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The interaction between tylophorine B and TMV RNA

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Abstract—Tylophorine B exhibits 60% inhibition against tobacco mosaic virus (TMV) at a concentration of 1.0×10^{-6} g/ml. In our study, high affinity for TMV RNA and assembly origin of TMV RNA (oriRNA) was revealed, accompanied by the conformational change of RNA. Considering that TMV assembly begins with the specific recognition by the coat protein aggregate of oriRNA, and that tylophorine B has favorable interaction with oriRNA, we speculate that tylophorine B likely exerts its virus inhibition by binding to oriRNA and interfering with virus assembly initiation. This work may shed light on the possible molecular inhibition mechanism against TMV by tylophorine B, and provide clues in rational design of sequence-specific RNA binding antivirus drugs.

Tylophorine B (autofine) (Fig. 1) and its analogs are phenanthroindolizidine alkaloids. 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.[1] Tylophorine B was reported with remarkable biological activity to tobacco mosaic virus (TMV),[2] it exhibited 60% inhibition against TMV at a concentration of 1.0×10^{-6} g/ml, in contrast, a commercial antivirus DHT only exhibited 50% inhibition at 5×10^{-4} g/ml under the same conditions.

TMV consists of a single, positive strand RNA of 6395 nucleotides encapsidated in a helical virion by about 2130 identical coat protein (TMV CP) subunits.[3,4] TMV particle could be reconstituted in vitro from purified RNA and coat protein under plausible physiological conditions.[5–7] Virion assembly is initiated by a specific reaction of coat protein aggregates with an internal region of TMV RNA known as the assembly origin which is capable of forming a putative hairpin loop structure (Fig. 2).[8,9] More recently, we showed high order of molecular recognition to bulged hairpin DNA afforded by tylophorine B,[10] while assembly origin of TMV RNA (oriRNA) also possesses hairpin loop structures, which inspired us to reveal the possible inhibition mechanism of tylophorine B on TMV.

A better understanding of its interaction mode with virion components including RNA and CP is important for us to determine tylophorine B's basic mode of action on TMV. So we isolated TMV by the polyethylene glycol precipitation method of Leberman.[11] TMV coat protein was prepared by the acetic acid method.[12] TMV RNA was prepared from stored virus applying the RNAgents total isolation system (Promega) according to manufacturer's protocol. To get oriRNA, TMV RNA was reverse transcribed using primer P1 (5′-TCGACATAG G GACATCTTC-3′) in 50 mM Tris–HCl (pH 8.3), 8 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, and 1 mM dNTPs, 0.5 U/μl AMV reverse transcriptase (TARAK A), 1U/μl RNase inhibitor (TARAKA) for 1.5 h at 42 °C. The cDNA molecules were then amplified by PCR, using primers P1 and P2 (5′-TAATACGACTCA CTATAGGAGACGGAGGGCCCATG-3′, T7 RNA polymerase promoter is underlined). The dsDNA of correct length was purified by electrophoresis and eluted. The purified DNA was transcribed in vitro to synthesize oriRNA in 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 5 mM DTT, 2 mM each of rNTP and 0.5 U/μl of T7 RNA polymerase (Promega), 1 U/μl RNase inhibitor. The reaction was carried out at 37 °C for 10 h. 0.5 U/μl DNase I (RNase-free) was added to digest DNA template at the end of transcription. The transcription reaction was examined by 8% PAGE (7 M urea) to get one clear band in the correct length. The transcripts were purified

Abbreviations: TMV, tobacco mosaic virus; oriRNA, assembly origin of TMV RNA; CP, coat protein.

Keywords: Tobacco mosaic virus; TMV RNA; Assembly origin; Tylophorine B; RNA-small molecule interaction.

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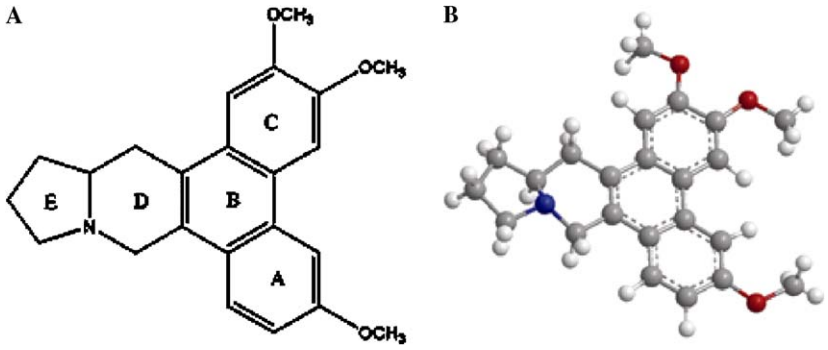

Figure 1. (A) 2D structure of tylophorine B. (B) 3D model of tylophorine B.

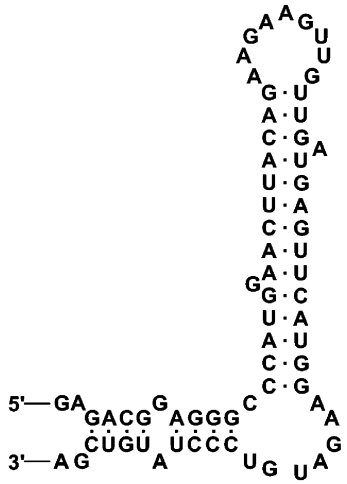

Figure 2. The secondary structure of oriRNA.

by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation.

The strong characteristic fluorescence of tylophorine B affords a sensitive spectroscopic handle to investigate its interaction with biomacromolecule. Figure 3A shows the effect of addition of 0–33.9 nM TMV RNA on the emission spectra of 59.6 nM tylophorine B. It is seen that increasing the concentration of TMV RNA results in a gradual decrease in fluorescence intensity of tylophorine B up to a maximum of ca. 24%, without any perceptible shift in fluorescence maximum 380 nm at the excitation wavelength of 310 nm. The changes in the emission spectra indicate the formation of tylophorine B–RNA complex. From the fluorescence titration curve (Fig. 3B), the point of 50% inhibition (IC_{50}) of the fluorescence maxima was estimated as about 2.4 nM, indicating a strong affinity of tylophorine B for TMV RNA. The stoichiometry for RNA–drug binding can be estimated empirically from the intersection of fluorescence asymptotes.[13] to give 2.4 nM (Fig. 3B), corresponding to 1:25 stoichiometry of binding.

A similar fluorescence quenching pattern was found upon addition of oriRNA. Keeping the concentration of tylophorine B 44.8 nM, its fluorescence intensity progressively reduced with the addition of 0–0.378 μM oriRNA up to ca. 25% quenching (Fig. 4). Further evaluation of the titration data showed that the stoichiometry of the binding was about 1:1 (Fig. 4A). The dissociation constant K_d of tylophorine B binding to oriRNA was calculated from the fluorescence titration

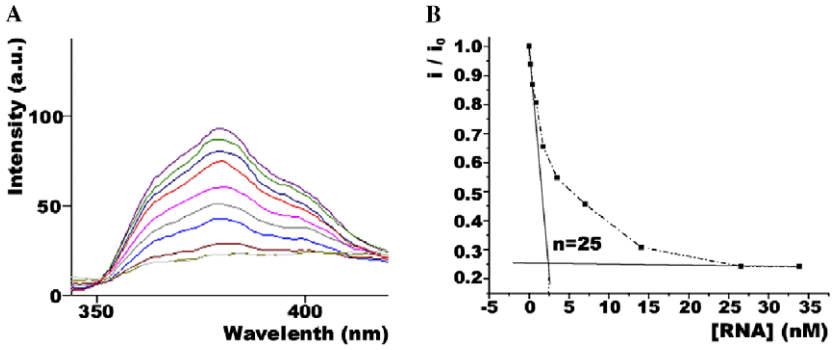

Figure 3. (A) Fluorescence quenching spectra of tylophorine B by TMV RNA. (B) Fluorescence titration curve. The intersection of fluorescence asymptotes is 2.4 nM, corresponding to 1:25 stoichiometry of binding. All fluorescence spectra in this paper were recorded on Fluorescence Spectrophotometer (VARIAN CARY Eclipse) at 20 °C. The emission spectra of tylophorine B were obtained using the excitation and emission slit widths of 20 and 10 nm, respectively, for RNA, or 10 and 20 nm for TMV CP in a total volume of 1.5 ml with a quartz cuvette of 1 cm pathlength. Measurements were taken in 10 mM phosphate buffer, pH 7.09, 50 mM NaCl, 0.1% acetonitrile, and fluorescence titration curves were corrected for the background intensity of the buffer and for dilution.

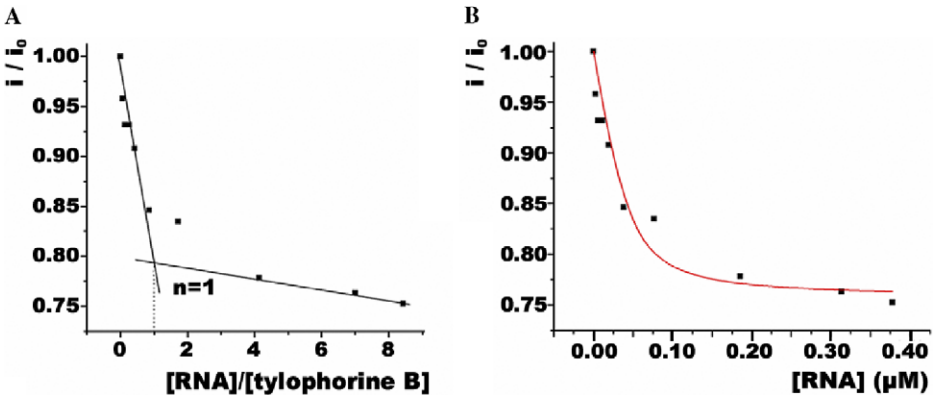

Figure 4. (A) Fluorescence titration curve of tylophorine B by oriRNA. The binding stoichiometry in terms of the number of RNA/tylophorine B molecules is the value at the intersection of fluorescence asymptotes, giving the value of ca. 1. (B) Fluorescence titration curve, from which the K_d of tylophorine B binding to oriRNA was calculated according to Eq. 1 using OriginPro 7.0 software, giving K_d value of 9 nM.

curve in Figure 4B according to Eq. 1 fitting with 1:1 binding mode, giving K_d value of 9 nM.

$$i/i_0 = 1 + \Delta i/2i_0 \times \left[(R_0 + T_0 + K_d) - \sqrt{(R_0 + T_0 + K_d)^2 - 4R_0T_0}\right] \quad (1)$$

where i and i_0 are the fluorescence intensities with and without oriRNA, respectively, Δi is the changed fluorescence intensity upon unit concentration of tylophorine B binding to RNA, R_0 and T_0 are the total concentrations of RNA and tylophorine B, respectively.

TMV CP 20S disk is believed to be necessary for virus assembly initiation and elongation, which was prepared by incubating 10–20 mg/ml freshly purified CP in ionic strength 0.1 M sodium phosphate buffer, pH 7.0, at 24 °C for 15 h. However, fluorescence titrations showed that it was not a good fluorescence quencher for tylophorine B (Fig. 5). The fluorescence intensity of tylophorine B did not vary even at 14.5-fold excessive concentration of TMV CP, implying the non or very weak binding to TMV CP by tylophorine B.

CD spectroscopy, which measures the differences between the absorbance of right-handed and left-handed circularly polarized light, is very sensitive to the changes in nucleic acid backbone conformation. It is extensively used to monitor conformational transitions of nucleic acids after binding ligand.[14–16] To gain insight into the effect of tylophorine B on the conformation of TMV RNA and oriRNA, the CD spectra of RNA with and without added tylophorine B were determined. The spectral properties of free TMV RNA showed the same spectra as the reported TMV RNA spectra,[17] with a maximum at 263.4 nm and a crosspoint at 246.9 nm (Fig. 6A). TMV RNA was found to undergo critical conformational changes upon addition of tylophorine B. The interaction of tylophorine B with TMV RNA elicited the increase in RNA CD spectrum at the maximum by 13%, with a shift of the crosspoint. The CD spectrum of oriRNA hairpin (Fig. 6B) is consistent with an A-form RNA helix[18,19] with a large positive band of 250–300 nm, a large negative band at 214 nm, and a small negative band at 234 nm. The addition of tylophorine B changed CD spectrum of oriRNA slightly, showing a very small increase in the negative peak accompanied by a slight widening of the negative band without peak shift; positive band was almost unchanged. The larger changes in CD of TMV RNA than oriRNA imply that the oriRNA holds much tighter tertiary structure with less disturbing effect by the addition of tylophorine B. The increases of CD peak values of RNA induced by tylophorine B are indicative of interaction between RNA and drug,[15,20,21] which might involve altered microenvironment and additional π–π stacking interactions associated with intercalation of the drug's aromatic ring into the bases.[15,20,21]

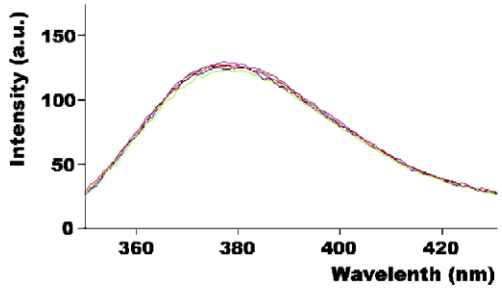

Figure 5. Fluorescence titration spectra of 87 nM tylophorine B by TMV CP 20S disk.

Tylophorine B's crystal structure as the benzene solvate has been reported before,[22] the aromatic rings lie almost in the same plane (Fig. 1). It tends to insert the planar, aromatic moiety between the nucleic acids base pairs. Our previous study showed that it has preferential molecular interaction with bulged hairpin DNA, especially one base-bulged structure.[10] Fluorescence titrations here demonstrate that the target of tylophorine B is not TMV CP, and more possibly TMV RNA. TMV RNA is capable to fold into a broad range of different structures, such as bulge, hairpin, loop, pseudoknot, and so on.[23,24] These motifs create a number of binding pockets, and tylophorine B could interact selectively and tightly with target sites by embedded in these pockets,

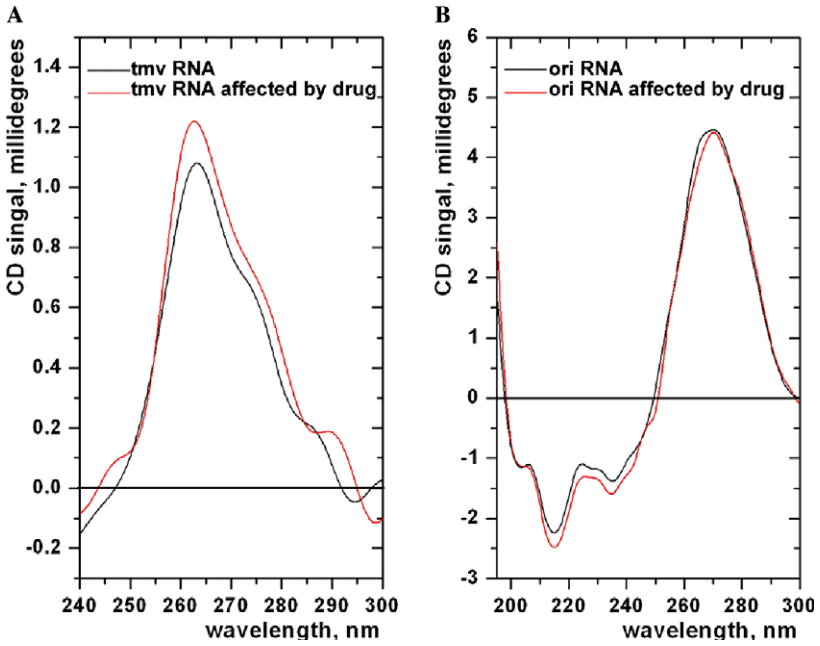

Figure 6. CD measurements were conducted at 25 °C on a J-715 CD spectropolarimeter (Jasco). Experiments were performed in 10 mM phosphate buffer, pH 7.09, 50 mM NaCl with a 1 mm pathlength cuvette in a total volume of 200 μl. Data were collected at a 0.2 nm resolution, 1 s response time, and 20 nm/min speed. (A) CD spectra of TMV RNA (21 μg/ml) alone and TMV RNA in the presence of tylophorine B (3.35 μM) after subtracting the signal of free drug. (B) CD spectra of oriRNA (0.96 μM) alone and oriRNA in the presence of tylophorine B (78.3 μM) after subtracting the signal of free drug.

which is possibly responsible for the high affinity of tylophorine B to TMV RNA with multiple binding sites. Assembly origin of TMV RNA is exactly one binding site of tylophorine B, the stable hairpin loop structure containing base bulges shows fairly high affinity for tylophorine B with K_d of 9 nM. TMV RNA is not only the passive carrier of genetic information of virus; it also performs biological function based on folding of the molecule into certain three-dimensional conformations. The CD results suggest that binding of tylophorine B disturbs the conformation of TMV RNA and oriRNA, which may interfere with RNA to carry out normal physiological function.

The conformational recognition and noncovalent binding have an important function in life process at the molecular level, which are implicated in many biological processes to regulate some important cellular processes. TMV assembly begins with the specific recognition by the coat protein aggregate of oriRNA; meanwhile tylophorine B was proved to have fairly high affinity for oriRNA, the targeting of drug to the specific hairpin loop structure offers the possibility of regulating the biological activity of the TMV RNA. Based on the evidences reported here, we speculate that tylophorine B likely exerts its virus inhibition by binding directly to assembly origin of TMV RNA, and interfering with virus assembly initiation. The study to test our hypothesis of tylophorine B's TMV inhibition mechanism through TMV assembly by tylophorine B is in progress in our laboratory and will be reported in due course.

Nucleic acids can have richly diverse secondary structures, including hairpins, knots, pseudoknots, loops, helical junctions, and bulges, which are of general biological significance and have been proposed as intermediates in a multitude of processes.[25] RNA bulged hairpin loop has been suggested as necessary motifs involved in important biological processes, such as the TAR region of HIV-1,[26,27] the conserved hairpin structure in Alfamovirus and Bromovirus,[28] 3′ untranslated region stem-loop (domain D) of bamboo mosaic potexvirus.[29] Compounds capable of binding to bulged hairpin loop structures could have significant therapeutic and antiviral potential. However, despite these obvious ramifications, few previous attempts have been made to prepare compounds with affinity for bulged hairpin loop sequences,[30,31] especially very few for such significant RNA motifs in viral biological processes. The present study demonstrates tylophorine B has favorable molecular interaction with TMV RNA bugled hairpin loop region, it is a good lead compound with molecular recognition for secondary structure. These findings may shed light on possible mechanism of inhibition against TMV by tylophorine B, and provide clues in rational design of sequence-specific RNA binding molecules.

Acknowledgments

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