDNA was carried out using the Stern-Volmer equation. The Stern-Volmer plots of daphnodorin B and daphentin binding to DNA were obtained at 298, 303 and 308 K. The plots of F_0/F *versus* [Q] are shown in Fig. 4 and the quenching constant K_{SV} are listed in Table-1. Obviously, the quenching constant of daphnodorin B and daphentin quenching procedure initiated by ctDNA decrease along with the temperature increases. This means that the quenching of daphnodorin B and daphentin to DNA is not controlled by dynamic collision but from the formation of a complex.

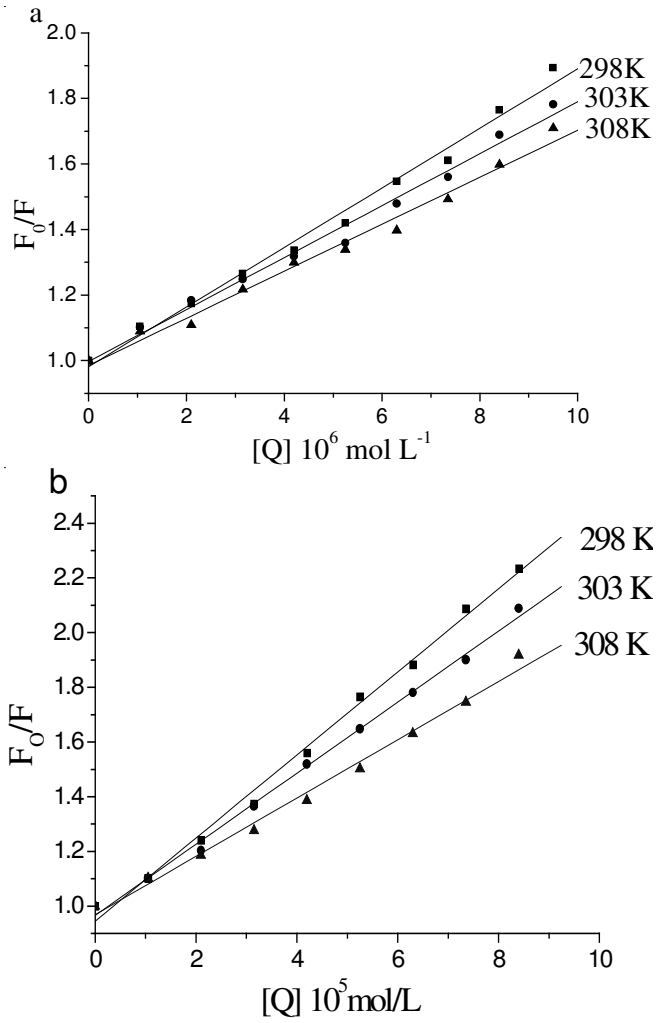

Fig. 4. Sterm-Volmer curves at 298, 303 and 308 K of daphnodorin B (a) and daphentin (b)

TABLE-1
QUENCHING CONSTANTS AT DIFFERENT TEMPERATURES

	T (K)	K_{sv} (L mol^{-1} s^{-1})	R
Daphentin	298	1.518×10^4	0.9973
	303	1.298×10^4	0.9982
	308	1.161×10^4	0.9954
Daphnodorin B	298	9.201×10^4	0.9924
	303	8.342×10^4	0.9943
	308	7.176×10^4	0.9913

Binding parameters: When small molecules binding dependently to a set of equivalent sites on a fluorescence molecule, the equilibrium between free and bound molecule is given by the equation[28]:

$$\log (F_0\text{-}F)/F = \log K + n \log [Q] \quad (5)$$

where K and n are the binding constant and the number of binding sites, respectively. The values of K and n were calculated and shown in the Table-2. It was noticed that about 1 daphnodorin B and daphentin molecule combine with 1 ctDNA molecule.

In orde to illustrate the interaction of daphnodorin B and daphentin with ctDNA, the thermodynamic parameters were calculated from the van't Hoff plots. There are several acting force between small molecular and biomacromolecule, such as hydrogen bond, van der Waals, electrostic force, hydrophobic force. When there is little change of temperature, the enthalpy change can be seen as a constant. The formulae which reflect the relationship to the change of enthalpy, free energy and entropy are follows:

$$\ln K = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (6)$$

$$\Delta G^\circ = \Delta H\text{-}T S^\circ = -RT \ln K \quad (7)$$

where K and R are binding constant and gas constant, respectively. The temperatures used were 298, 303, 308 K. The enthalpy change (ΔH°) and entropy change (ΔS°) were obtained from the slope of the van't Hoff relationship and listed in Table-2.

It can be seen from the results obtain, that for the binding system of daphnodorin B and daphentin, both of their ΔG° are negative values which reveled the interaction process are spontaneous. For the daphentin system, $\Delta H^\circ > 0$, $\Delta S^\circ > 0$, the mainly force is hydrophobic; the daphnodorin B system, $\Delta H^\circ < 0$, $\Delta S^\circ < 0$, the mainly acting force is van der Waals or hydrogen bond[29]. Therefore, the hydrophobic forces is the main acting force in the binding of daphnodorin B and ctDNA, but van der Waals or hydrogen bond plays a major role in the binding of daphentin and ctDNA.

Fluorescence polarization experiment: Small molecules are weakly polarized due to the rapid tumbling motion in aqueous media. However, when small molecule intercalates into the helix, its rotational motion is restricted and therefore the fluorescence polarization of the bound chromophore is increased. In contrast, binding solely to the phosphate backbone or to the DNA grooves does not result in enhanced fluorescence polarization[30]. As shown in Table-3, an increase in ctDNA resulted in an increase in fluorescence polarization of the daphnodorin B system, but the fluorescence polarization of the daphentin system had no significant change. These data prove that daphnodorin B intercalates into the helix and results with lower rotation rate and higher polarization, while the interaction of daphentin and DNA is groove binding and results without effective change of the rotation rate thus the polariztion.

TABLE-2
BINDING CONSTANTS OF DAPHNODORIN B AND DAPHENTIN WITH ctDNA AND THE THERMODYNAMIC PARAMETERS

	Temp. (K)	N	K (L mol^{-1})	R	ΔG (KJ mol L^{-1})	ΔH (KJ mol L^{-1})	ΔS (Jmol $^{-1}K^{-1}$)
Daphnodorin B	298	0.9264	6.282×10^5	0.9936	-34.19	-51.12	-282.6
	303	0.9567	5.018×10^5	0.9948	-33.06		
	308	1.062	3.166×10^5	0.9927	-31.37		
Daphentin	298	1.271	1.154×10^4	0.9934	-23.94	27.52	173.2
	303	1.124	2.141×10^4	0.9968	-25.12		
	308	1.082	3.043×10^4	0.9921	-25.67		

TABLE-3
FLUORESCENCE POLARIZATION VALUES OF DAPHNODORIN B AND DAPHENTIN IN PRESENCE OF ctDNA

DNA (1×10^{-5} mol L^{-1})	0	0.4	0.8	1.2	1.6	2.0	2.4
Daphnodorin B	0.01956	0.02895	0.03445	0.03725	0.04081	0.04372	0.04968
Daphentin	0.09708	0.09881	0.10900	0.09814	0.10910	0.09964	0.09973

Studies of DNA melting temperature: When DNA solutions are exposed to extremes of pH or heat, the double helical structure of DNA undergoes a transition into a randomly single-stranded form at the melting temperature (T_m). The intercalation of small molecules into the double helix is known to increase the DNA melting temperature[16]. Intercalation binding can stabilize the double helix structure causing T_m to increase by about 5-12 ºC, but non-intercalation binding has no significant effect on T_m[17]. Thus, this parameter is most useful in analyzing the mode of binding interaction[31].

The T_m of the complexes of DNA-daphentin and DNA-daphnodorin B system were determined by monitoring the UV-VIS absorption spectra. The absorption at 260 nm was monitored every 5 ºC between 40 and 95 ºC. As shown in Fig. 5, f_{ss} was defined as $(A-A_0)/(A_f-A_0)$, in which A_0 and A_f are the initial and final absorbance[32]. f_{ss} was plotted with temperature and T_m was determined as $f_{ss} = 0.5$. T_m of DNA system in the absence of small drug molecules is 75.2 ºC, but the Tm of DNA-daphnodorin B and DNA-daphentin system are 82.5 and 77.8 ºC, which corresponded to an increase of 7.3 and 2.6 ºC, respectively. The data suggests that the binding modes of daphentin and daphnodorin B with DNA are different, where the interaction of daphnodorin B with DNA is intercalative binding, but the binding mode of daphentin with DNA is groove binding.

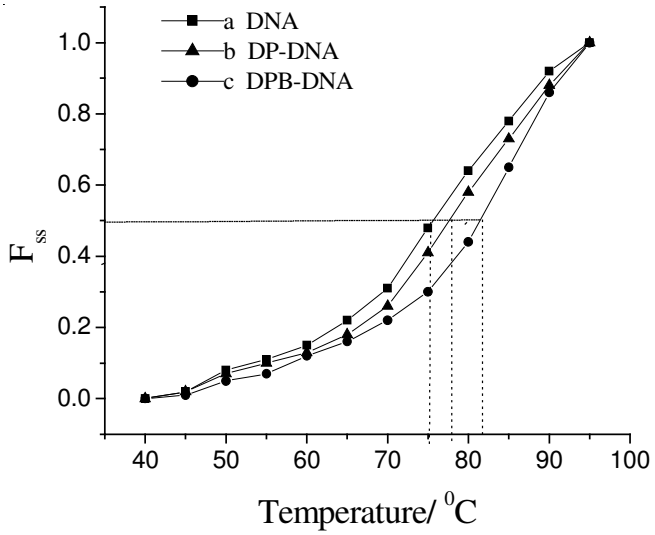

Fig. 5. Thermal melting curves for DNA in the absence (a) and presence of daphentin (b) and daphnodorin B (c) $C_{DNA} = 2.10 \times 10^{-5}$ mol L^{-1}; $C_{(Daphnodorin\ B)} = C_{(Daphentin)} = 1 \times 10^{-5}$ mol L^{-1}

KI quenching experiment: To study the interaction pattern of small molecule with DNA, fluorescence quenching experiments were performed. Some anion can quench fluorescence efficiently, such as[33,34] $Fe(CN)_6^{4-}$ and I^-. In this paper, I^- was chosen to determine the accessibility of sample to anionic quencher in the absence and presence of ctDNA.

Intercalation protected the bound molecules from the anionic quencher, due to the bases above and below the intercalator. Groove binding exposes the bound molecules to the solvent surrounding the helix. Compared with intercalative binding, grooving binding provides much less protection for the chromophore. If small molecule binds to DNA in the groove, the magnitude of K_{SV} of the bound small molecule should be higher than that of the free small molecule[35]. In contrast, if small molecule is intercalated into the DNA base pairs, the magnitude of K_{SV} of the bound small molecule should be lower than that of unbound small molecules[32]. As shown in Fig. 6, binding to DNA resulted in decreased quenching of the fluorescence intensity. The K_{SV} values are summarized in the Table-4. When the concentration of I^- was increased, the corresponding K_{SV} of daphentin system was lower than the K_{SV} of daphentin-DNA system, but the K_{SV} of daphnodorin B system was higher than the K_{SV} of daphnodorin B-DNA system. These results further demonstrate that the binding modes of daphnodorin B and daphentin with ctDNA are intercalative and groove binding, respectively.

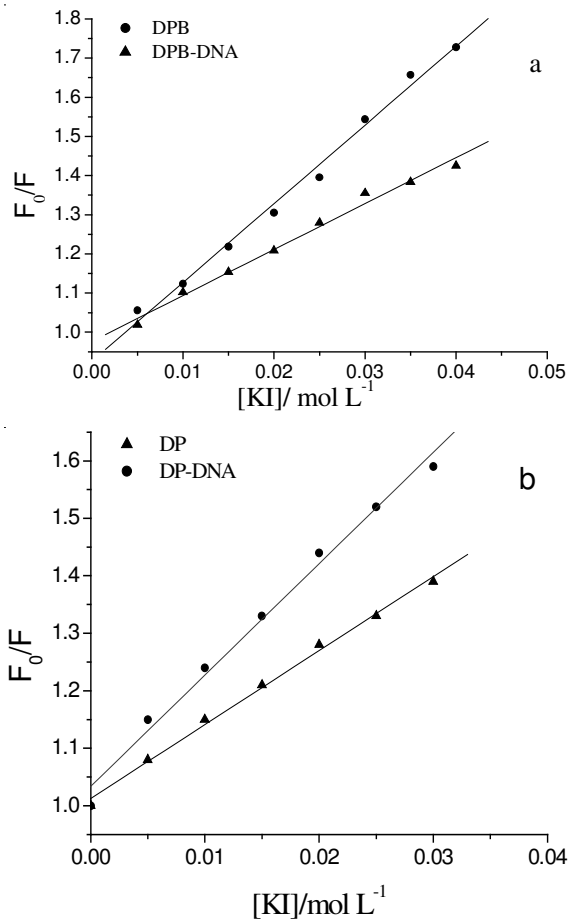

Fig. 6. Stern-Volmer plots of KI quenching effect on daphnodorin B (a) and daphentin (b) $C_{(Daphnodorin\ B)} = C_{(Daphentin)} = 5 \times 10^{-6}$ mol L^{-1}; $C_{DNA} = 2.10 \times 10^{-5}$ mol L^{-1}

TABLE-4
STERN-VOLMER EQUATIONS OF DAPHNODORIN B AND DAPHENTIN QUENCHED BY KI

Compound	Stern-Volmer equations	K_{SV} (L mol^{-1})	r
Daphnodorin B	Y = 0.9263 + 20.09 [Q]	20.09	0.9958
Daphnodorin B-DNA	Y = 0.9767 + 11.73 [Q]	11.73	0.9945
Daphentin	Y = 1.0120 + 12.86 [Q]	12.86	0.9972
Daphentin-DNA	Y = 1.0420 + 19.36 [Q]	19.36	0.9948

Effect of ionic strength: DNA is an anionic polyelectrolyte with phosphate groups. The study of ionic strength effect is also an efficient method to distinguish binding mode between molecules and DNA. As a controller of ionic strength of solution, Na^+ act as counter ions to decrease the unwinding tendency for electrostatic repulsion between the negatively charged phosphate groups on adjacent nucleotides[36]. When NaCl is present in the system, the electrostatic repulsion between the negatively charged phosphate skeletons on adjacent nucleotides reduces with increasing concentration of Na^+. Nevertheless, when small molecule binds to DNA in the mode of groove binding, it is exposed in the solution much more than it does in the mode of the intercalation. The effect of NaCl on the fluorescence intensity ratio (F/F_0) is shown in Fig. 7. With increasing concentration of NaCl, there was no significant change observed in the fluorescence intensity of daphnodorin B system, but the ratio of F/F_0 for the daphentin system was increased. This phenomenon shows that the electrostatic repelling interaction among the negative charges of DNA phosphate backbone in the daphentin system changed in the presence of NaCl but not affected in the daphnodorin B system, thus providing another evidence that daphentin interacts with ctDNA by groove binding, which makes the daphentin-DNA system much more sensitive to the surrounding ionic changes.

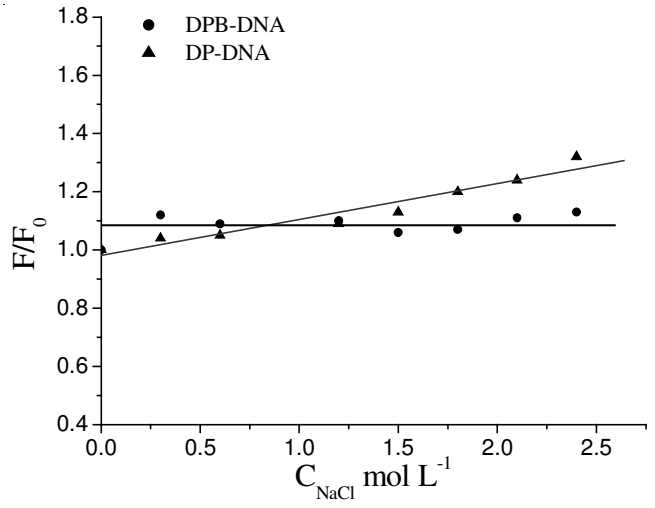

Fig. 7. Effects of ionic strength on the fluorescence intensity of DNA-daphnodorin B and DNA-daphentin

Conclusion

The interactions of daphnodorin B and daphentin with ctDNA had been studied by using various experimental techniques. The binding modes of daphnodorin B and daphentin with ctDNA were determined to be intercalative and groove binding, respectively. The structure of daphentin shows that it contains an rigid plane benzopyran with delocalized electrons and no group available to interact with DNA bases above and below the plane for hydrogen bonding, thus it might lead to groove binding through hydrophobic effect among entry into ctDNA hydrophobic region. However, daphnodorin B has a hydroxyl and two pairs of phenyl hydroxyl groups around the rigid plane benzopyran and the three groups can easily form hydrogen bond with DNA bases. It is possible that these groups form hydrogen bonds with DNA bases and make daphnodorin B embedding into DNA chains more stable. It is obvious that the interaction strength of hydrogen bond is much greater than electrostatic interaction and hydrophobic effect. Hence, the interaction strength of daphnodorin B with ctDNA is greater than daphentin with ctDNA. In addition, the difference in anticancer activity owning to their different interaction modes and binding strength was confirmed by anticancer activity screening test.

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