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RNA specific molecules: Cytotoxic plant alkaloid palmatine binds strongly to poly(A)

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Abstract—The cytotoxic plant alkaloid palmatine was found to bind strongly by partial intercalation to single stranded poly(A) structure with binding affinity (K_a) of $(8.36 \pm 0.26) \times 10^5$ M^{-1}. The binding of palmatine was characterized to be exothermic and enthalpy driven with one palmatine for every two adenine residues. On the other hand, the binding to the double stranded poly(A) has been found to be significantly weak. This study identifies poly(A) as a potential bio-target for the alkaloid palmatine and its use as a lead compound in antitumor drug screening.

RNAs are versatile molecules that can fold into diverse structures and conformation, and these structures can serve as receptors for specific drug recognition sites.[1,2] The complex diversity of RNA molecules has hindered the development of small molecules that can specifically target RNA molecules. Given the essential role played by RNA in many biological processes and ever since the knowledge that several serious diseases like HIV, AIDS, hepatitis C, etc. are indeed caused by RNA viruses, concerted effort has now been directed in designing RNA targeted new class of therapeutics.[3–7] A rational design of RNA binding compounds, however, requires a detailed knowledge of their mode and mechanism of action. New drugs developed must be able to bind to unique structural regions in mRNA to regulate gene expression. Invariably all mRNAs in eukaryotic cells have a polyadenylic acid [poly(A)] chain of about 200–250 bases (~70–90 in yeast) at the 3′-end that essentially confers them the stability and influence translation and transcription process.[8] Catalyzed by the enzyme poly(A) polymerse (PAP), polyadenylation of mRNA is a critical cellular event in the maturation of all eukaryotic mRNAs. Very recently it has been identified that Neo-poly(A) polymerse (PAP), a human PAP, is significantly over expressed in human cancer cells and may be a potential tumor target.[9,10] Further, poly(A) has the unique distinction to exist in single stranded partially stacked helical or double stranded parallel helix depending on a narrow pH range.[11,12] Thus, molecules capable of recognizing and binding to poly(A) tail of mRNA could switch off protein synthesis and would represent a new class of potential therapeutic agents.

In contrast to extensive studies on the interaction of drugs to double stranded DNA, little is known about small molecules binding to adenine-rich single stranded RNAs. It appears that very few drugs bind to single stranded nucleic acids. Our laboratory was the first to report that berberine, an isoquinoline alkaloid with antitumor activity, selectively targets single stranded (ss) poly(A) for binding.[13] Palmatine (Fig. 1) is a protoberberine alkaloid and a close structural analog of berberine that has been shown to exhibit significant antitumor activity against HL-60 leukemic cells.[14] Inhibition of reverse transcriptase has been suggested to be one of the many reasons for the antitumor activity of palmatine.[15]

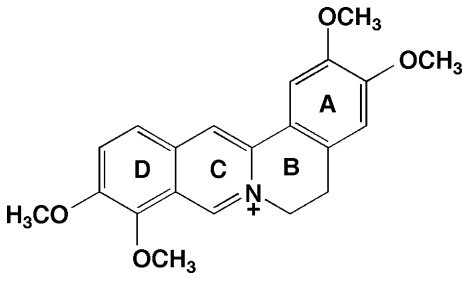

Figure 1. Chemical structure of palmatine.

Keywords: Palmatine; RNA; Poly(A); Binding; Spectroscopy; ITC.
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Binding studies of palmatine to nucleic acids have been scanty.[16,17] In our ongoing investigation elucidating the structure–activity relationship of isoquinoline alkaloids, we report here from a multifaceted spectroscopic and thermodynamic study that the plant alkaloid palmatine can specifically and strongly bind to ss poly(A) sequences.

Figure 2 shows the results from spectrofluorimetric titration of seven different nucleic acid samples with palmatine in 10 mM citrate–phosphate buffer, pH 7.1.[18] Palmatine has a weak intrinsic fluorescence with maximum around 525 nm when excited at 350 nm that enhances on binding with nucleic acids. The striking result that emerges from this experiment is the pronounced enhancement of the fluorescence intensity of palmatine on binding to ss poly(A). The fluorescence enhancement of palmatine by double stranded (ds) DNA and t-RNA was significantly lower compared to ss poly(A) and negligible with other ss RNAs like poly(U), poly(C), and ds poly(A), and ds RNA like poly(C)·poly(G). This study clearly underscores the higher affinity of palmatine to ss poly(A) structure. The binding of palmatine to ss poly(A) was further examined by absorption spectral studies,[19] the result of which is presented in Figure 3. Hypochromic and bathochromic effects were observed in both the visible absorption bands of palmatine with three sharp isosbestic points (shown by arrows in Fig. 3) indicating clearly equilibrium between free and bound alkaloid molecules in the binding process The bathochromic and hypochromic effects of palmatine–ss poly(A) complex were similar to those observed for intercalative ligand–DNA complexation and are suggestive of strong intermolecular interaction involving effective overlap of the π electron cloud of palmatine with the adenine bases. Additionally, polarity effects of the polymer and electron transfer from the bases may also contribute to this effect on the absorption spectrum, although to a minor extent.[20] The binding affinity of the alkaloid estimated from Scatchard plots fitting McGhee and von Hippel analysis[21] using SCATPLOT program (inset in Fig. 3) yielded an intrinsic binding constant (K) of $(6.40 \pm 0.25) \times 10^5\ M^{-1}$ (Table 1). On the other hand, with the ds poly(A), no absorption spectral changes were manifested indicating the lack of affinity of palmatine to the ds conformation. The binding affinity from fluorescence analysis yielded a K value of $(6.90 \pm 0.20) \times 10^5\ M^{-1}$ in excellent agreement with the spectrophotometric data. It may be noted that the binding affinity of palmatine to ss poly(A) reported here is much high and almost about fifty times higher than the value of $1.36 \times 10^4\ M^{-1}$ reported by Hirakawa et al. for its binding to ds calf thymus DNA under identical [Na^+] concentrations.[17]

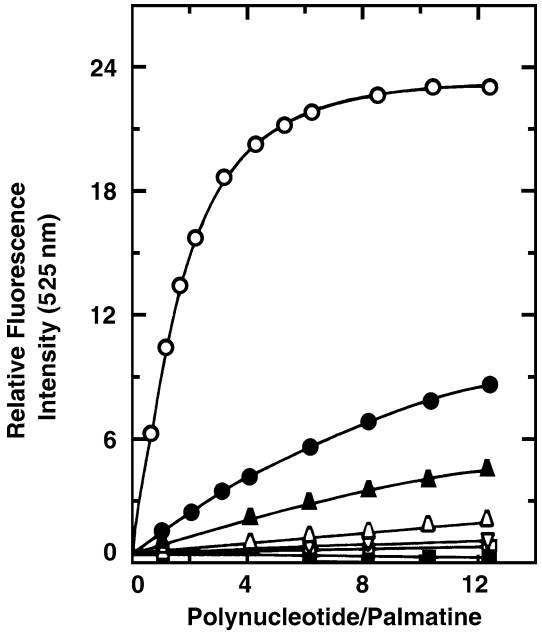

Figure 2. Increase of steady state fluorescence intensity of palmatine (10.5 μM) at 525 nm in presence of increasing concentration of (O–O) ss poly(A); (●–●) Herring testis DNA; (▲–▲) t-RNA; (▽-▽) poly(C)·poly(G); (□–□) ss poly(U); (■–■) ss poly(C) in citrate-phosphate buffer, pH 7.1 and (Δ-Δ) ds poly(A) in 10 mM citrate-phosphate buffer pH 4.5 at 20 °C, excited at 350 nm. Each point was an average of four sets of experiment.

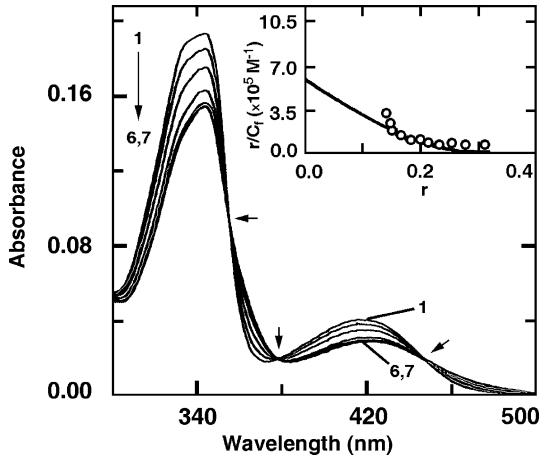

Figure 3. Absorption spectral changes of palmatine in presence of ss poly(A). Curves (1–7) denote absorption spectrum of palmatine (7.5 μM) treated with 0, 7.50, 15.0, 22.50, 33.75, 41.25 and 48.75 μM of ss poly(A) respectively in 10 mM citrate-phosphate buffer, pH 7.1 at 20 °C. Inset: Scatchard plot of binding of palmatine to ss poly(A). The solid line is the non-linear least square best fit of the experimental points to the McGhee-von Hippel equation[21] within the regions of the Scatchard plot ranging from 30% (lower limit) and 90% (upper limit).

The effect of ionic strength on the ss poly(A)–palmatine complexation was studied using absorbance and fluorescence spectroscopy at four different [Na^+] ion concentrations between 4 and 50 mM. It was observed that the extent of hypochromic and bathochromic effects in the absorption spectra and the enhancement of fluorescence intensity decreased as the ionic strength increased. The variation of the intrinsic binding constant (K) with [Na^+] ion concentration obtained from both absorption and fluorescence data varies linearly and falls by about six times on increasing the [Na^+] ion concentration from 4 to 50 mM. Palmatine is a polycondensate molecule with a positive charge in the C ring. It is therefore

Table 1. Comparative binding and thermodynamic parameters for palmatine–ss poly(A) complexation obtained from spectrophotometric, spectrofluorimetric, and isothermal titration calorimetric (ITC) study in citrate–phosphate buffer of 10 mM [Na^+] molarity at pH 7.1

Technique	T (°C)	$K \times 10^5$ (M^{-1})[a]	n[b]	$-\Delta G^o$ (kcal/mol)	$-\Delta H^o$ (kcal/mol)	$-\Delta S^o$ (cal deg^{-1} mol^{-1})
Spectrophotometry	20	6.40 ± 0.25	2.96 ± 0.02	7.83 ± 1.05	—	—
Spectrofluorimetry	20	6.90 ± 0.20	2.65 ± 0.03	7.87 ± 1.65	—	—
ITC	20	8.36 ± 0.26	—	7.99 ± 1.20	8.61 ± 1.40	2.27 ± 1.50

[a] Average of four determinations. Here K stands for K the intrinsic binding constant from spectroscopic analysis or K_a the binding affinity from ITC.
[b] Number of occluded sites from McGhee–von Hippel analysis.

reasonable to assume that electrostatic attraction between the positive charge on the alkaloid and the negative charge on the phosphate group contributes to the affinity of palmatine to ss poly(A) interaction.

The characteristic circular dichroic (CD) spectrum of the ss poly(A) was remarkably perturbed in the presence of palmatine (Fig. 4) resulting in rapid decrease of the 266 and 210 nm positive, and the 220 nm negative bands.[22] Interestingly, concomitant with the changes in the intrinsic CD in the UV region, there appeared a conservative induced CD spectrum in the 300–400 nm region for the bound alkaloid molecules, the ellipticity of which increased as the binding progressed. It is pertinent to note that palmatine is an optically inactive compound with no intrinsic CD spectrum but acquires induced CD due to its strong association on the helical organization of poly(A). On the other hand, the CD spectrum of the ds poly(A) was only marginally affected with increasing concentration of palmatine. Significantly, no induced CD bands were observed for the bound palmatine molecules in the presence of ds conformation, reiterating the strong association of palmatine to the ss poly(A) conformation. Temperature dependent CD experiments on palmatine-ss complex indicated that the bound palmatine significantly affected the denaturation and renaturation path of ss helical poly(A) confirming the strong association of palmatine.

The mode of binding of palmatine to helical ss poly(A) structure was investigated from viscosity measurements.[7,19,23] Hydrodynamic measurements are sensitive to length changes and are regarded as the most critical test for elucidating the binding mode to nucleic acids in solution.[24] The relative specific viscosity of the poly(A)–palmatine complex increased as the palmatine/poly(A) ratio increased and leveled of at a [drug]/[polynucleotide] > 0.5, suggesting an intercalation type of insertion of the alkaloid into the helical ss poly(A) structures. No viscosity enhancement was, however, observed in ds poly(A). These data unequivocally established that palmatine formed a partial intercalation complex with ss poly(A).

Isothermal titration calorimetry (ITC) was used to thermodynamically characterize the binding of palmatine to ss poly(A) under identical buffer conditions. Figure 5 shows the representative raw ITC profile resulting from a typical ITC experiment in which palmatine was titrated into the ss poly(A) solution.[25] To extract the binding and thermodynamic parameters of palmatine–ss poly(A) interaction, the thermogram was fitted to a single site model (inset) using Origin Software and the thermodynamic parameters have been estimated from the best fit to the observed heat release. The data were analyzed with several different initial guess and the resulting fits gave consistent values of the parameters, $K_a = 8.36 \pm 0.26 \times 10^5\ M^{-1}$, $\Delta H^o = -8.61 \pm 1.40$ kcal/mol; $\Delta S^o = -2.27 \pm 1.50$ cal deg^{-1} mol^{-1} and a binding site size of ~1.57 nucleotides (1/n) (Table 1). The small

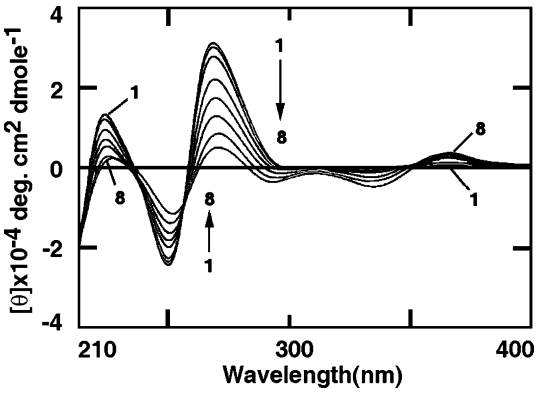

Figure 4. CD spectral changes of ss poly(A) on interaction with palmatine. Curves (1–8) denote 62.12 μM ss poly(A) treated with 0, 3.11, 6.21, 12.42, 18.64, 24.85, 31.06 and 37.27 μM of palmatine respectively in 10 mM CP buffer, pH 7.1 at 20 °C.

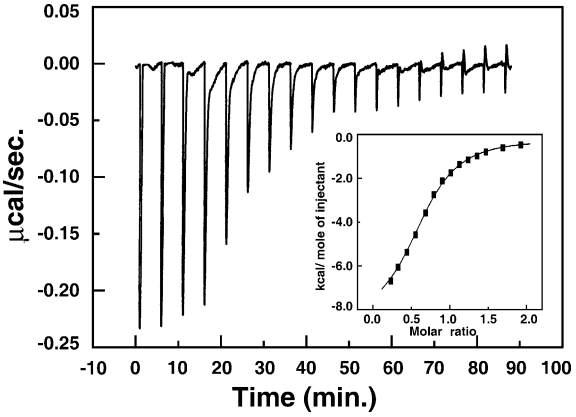

Figure 5. Representative ITC profile for the binding of palmatine to ss poly(A) at a 20 °C in 10 mM citrate-phosphate buffer, pH 7.1. Each peak shows the raw data for sequential (10 μL of 150 μM) injection of palmatine into ss poly(A) solution (10 μM). Inset: the plot of heat evolved (kcal) per mole of palmatine added, corrected for the heat of palmatine solution dilution against the molar ratio of palmatine to ss poly(A). The data (filled rectangle) was fitted to a single set of identical sites model and the solid line represent the best fit of the data.

entropy term ($T\Delta S^{o} = -0.665$ K cal mol^{-1}) suggested the binding of palmatine to poly(A) to be predominantly enthalpy driven. A binding free energy of −7.83 and −7.87 kcal/mol, respectively, was determined from spectrophotometric and spectrofluorimetric data using the standard equation $\Delta G = -RT\ln K$. The binding of palmatine to ss poly(A) is an exothermic process and the binding free energy arises from the large negative enthalpy. It is significant to observe that there is close similarity between the binding constant evaluated by the spectroscopic and calorimetric techniques. Recently, a study by Xing et al.[26] also proposed enthalpy driven binding of coralyne to ss poly(A), while Maiti and coworkers previously reported[13] the binding of berberine to poly(A) to be endothermic and entropy driven. It is likely that the interaction of palmatine likewise coralyne may involve a variety of non-covalent interactions each of which may contribute to the negative enthalpy. Although palmatine is structurally closer to berberine, the energetics of its interaction to poly(A) appears to be different from that of berberine. The single stranded poly(A) helix is stabilized by pairwise stacking of the adenine residues, while in the double stranded helix the adenines base pair through C6–$NH_2\cdots$N7 and two C6–$NH_2\cdots{}^{-}O_2P$═hydrogen bonds, and the phosphate interacts electrostatically with the proton bound to the N1 of adenine in the opposite chain. The resulting double stranded structure has two parallel strands and a single groove that is stabilized by the partial protonation of the base pairs.[11,12] It is likely that the positively charged palmatine molecules are unable to bind to the protonated ds poly(A) structure due to the repulsion of the protonated adenine residues.

In summary, this study has demonstrated that the natural product and isoquinoline alkaloid, palmatine strongly bound to ss poly(A) molecules and assumed a helical arrangement after complexation. The binding process is exothermic and enthalpy driven. As ss poly(A) plays significant role in control of eukaryotic cell processes, these results suggest a potential mechanism by which the alkaloid can inhibit the process of gene expression and gene transcription leading to its usefulness as a therapeutic agent.

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controller and thermal programmer (PTC343). The reported spectra are averages of four successive scans measured under condition of stirring and are base line corrected and smoothed. For reference, see Ray, A.; Kumar, G. S.; Das, S.; Maiti, M. *Biochemistry* **1999**, *38*, 6239.

23. A Cannon-Manning semimicrodilution viscometer (Type 75) mounted vertically in a constant temperature bath (Cannon Instruments Co., State College, PA, USA) maintained at 20 ± 0.5 °C was used for flow time measurements. Flow times of sample alone and sample with different ratios of alkaloid were measured in triplicate by an electronic stopwatch model HS-30 W (Casio Computer Co. Ltd, Japan) with an accuracy of ±0.01 s. Viscosity experiments were carried out with 430 and 340 μM of ss and ds poly(A) sample, respectively. For reference, see Maiti, M.; Nandi, R.; Chaudhuri, K. *FEBS Lett.* **1982**, *142*, 280.
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