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Binding interaction of phenothiazinium dyes with double stranded RNAs: Spectroscopic and calorimetric investigation

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ABSTRACT

RNA targeting through small molecules is an emerging and promising therapeutic route that necessitates identification of small molecules that can selectively target specific RNA structures. In this context a comparative study of the interaction of two phenothiazinium dyes thionine (THN) and toluidine blue O (TBO) with three double stranded RNA polynucleotides (ds RNAs) viz. poly(I).poly(C), poly(A).poly(U) and poly(C).poly(G) was conducted by various biophysical techniques. A higher binding of THN with poly(I).poly(C) over poly(A).poly(U) and poly(C).poly(G) was observed. The intercalative binding and RNA induced fluorescence quenching of the dyes through a static mechanism was confirmed by viscosity studies and steady state polarization anisotropy experiments. Binding induced structural perturbation in the RNA polynucleotides was confirmed from circular dichroism spectroscopy. DSC and thermal melting experiments confirmed that the binding resulted in strong thermal stabilization. The binding affinity of THN with poly(I).poly(C) was the highest followed by that to poly(A).poly(U) and poly(C).poly(G). The trend was the same for TBO also, but THN bound stronger than TBO. The binding of the dyes was characterized by strong negative enthalpy changes with minimum positive entropy changes indicating typical intercalative interaction. The results presented here may be useful to design new types of RNA binding antitumor, antibacterial and anticancer agents.

1. Introduction

RNA viruses cause infections like HIV, Ebola hemorrhagic fever, SARS, influenza, hepatitis C, West Nile fever, polio, and measles. Antiviral compounds can be introduced that can specifically binds to RNA molecules for the inhibition of these viruses [1]. RNA molecules can change their conformation and structures and acts as specific drug recognition sites for many small molecule drugs [2]. In recent years, post transcriptional gene silencing by the RNA interference (RNAi) or double-stranded RNA (ds RNA) is considered as a tool for gene regulation [3]. Double-strand RNA is a signal for gene-specific silencing of expression in a number of organisms. The versatile activity of RNAs is exhibited in their ability to regulate many types of cellular processes. Double stranded RNAs are produced in many viruses mainly during the process of replication and it is activated by NF-kB and interferon-inducible protein kinase, PKR [4]. In cells ds RNAs are produced by gene expression. To produce antiviral, antibacterial, antitumor and anticancer compounds that are targeted to RNA the exact mode and mechanism of binding needs to be known.

Thionine (3,7-diamino-5-phenothiazinium, THN hereafter) is a unipositive charged planer tricyclic heteroaromatic molecule that can effect photoinduced mutagenic actions [5,6]. THN has the potential to inactivate frog sperm nucleus [7]. It can cause toxicity in anaerobic glycolysis [8], induce structural changes developed in rat mast cells and block mast cell damage by inhibiting cell metabolism [9]. Toluidine blue O (2-methyl-3-dimethylamino-7-amino-phenothiazin-5-iumchloride) is also a planar cationic dye with anti tumoral activity [10]. TBO has some activity on selective cancer cells and has the potential to be developed as an anticancer agent [11]. TBO is widely known to have antimicrobial properties when used as a photosensitizers [12]. TBO is an effective photosensitizer against planktonic bacterial and fungal growth and can reduce the cell viability of many microorganisms [13]. It has great application in the field of gynecology during colposcopy and detection of oral premalignant and malignant lesions [14,15]. It is also used as sensor, biosensor, polymerisation inhibitor, staining agent and photosensitizer to determine the action of photoactivated microorganisms etc. [16–19]. Studies on the interaction of THN and TBO with DNA polynucleotides have already been reported [20–24].

Now, we are interested to understand the interaction of these dyes with double stranded ribonucleic acids (ds RNAs) to develop them as therapeutic agents targeted to RNA structures of viruses. Hence, comparative binding interactions of THN and TBO with three sequence

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specific ds RNAs were performed by various spectroscopic and calorimetric tools to delineate the detailed interaction aspects.

2. Materials and Methods

2.1. Materials

Thionine acetate (CAS No. 78338-22-4, purity 90%), TBO (CAS No. 92-31-9, purity 80%) (Fig. 1) and the double stranded RNAs, poly(I).poly(C), poly(A).poly(U) and poly(C).poly(G) were obtained from Sigma-Aldrich Corporation, USA. The dyes were refined by repeated recrystallization to upgrade its purity. All other reagents like KCl, KI etc. used were of analytical grade. Double stranded RNAs were dissolved in 50 mM citrate-phosphate (CP) buffer of pH 7.0, and stored at 4 °C with gentle stirring overnight. The sonication was done in a 2000 Labsonic sonicator to a uniform size of ~(280 ± 40) base pairs. Their concentration in base pairs was evaluated by use of molar absorption coefficients (ε) as described in the literature [25–28].

2.1.1. Preparation of the Dye Solutions

THN and TBO were prepared in CP buffer where these compounds were fairly soluble. Each day these solutions were prepared afresh and stored in the dark to avoid unwanted photochemical damages. To avoid aggregation of the dyes, the concentration of the dye solutions was prepared always lowest as possible. The molar absorptivity (ε) of THN and TBO are 54,200 M^{-1} cm^{-1} and 28,000 M^{-1} cm^{-1}, respectively, at λ_{max} 598 nm and 633 nm [12,20–24]. At the range of concentration used here no deviation of Beer's law was noticed.

2.2. Methods

2.2.1. Spectroscopic Measurements

Jasco V660 spectrophotometer (Hachioji, Japan) was used to carry out the absorption study and the instrument was attached to a thermo electrical controlling cell holder. The experiments were conducted in quartz cuvettes (Hellma, Germany) of 1 cm path length at 293 ± 0.5 K temperature. The titration procedures were described in details previously [22,23,29].

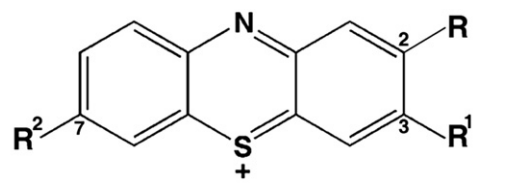
Fluorescence titrations were conducted in a Shimadzu RF5301-PC spectrofluorimeter (Kyoto, Japan) and in a fluorescence free quartz cuvette (1 cm) as described [22,29].

2.2.2. Analysis of Spectroscopic Data and Calculation of Binding Constants

In both absorption and fluorescence spectral titrations, ds RNAs concentration was varied and added into a fixed concentrated dye solution till saturation was achieved. From the spectral data the equilibrium constants were estimated by the Benesi–Hildebrand plots as,

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{1}{K_{BH}(\Delta A_{max})} \times \frac{1}{[M]} \quad (1)$$

Here [M] is the RNA concentration and ΔA is the difference in absorbance of RNA itself and dye-RNA complex at the λ_{max}.



R=H, $R^1=NH_2$, $R^2=NH_2$ for Thionine

R=CH_3, $R^1=NMe_2$, $R^2=NH_2$ for TBO

Fig. 1. Chemical structure of THN and TBO.

2.2.3. Fluorescence Quenching Study

Potassium iodide (KI) was used to carry out the fluorescence quenching experiment. The experiment was operated by mixing various concentrations of KCl and KI solutions. The stable P/D ratio was retained monitoring the fluorescence intensity as a function of KI [30,31]. The results of relative fluorescence intensity (F_o/F) against [KI] were represented as Stern-Volmer plots.

2.2.4. Fluorescence Polarization Anisotropy Study

Fluorescence anisotropy of thionine and TBO with RNA duplexes was conducted on a Horiba PTI QM-400 unit (Horiba PTI, Canada) as suggested by Larsson and colleagues [32]. The steady state polarization anisotropy 'A' is described as

$$A = (I_{vv} - I_{vh} - G)/(I_{vv} + 2I_{vh}.G) \quad (2)$$

where I_{vv}, I_{vh}, I_{hv} and I_{hh} depicted the excitation and emission fluorescence signal with the polarizer positions set at (0°, 0°), (0°, 90°), (90°, 0°) and (90°, 90°), respectively. G is the ratio I_{hv}/I_{hh} to correct the instrumental error.

2.2.5. Viscosity Measurements

The flow times were counted by a semi micro capillary viscometer of Cannon-Manning Type 75 from Cannon Instruments Co. (State College, PA, USA). It was aligned in a vertical position in a constant temperature bath at (293.15 ± 1) K. Flow times of dye-RNA complexes were noted with the help of electronic stopwatch model HS-30W (Casio Computer Co., Japan) with an accuracy of ±0.01 s [26,29].

2.2.6. UV Melting Study

Thermal melting of RNA itself and dye-RNA complexes were conducted on a Shimadzu Pharmaspec 1700 unit together with a TMSPC-8 Peltier controller [31]. To carry out this experiment 40 μM of RNA aliquots with varying concentration of dye solutions were used in the eight chambered micro cuvette. At 260 nm the melting experiment was examined and the rate of increase of temperature was 1 K/min throughout the experiment. Half dissociation of the polynucleotide obtained at a distinct temperature, called transition temperature (T_m), is calculated from the first derivative curve of absorbance against temperature (∂A/∂T). The melting temperature of poly(C).poly(G) and its dye complexes were not performed as no melting was seen even when the temperature reached >383.15 K under the conditions of our study [33].

2.2.7. DSC Study

The residual heat capacity of the double helix transition of polynucleotide and their dye complexes were measured from the DSC study. The experiment was conducted on a MicroCal LLC, VP-DSC unit, USA (now Malvern Instruments, Malvern, UK). By repeating the buffer scanning, the machine was settled thermally stable with scanning rate of 60 K per hour and at 25 psi pressure. Baseline was deducted from the complex thermograms. The DSC profiles were scanned by Origin 7.0 software to provide the model independent calorimetric transition enthalpy (ΔH_{cal}) [34]. By analyzing the shape of the DSC profile the model-dependent van't Hoff enthalpy (ΔH_v) was acquired. For a truly cooperative reversible transition, $\Delta H_{cal}/\Delta H_v$ is always unity or near to unity [25,41]. The reversibility of the transition was confirmed by the cooling of the RNA-dye samples from 373.15 K to 298.15 K and performing a scanning on the renatured sample under identical scanning condition.

2.2.8. Circular Dichroism Study

The Jasco spectropolarimeter J815 equipped with a temperature controller was used to carry out the CD experiments. The experiment was directed by the thermal programmer (425L/15) of Jasco software at 293.15 ± 0.5 K [26,28]. CD spectra were registered in the 190–800 nm