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# Selective interaction between tylophorine B and bulged DNA

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**Abstract**—Tylophorine B exhibits pronounced cytotoxicity and antitumor activity. In order to survey the structure selectivity to DNA afforded by tylophorine B, we have synthesized a variety of duplex, bulge- and hairpin-containing oligodeoxyribonucleotides. Their binding to tylophorine B has been assayed by fluorescence spectroscopy and thermal melting experiments. The results indicate that oligonucleotides interact with tylophorine B at submicromolar concentration, and the affinity for DNA bulge is optimal (with $K_d$ of 0.018 μM). In addition, the bulged hairpin oligonucleotides are stabilized by binding to tylophorine B. These findings may shed some light on tylophorine B's mode of action in biological systems and result in the rational design of sequence-specific DNA binding molecules.


Tylophorine B (autofine) (Fig. 1a) and its analogs are phenanthroindolizidine alkaloids, which are characteristic constituents of *Cynanchum*, *Pergularia*, *Tylophora*, and some other genera of the *Asclepiadaceae*[1,2] These compounds exhibit pronounced cytotoxicity and antitumor activity,[3–11] and inhibit enzymes involved in the synthesis of DNA[5,6] In traditional Chinese medicine, tylophorine B is used for pain relief, invigorating the circulation of the blood, treating insomnia and asthma, and the removal of strains. In vitro study has suggested that tylophorine B has high cytotoxicity against drug-sensitive KB-3-1 and a multidrug-resistant KB-V1 cancer cell line with $IC_{50}$ values of 16 and 14 nM, respectively.[9] It has also shown significant inhibition against tobacco mosaic virus.[12] These findings emphasize the potential of tylophorine B as a therapeutic lead. A better understanding of its interaction mode with possible cellular targets such as nucleic acid is important to determine its basic mode of action on biological systems. Much work has been focused on the elucidation of noncovalent interactions with DNA by small natural products and their synthetic derivatives. A quantitative understanding of factors that determine recognition of DNA sites would be valuable in the rational design of sequence-specific DNA binding molecules for application in medicine and in the development of tools for biotechnology. In this paper the interaction of tylophorine B to bulged DNA was determined using fluorescence spectroscopy and thermal melting experiments, which show that tylophorine B has favorable molecular interaction with bulged DNA. This is the first detailed report on the interaction between phenanthroindolizidine alkaloids and DNA.

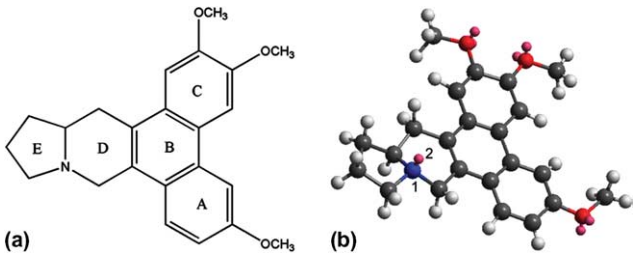


**Figure 1.** (a) 2D structure of tylophorine B generated by ChemDraw; (b) 3D model of tylophorine B generated by Chem3D, ball 1 is nitrogen and ball 2 is lone-pair electron.

HPLC analysis (Fig. 2) suggests that tylophorine B has two isomers in the solution buffer under experimental conditions (pH 4.75, 7.20, and 8.06). The absorbance spectra of two possible isomers (denoted as **A** and **B** in Fig. 2) exhibited extremely slight difference in the range 240–250 nm. However, the reinjection of each HPLC

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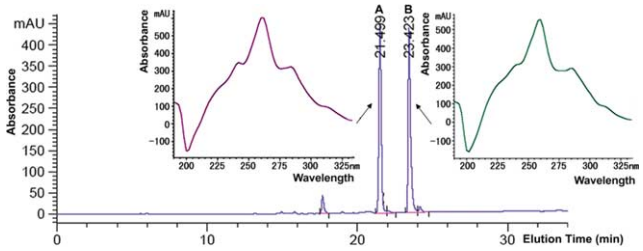


**Figure 2.** HPLC profile for tylophorine B eluted by linear gradient of 0–100% methanol in 10 mM acetate ammonium, pH 4.75. The insets are the UV absorption spectra of isoform A and B as denoted. The result was homologous at pH 7.20 and 8.06 (data not shown).

peak isolate gave same two peaks again on HPLC (data not shown). A 3D model study (Fig. 1b) shows that the lone-pair electron on the nitrogen atom costs very low energy to flip over, which may be the reason for the observed phenomena that this compound did not stably exist in only one isoform. Further, the two isoforms are always in equilibrium in solution. Isomer **B** tends to be more stable than isomer **A** at high pH conditions, as the ratio of the integral area of **A** to **B** in the HPLC is 1:1 at pH 4.75, and increases to 1:2.5 at pH 7.20 and 8.06. The contribution of the lone-pair electron isomerization on the nitrogen atom in tylophorine B to its biological properties is still in need of clarification.

The conformational recognition of DNA has been implicated in many biological processes, which regulate some important cellular processes, such as transcription and translation. In order to survey the structure selectivity afforded by tylophorine B, we have synthesized a variety of duplex, bulge- and hairpin-containing oligodeoxyribonucleotides by DNA Synthesizer (Expedite 8909) using standard phosphoramidite chemistry, and their putative fold patterns are shown in Figure 3. Annealing of strands to form duplexes containing bulges was accomplished by heating the solution at 95 °C for 5 min and then slowly cooling to room temperature, followed by refrigeration for 30 min to ensure the formation of bulges.[13]

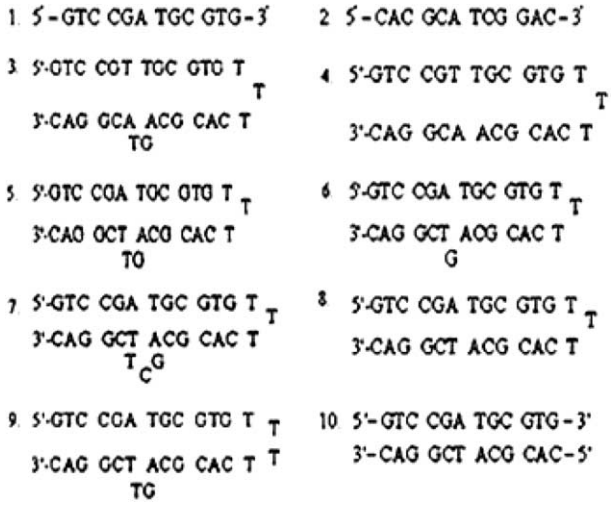


**Figure 3.** Sequences of 10 DNA strands and their tentative secondary structures.

The strong characteristic fluorescence of tylophorine B provides a sensitive spectroscopic handle to study its interaction with DNA. Figure 4a shows the effect of addition of synthetic oligonucleotide **6** on the emission spectrum of tylophorine B. Increasing the concentration of the oligonucleotide results in a gradual decrease in the fluorescence intensity of tylophorine B, without any perceptible shift in the fluorescence maximum of 380 nm at the excitation wave length of 310 nm. A similar fluorescence quenching pattern was found upon addition of the other nine strands of DNA (data not shown). The changes in the emission spectra indicate the formation of a tylophorine B–DNA complex. The fluorescence intensity of tylophorine B tends to be constant at a high concentration of DNA, which suggests the binding reaches saturation. The binding stoichiometry of the tylophorine B–DNA complex is ca. 1:1, which is estimated from Figure 4b by the intersection point of the straight lines in a plot of increase in fluorescence against the molar ratio of DNA to tylophorine B.[14] For 1:1 binding, tylophorine B and DNA satisfy the following equation:

$$\text{tylophorine B–DNA} \overset{K_d}{\leftrightarrows} \text{tylophorine B} + \text{DNA} \quad (1)$$

The dissociation constant $K_d$ of tylophorine B to DNA is calculated from the fluorescence titration curves in Figure 4c according to Eq. 2:[15]

$$\frac{i}{i_0} = 1 + \frac{\Delta i}{2i_0} \times \left\{ (D_0 + T_0 + K_d) - \left[ (D_0 + T_0 + K_d)^2 - 4D_0T_0 \right]^{\frac{1}{2}} \right\} \quad (2)$$

where $i$ and $i_0$ are the fluorescence intensities in the presence and in the absence of DNA, respectively; $\Delta i$ is the changed fluorescence intensities upon unit concentration of tylophorine B binding to DNA, $D_0$ and $T_0$ are the total concentration of DNA and tylophorine B, respectively.

Table 1 lists the $K_d$ values. Clearly, the affinities of tylophorine B to the 10 strands of oligonucleotides are strong, most $K_d$ values are less than $10^{-6}$ M. As can be seen in Table 1, tylophorine B has remarkable selective recognition of both primary and secondary structural features of nucleic acids. Tylophorine B binding is most favorable to bulge containing duplex regions. The binding to single stranded oligonucleotide **1** and **2** is relatively weak, with $K_d$ of 0.97 and 3.25 μM. As expected, the affinity for 12-mer duplex oligonucleotide **10** was also relatively low (0.24 μM), since the thermal stability of short duplexes is lower than that of their hairpin counterparts **4** and **8** (0.18 and 0.16 μM, respectively). Nevertheless the $K_d$ value of **10** is still lower than those of **1** and **2**, suggesting that affinity for the duplex is preferable. Affinity for bulge- and hairpin-containing oligonucleotides is better than single and duplex oligomers. The binding to one base bulged DNA is optimal. With the increase of bulge base number, the binding

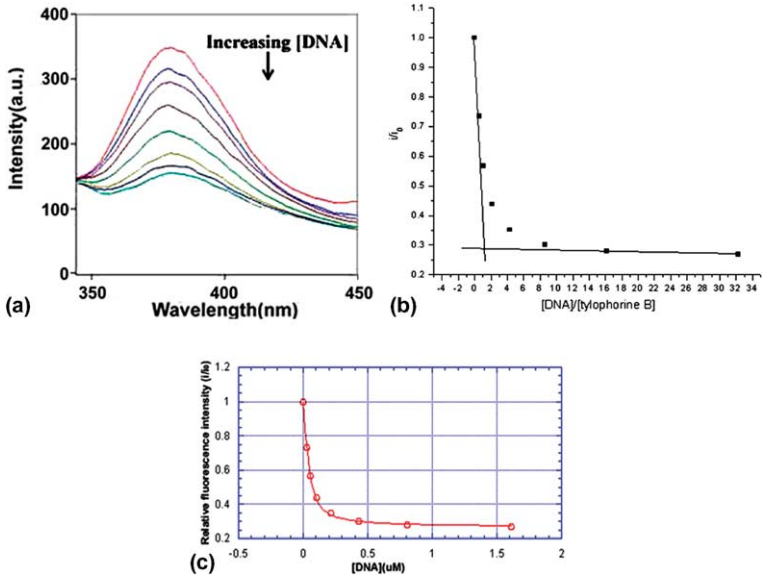


**Figure 4.** (a) Fluorescence emission spectra were measured on a fluorescence spectrophotometer (VARIAN CARY Eclipse). The emission spectra of tylophorine B were obtained in the range 340–450 nm upon excitation at 310 nm at 17.0 °C, using a slit width of 20 nm each. The fluorescence titration experiment was performed by keeping tylophorine B concentration (0.05 μM) constant and varying the concentrations of oligomer **6** from 0, 0.027, 0.054, 0.11, 0.22, 0.43, 0.81 to 1.6 M containing 10 mM phosphate, 50 mM NaCl, pH 7.09. (b) The binding stoichiometry in terms of the number of DNA/tylophorine B molecules is the value at the intersection of the two straight lines, giving the value of ca. 1. (c) The dissociation constant ($K_d$) of tylophorine B binding to oligomer **6** was calculated from the fluorescence titration curve according to Eq. 2 using KaleidaGraph software, giving the value of 0.018 μM. Other fluorescence emission spectra and $K_d$ values were obtained in the same way.

**Table 1.** Dissociation constants ($K_d$) of DNA–tylophorine B

| Sequence | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|
| $K_d$ (μM) | 0.97 | 3.25 | 0.11 | 0.18 | 0.052 | 0.018 | 0.095 | 0.16 | 0.046 | 0.24 |

ability reduces. Oligonucleotide **6** containing one bulge base G possessed the best binding capability, with a $K_d$ of 0.018 μM. Increasing by only one bulge base (oligonucleotide **5**) induces binding ability to decrease almost 3-fold (0.052 μM). Further, additional two bulge bases (oligonucleotide **7**) decreases binding by more than 5-fold (0.095 μM). Oligonucleotide sequences without any bulge base exhibit even lower binding capability than any other hairpin sequences containing a bulge, their dissociation constants (**4**, **8**) increase by ca. 10-fold. Also, the recognition of tylophorine B by oligonucleotide is affected by the sequence neighboring the bulge motif. The sequence with A–T and T–A base pairs nearby the bulge showed better binding ability than the sequence with T–A and T–A base pairs. Oligonucleotide **3** and **5** both contain two bulge bases, but replacement of the T–A base pair adjacent to the bulge site in **5** with an A–T base pair as in **3** leads to 1-fold loss in binding ability. The analogous sequence selectivity was found in the interaction between neocarzinostatin chromophore (NCSi-gb) and bulge DNAs.[16] In addition, the number of looped bases in the hairpin exhibited little effect on binding ability. Compounds **5** and **9** had the same sequence except for the looped base number, and their $K_d$ values are almost same.

Promoted by the high affinity and specificity of tylophorine B binding to DNA, the effect of DNA thermal stability afforded by tylophorine B was also examined. Ultraviolet absorptions of 1–2 μM oligonucleotide **5** to **9** were measured by UV–visible spectrophotometer (VARIAN CARY 100 BIO) with heating up at 0.5 °C/min in phosphate buffer containing 10 mM phosphate, 50 mM NaCl, pH 7.09. $T_m$ values of DNA in the presence of tylophorine B were determined at the condition that the concentration of drug was 2-fold more than that of DNA, and calculated with the derivative method supplied in Cary WinUV software package for $T_m$ calculation. As shown in Table 2, $T_m$ of oligonucleotides increases upon binding by tylophorine B, which means that their secondary structures are stabilized by the interaction with tylophorine B. The changed $T_m$ values ($\Delta T_m$) are generally affected by the oligonucleotides' dissociation constant and the stability of their own secondary structure. The stronger the interaction between DNA and tylophorine B, the more the $T_m$ value increases. On the other hand, the more stable the hairpin structure itself is, the less the $T_m$ value increases. The sequence with a lower number of bulge bases has its hairpin secondary structure most stabilized by tylophorine B. Thus, oligomer **6**'s $K_d$ value was the lowest; however its $\Delta T_m$ is not the highest. Comparatively, **9** with two bulged bases

**Table 2.** $T_m$ of oligomers and $\Delta T_m$ by tylophorine B

| Sequence | **5** | **6** | **7** | **8** | **9** |
|---|---|---|---|---|---|
| $T_m$ (°C) | 64.9 | 70.2 | 63.3 | 80.2 | 64.5 |
| $\Delta T_m$ (°C) | 1.2 | 1.2 | 1.0 | 0.4 | 1.6 |

is stabilized most by tylophorine B, and **8**, which has highest $K_d$ and $T_m$ value in Table 2, is stabilized the least.

Most drugs tend to interact noncovalently with DNA through two general modes: in a groove-bound fashion stabilized by a mixture of hydrophobic, electrostatic, and hydrogen-bonding interactions[17,18] and through an intercalative association[19,20] in which a planar, heteroaromatic moiety slides between the DNA base pairs. Tylophorine B's crystal structure as the benzene olvate has been determined,[21] the aromatic rings lie almost in the same plane, with dihedral angles of only 1.7° (A/B), 2.8° (B/C), 2.2° (A/C), 6.7° (D/E), 7.3° (B/D) (Fig. 1). We speculate that the interaction between the planar molecule tylophorine B and oligonucleotide largely tends to be in the latter mode. Tylophorine B's crystal structure[21] and the solution NMR structure of the neocarzinostatin chromophore–one base bulge DNA complex[22] help us to formulate the schematic binding model of the tylophorine B–one base bulge oligonucleotide **6** complex (Fig. 5). The bulge motif creates the exact binding pocket to accommodate the planar tylophorine B molecule, and is likely responsible for the expressed affinity of different oligomers with a varying number of bulge bases. Tylophorine B stabilizes DNA through π–π stacking weak interactions associated with intercalation of the aromatic ring between the base pairs. As exhibited in the model, the base pairs adjacent to tylophorine B with A–T and T–A pairs fit well, which afford larger surfaces than T–A and T–A pairs for stacking. This might be the reason why tylophorine B binds preferably at A–T and T–A pairs. Also, the purine bulge base helps to form a hydrophobic pocket with additional stacking to tylophorine B, further stabilizing the intercalative binding. Determination of the solution structure of tylophorine B-oligonucleotide **6** complex by NMR, which will clarify the detailed binding mode and help to understand the binding contribution by the lone-pair electron isomerization on the nitrogen atom in tylophorine B, is currently underway.

Bulged regions of nucleic acids are important structural motifs, whose function has been linked to a number of key nuclear processes. Additionally, bulged intermediates have been implicated in the etiology of several genetic diseases and as targets for viral regulation.[23] By screening a library of bulge-containing oligodeoxynucelotides, correlations between DNA structure and affinity by tylophorine B can be drawn. The striking differences in affinity result from subtle modification in DNA sequence, confirming the high order of molecular recognition that tylophorine B imparts on DNA. These findings may shed some light on tylophorine B's mode of action in biological systems and lead to the rational design of sequence-specific DNA binding molecules.

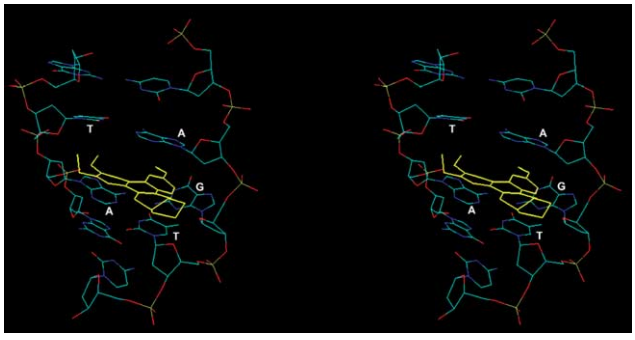


**Figure 5.** Schematic model (stereoview) of tylophorine B–one base bulge oligonucleotide **6** complex is formulated using DOCK module in SYBYL program according to tylophorine B's crystal structure[21] and the solution NMR structure of neocarzinostatin chromophore–one bulge oligonucleotide complex.[22]

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