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Utilization of circular dichroism and electrospray ionization mass spectrometry to understand the formation and conversion of G-quadruplex DNA at the human *c-myb* proto-oncogene

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ABSTRACT

G-quadruplex DNAs are involved in a number of key biological processes, including gene expression, transcription, and apoptosis. The *c-myb* oncogene contains a number of GGA repeats in its promoter which forms G-quadruplex, thus it could be used as a target in cancer therapeutics. Several in-vitro studies have used Circular Dichroism (CD) spectroscopy or electrospray ionization mass spectrometry (ESI-MS) to demonstrate formation and stability of G-quadruplex DNA structure in the promoter region of human *c-myb* oncogene. The factors affecting the *c-myb* G-quadruplex structures were investigated, such as cations (i.e. K^+, NH_4^+ and Na^+) and co-solutes (methanol and polyethylene glycol). The results indicated that the presence of cations and co-solutes could change the G-quadruplex structural population and promote its thermodynamic stabilization as indicated by CD melting curves. It indicated that the co-solutes preferentially stabilize the *c-myb* G-quadruplex structure containing both homo- and hetero-stacking. In addition, protopine was demonstrated as a binder of *c-myb* G-quadruplex as screened from a library of natural alkaloids using ESI-MS method. CD spectra showed that it could selectively stabilize the *c-myb* G-quadruplex structure compared to other six G-quadruplexes from tumor-related G-rich sequences and the duplex DNAs (both long and short-chain ones). The binding of protopine could induce the change in the G-quadruplex structural populations. Therefore, protopine with its high binding specificity could be considered as a precursor for the design of drugs to target and regulate *c-myb* oncogene transcription.

1. Introduction

Human *c-myb* is the homologue of *V-mybs* oncogenes found in avian leukosis viruses. The *c-myb* proto-oncogene encodes a key transcription factor principally involved in proliferation, differentiation, and maturation during haematopoiesis [1]. *c-myb* plays critical roles in the determination of cell fate in hematopoietic cells [2], epithelial cells of the colon [3,4], kidney [5], and mammary gland [6], smooth muscle cells [7], and neural stem cells [8]. An important role of *c-myb* in leukemogenesis is the maintenance of cell proliferation and prevention of cell differentiation [9]. Further the *c-myb* promoter comprises multiple GGA repeats, that are known to form G-quadruplexes. Hence, the *c-myb* gene could be used as a potential target for the leukemia and cancer therapeutics [10].

G-quadruplexes are four-stranded DNA or RNA secondary structures formed as a result of hydrogen-bonds and π-π stacking interactions in a stretch of G-rich sequences [11]. G-rich sequences are widely distributed through the human genome, such as in promoters, transcription start sites, untranslated regions of mRNA, ribosomal DNA (rDNA) and at telomeres [12,13]. Formation of G-quadruplex is reported in promoters of a number of human proto-oncogenes, like *C-myc*, *PIM1*, *KRAS*, *NRAS*, *and VEGF* [14–18], and plays an important function in regulating gene expression, transcription and apoptosis [19]. Yuan's group focuses on G-quadruplex function, recognition and transcription regulation in *c-myb* gene, revealing a novel regulatory element in *c-myb* proximal promoter region, and its transcription regulation by G-quadruplex binders [20,21]. Their study revealed *c-myb* G-quadruplex structures, which were of great importance in the regulation of *c-myb* function [22]. A number of natural alkaloids that alter the stability of G-quadruplex, such as tetrandrine and Sanguinarine, show anticancer activity and thus could be used in chemotherapy for human cancers [23–25]. In our previous work we found that several natural alkaloids with flexible and chiral structures could bind to tumor-related G-quadruplex DNAs [26–28]. Therefore, it is necessary to further explore the anti-cancer mechanism of these natural binders at the molecular level [29,30].

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To date, structural polymorphism of biomolecular topology and the binding of small molecules with biomolecules have been evaluated by various techniques, including isothermal titration calorimetry (ITC) [31,32], circular dichroism (CD) [33,34], fluorescence measurements [35,36] and electrospray ionization mass spectrometry (ESI-MS) [37,38]. In the current study we have utilized electrospray ionization mass spectrometry (ESI-MS) as well as circular dichroism (CD) to investigate G-quadruplex formation by a 25-nucleotides long Guanine rich sequence, located downstream of *c-myb* gene promoter. Of the five natural alkaloid molecules analyzed in this study (P1-P5, Fig. 1), protopine (P1) shows remarkable binding affinity to *c-myb* G-quadruplex structure. CD results also demonstrated that the binding of P1 could induce a structural change in the strand direction of the guanine-rich tracts from parallel to antiparallel.

2. Materials and Methods

2.1. Materials

The G-rich sequence in the *c-myb* promoter region (S1, 5′-AGGGAG TCGGGCAGGGGTGCTGGGA-3′) (MW = 7884.14), 12-mer complementary sequences for 12 bp duplex DNA (ss1: 5′-AGGGAGTCG GGC-3′, MW = 3751.50; ss2: 5′-GCCCGACTCCCT-3′, MW = 3542.36) and six other tumor-related G-rich sequences were synthesized and at Sangon Biotech Co., Ltd. (Beijing, China) and was them purified by high-performance liquid chromatography (HPLC). CtDNA was purchased from Sigma Aldrich, USA. The oligos re-suspended in ultrapure Milli-Q water (100 μM) and, stored in −20 °C.

The ctDNA solution was diluted in 50 mM NH_4OAc (pH 7.0) and placed at 4 °C overnight. The UV absorbance ratio A_{260}/A_{280} of ctDNA solution was found to be >1.8, suggesting that there's no protein contamination in ctDNA sample [39]. The final concentration of the ctDNA solution was measured using molar excitation coefficient of 6600 M^{-1} cm^{-1} at 260 nm [40]. For 12 bp duplex DNA, two complementary single-stranded oligonucleotides, ss1 and ss2, were mixed in equimolar proportions (500 μM) in 500 mM NH_4OAc solution, annealed at 85 °C in 10 min and slowly cooled to room temperature (over 4 h) to allow the formation of the duplex (dsDNA).

The five alkaloids utilized in this study were obtained from NIFDC (National Institutes for Food and Drug Control Beijing, China). These alkaloids were resuspended in ultrapure water to a concentration of 0.5 mM. The polyethylene glycol 200 (PEG 200, the average molecular weight = 200) was brought from Sigma-Aldrich (China). All the materials used in the experimental are analytical pure.

2.2. ESI Mass Spectrometry

A LCQ DECA XP Plus ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was used for ESI-MS analyses. Negative ion data collection mode was utilized, with a 2.7 kV capillary voltage at a temperature of 120 °C. The samples for mass spec were prepared in 50 mM NH_4OAc (pH 7.0), 25% CH_3OH and 5 μM DNA, for 4 h at 37 °C. A flow rate of 2.0 μL/min was used to inject the samples into the mass spectrometer.

2.3. Circular Dichroism Spectroscopy

Chirascan CD Spectrometry (Applied Photophysics Ltd., England) was used to perform CD experiments. The samples for CD were prepared in 30 mM Tris–HCl (pH 7.4) and 2.5 μM DNA and varying concentration of KCl, or in NH_4OAc solutions (25% CH_3OH). The DNA used in this study was heated to 90 °C for 5 min and then cooled to 4 °C for 6 h to facilitate annealing. To obtain an average spectrum the samples were scanned three times in a cuvette (0.1 cm path-length). The wavelength of the scan was 220 nm to 350 nm. The CD melting experiments were preformed from 20 °C to 90 °C with the rate of 2.0 °C/min and samples were scanned three times to obtain the average peak intensities at 290 nm.

3. Results and Discussion

3.1. The c-myb Promoter Intramolecular G-quadruplex DNA Formation

The G-rich DNA sequences can fold into G-quadruplexes upon exposure to cations, such as K^+ and NH_4^+. CD spectroscopy is useful to discriminate different types of G-quadruplex topologies according to the sequence of glycosidic bond angles (GBA) adopted by guanosines in the G-quadruplex stem [41,42]. A parallel-stranded G-quadruplex with the strands oriented in the same direction, containing stacked guanines of the same GBAs (*syn* − *syn* or *anti* − *anti*), possesses the

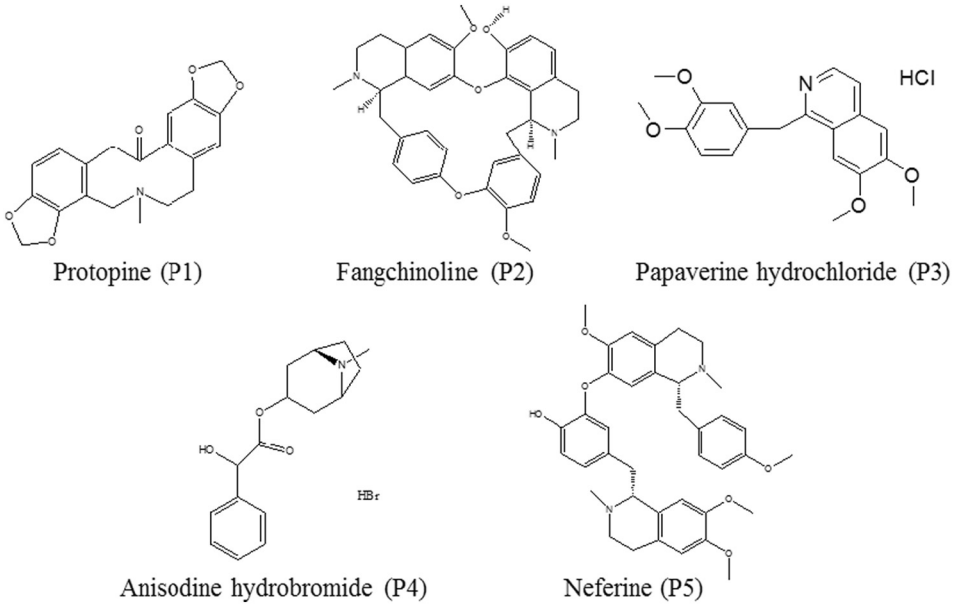

Fig. 1. Structure of alkaloid molecules (P1-P5) utilized in this study.