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Entropy driven binding of the alkaloid chelerythrine to polyadenylic acid leads to spontaneous self-assembled structure formation

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ABSTRACT

The binding thermodynamics and interaction of the putative anticancer alkaloid chelerythrine with polyadenylic acid were investigated by isothermal titration calorimetry, absorption and fluorescence spectroscopy, circular dichroism, differential scanning calorimetry and thermal melting experiments. The equilibrium binding constant was evaluated to be of the order of 10^7 M^{-1}. Strong positive entropic and favorable enthalpic contributions to the binding were revealed. The binding affinity was enhanced within (10 to 100) mM Na^+ concentration. Circular dichroism spectra confirmed that the increase in entropy change was caused by a strong conformational change in the RNA polynucleotide. Absorption and circular dichroism melting studies revealed that chelerythrine binding induced self-assembled duplex structure formation in poly(A) molecules resulting in a cooperative melting profile. This was further confirmed from differential scanning calorimetry data. The intercalation binding of the alkaloid involved strong energy transfer from the polynucleotide bases to the bound alkaloid molecules. The remarkably high entropy driven binding of the alkaloid induced spontaneous self-assembled structure formation in poly(A) and the associated binding affinity is the highest so far reported for a small molecule binding to poly(A).

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1. Introduction

The key and versatile role of RNA molecules in protein synthesis, mRNA splicing, transcription and replication make them prime targets for small molecule drugs intended for therapeutic intervention. The importance of RNA targeting [1,2] has become more relevant with the recent discovery of a number of small RNAs like si RNA and micro RNA that are found to exert significant control in many crucial cellular events. Furthermore, RNAs in viral and eukaryotic genomes are potential drug targets for inactivation. In recent times search for RNA targeted therapeutics has intensified and fundamental studies on RNA binding small molecules have been pursued with great enthusiasm. The discovery of Topalian *et al.*, that neo PAP, a human PAP, is over expressed in some human cancer cells compared to their expression in normal cells has led to interest in poly(A) tail of mRNA as a malignancy specific drug target [3]. The expression of mRNAs is strongly influenced by the 3′ poly(A) tail added during the post transcriptional modification. Binding of small molecules to poly(A) tail of mRNA could alter its conformation, prevent elongation or create alternate conformations, leading to switching off of protein synthesis. A number of alkaloids and other small molecules have recently been found to induce a unique self-assembled structure in poly(A) in vitro [4–12]. The effects of such transformations if mimicked in vivo may be very interesting for arresting cell division.

Chelerythrine (figure 1), a benzophenanthridine alkaloid, is derived from *Chelidonium majus* L. (*Papaveraceae*) plants [13]. Structurally, it is 1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride ($C_{21}H_{18}ClNO_4$) [14]. A number of biological applications of chelerythrine have been reported very recently. It is an inhibitor of protein kinase C (PKC) responsible for the maintenance of erythrocyte deformability [15–17]. It also stimulates the production of reactive oxygen species, which may deplete cellular antioxidants, provide a signal for rapid execution of apoptosis [18], and provoke cell death in a variety of tumour cells leading to potential application as an anticancer agent [16,19,20]. The DNA binding activity of chelerythrine was studied recently in our laboratory [21,22] to show that the binding occurs by intercalation with remarkable guanine-cytosine base pair specificity. A few other studies on its DNA binding have also been reported [23–25]. But no studies with RNAs are reported.

Targeting RNA molecules for drug design is a very challenging approach and advancement of our fundamental understanding of

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RNA-ligand interaction is necessary to realize the full potential of RNA targeted therapeutics. In the present study, we investigated the effect of chelerythrine on single stranded poly(A). The binding thermodynamics, the propensity to form self-assembled structure in the presence of the alkaloid, and related structural aspects have been elucidated. We report that chelerythrine exhibits entropy driven binding to poly(A) with remarkably high affinity (>10^7 M^{-1}), hitherto not reported with any other small molecule that binds poly(A).

2. Experimental

2.1. Materials

Chelerythrine chloride (here after chelerythrine), polyadenylic acid potassium salt (here after poly(A)), sodium chloride and sodium phosphate were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Poly(A) was dissolved in RNAase free, autoclaved 10 mM citrate–phosphate (CP) buffer containing 5 mM Na_2HPO_4, and 1.4 mM citric acid, pH 6.25. The sample was dialyzed extensively against the experimental buffer at $T = 278.15$ K. The provenance and mass fraction purity values of the samples used are listed in table 1. Concentration of poly(A) in terms of nucleotide phosphate (hereafter called nucleotide) was determined using a molar absorption coefficient (ε) value of 10,000 M^{-1} cm^{-1} [26,27]. Chelerythrine concentration was determined by absorbance measurements using ε value of 37,060 M^{-1} cm^{-1} at 316 nm [28]. All other chemicals and reagents used were of analytical grade. The maximum uncertainty in the estimation of the concentration of poly(A) and chelerythrine are 1.2%.

2.2. Methods

2.2.1. Isothermal titration calorimetry (ITC)

ITC measurements were performed in a MicroCal VP ITC unit (MicroCal LLC, Northamption, MA, USA). The reagents were dissolved in the buffer and degassed on the MicroCal's Thermovac unit at $T = 293.15$ K. Aliquots (10 μL) of chelerythrine solution were titrated into a solution of poly(A) kept in the instrument's cell. Each injection generated a heat spike that diminished in intensity as the reaction progressed. The titrations were continued past saturation. Blank experiments, where alkaloid was injected into buffer and buffer was injected into the poly(A) solution, were done to get the heat change due to dilution and these heats of dilution were appropriately subtracted to get the corrected thermogram. The thermograms were analyzed using the in-built Microcal LLC Origin 7.0 software to get the binding isotherm. The "one set of sites" model provided the best fit curve for the obtained data points at all salt conditions yielding the binding affinity (K), the binding stoichiometry (N) and the standard molar enthalpy of binding ($\Delta_{bind}H^o$). The standard molar Gibbs energy change ($\Delta_{bind}G^o$) and the standard molar entropy contribution to the binding ($T\Delta_{bind}S^o$) were subsequently calculated from standard relationships [29,30].The ITC unit was periodically calibrated and verified as per the criteria of the manufacturer. The combined standard uncertainty $u_c(x)$ for the calorimetric measurements was calculated using the relation $u_c(x) = \{(u_1(x))^2 + (u_2(x))^2 + (u_3(\underline{x}))^2 + \ldots +\}^{1/2}$ where $u_1(x), u_2(x), u_3(x), \ldots$ denote the individual uncertainties in the measurements. The isothermal calorimeter was calibrated electrically and calibrated via water–water dilution experiments as per criteria of the manufacturer that the mean energy per injection was <5.46 μJ and standard deviation was <0.063 μJ.

TABLE 1
Provenance and mass fraction purity of the substances used in this study.

Sample	Provenance	Mass fraction purity[a]
Poly(A)	Sigma–Aldrich	0.98
Chelerythrine	Sigma–Aldrich	0.95
Disodium hydrogen phosphate	Sigma–Aldrich	0.99
Citric acid	Sigma–Aldrich	0.99

[a] The mass fraction purities of poly(A), chelerythrine, disodium hydrogen phosphate and citric acid are based on information provided by the supplier Sigma–Aldrich. The purities of other samples were improved by re-crystallization.

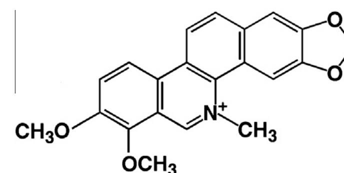

FIGURE 1. Chemical structure of the iminium form of chelerythrine.

2.2.2. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were acquired on a Jasco J815 unit (Jasco International Co. Ltd., Hachioji, Japan) as reported in details elsewhere [31]. Samples were placed in the 10 mm quartz cuvette and allowed to equilibrate at $T = 293.15$ K. The measurements were performed at room temperature in the wavelength range (200 to 400) nm. The reference CD spectrum was recorded and subtracted. The molar ellipticity values [θ] are expressed in terms of nucleotide phosphate.

2.2.3. Thermal melting experiments

In biochemical studies, heat denaturation of double stranded nucleic acids is also called melting. It is the process by which double-stranded nucleic acid unwinds and separates into single-stranded units through the breaking of hydrogen bonds between the base pairs and hydrophobic stacking attractions leading to a sigmoidal transition curve. The melting process is generally monitored at the wavelength maximum (257 nm) and characterized by melting temperature (T_{fus}) that actually denotes the temperature at which 50% of the nucleic acid exists as double strands and 50% as single strands. The melting or fusion temperature is determined by the maxima of the first derivative melting profiles. Absorbance versus temperature curves (optical melting profiles) were measured on a Shimadzu Pharmaspec 1700 unit equipped with a Peltier module TMSPC-8 model microcell controlling the temperature of the sample in the cuvette (Shimadzu Corporation, Kyoto, Japan) as reported previously [31]. The CD melting profiles were obtained on the Jasco 815 unit equipped with a Jasco temperature controller (PFD 425L/15) monitoring the CD signal at 257 nm. For the melting profiles, the ellipticity values are expressed in units of milli degrees.

2.2.4. Differential scanning calorimetry (DSC)

DSC measurements were performed on an ultrasensitive VP DSC micro calorimeter (MicroCal LLC) as described previously [29,30]. At first, the sample and reference cells were filled with the buffer solution, equilibrated at $T = 293.15$ K for 15 min, and scanned from $T = (293.15$ to $373.15)$ K at the rate of 40 $K \cdot h^{-1}$ to obtain a stable base line. Thereafter runs of poly(A) and (poly(A) + alkaloid) complexes were performed. Each experiment was repeated twice with separate fillings. DSC thermograms of excess heat capacity versus temperature were analyzed by using the Origin 7.0 software in order to obtain the calorimetric transition enthalpy ($\Delta_{cal}H$). The measured calorimetric transition enthalpy is obtained from the analysis of the area under the experimental heat capacity (c_p^o) curve whereas the van't Hoff enthalpy ($\Delta_{vh}H$) is obtained from the shape analysis of

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