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Quadruplex forming promoter region of c-myc oncogene as a potential target for a telomerase inhibitory plant alkaloid, chelerythrine

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ABSTRACT

Guanine rich sequences present in the promoter region of oncogenes could fold into G-quadruplexes and modulate transcription. Equilibrium between folding and unfolding of the quadruplexes in these regions play important role in disease processes. We have studied the effect of a putative anticancer agent chelerythrine on G-rich NHE III_1 present in the promoter region of c-myc oncogene. We have demonstrated the ability of chelerythrine, a telomerase inhibitor, to block the hybridization of Pu27 with its complementary strand via folding it into a quadruplex structure. Calorimetry shows that the association of Pu27 with chelerythrine is primarily enthalpy driven with high binding affinity (~10^5 M^{-1}). The association does not lead to any major structural perturbation of Pu27. The resulting 2:1 complex has enhanced stability as compared to free Pu27. Another notable feature is that the presence of molecular crowding agent like ficoll 70 does not change the mode of recognition though the binding affinity decreases. We suggest that the anticancer activity of chelerythrine could be ascribed to its ability to stabilize the quadruplex structure in the c-myc promoter region thereby downregulating its transcription.

1. Introduction

The human oncogene, c-myc, plays an important role in many cellular events and its overexpression is related to an increase of cellular proliferation in a variety of malignant tumors. Guanine-rich nuclear hypersensitive element III_1, present in its promoter region controls 80–90% of the transcription activity of c-myc [1–5]. Previous reports have shown that this 27 nucleotide guanine-rich sequence (Pu27, Table T1), downregulates c-myc transcription upon folding into a quadruplex structure which acts as a transcriptional repressor element [3,6–12]. Several small molecules which can induce and stabilize this quadruplex structure are, therefore, putative agents to downregulate c-myc expression [13].

A large number of plant alkaloids and their derivatives are known to possess anticancer activity and recent studies have shown that association with quadruplex DNA might be a possible mechanism [7,8,14–20]. Earlier reports from our laboratory have attributed the quadruplex binding potential of the plant alkaloids sanguinarine (SGR, Fig. 1A(i)) and ellipticine as one of the plausible mechanisms of their potentials as anticancer agents [16,17]. Among these two small molecules, SGR is structurally similar to chelerythrine (CHL) (Fig. 1A(ii)), the molecule studied in the current report. Here we have reported that association of CHL with Pu27 blocks its hybridization with the complementary strand leading to an inhibition of its extension by polymerase. We have studied the association of CHL with Pu27 and the subsequent structural alteration, if any, of the quadruplex. The ability of CHL to specifically inhibit protein kinase C is currently the proposed mechanism put forward for its anticancer activity [21]. Our results suggest that targeting quadruplex DNA formed by the sequence upstream of c-myc oncogene is an additional mechanism by which CHL might impart its anticancer activity.

A variety of biomolecules are present inside the living cells leading to a molecular crowded environment optimized for biomolecular functions [22–24]. The structure and stability of some types of G-quadruplexes are also altered in the presence of molecular crowding agents. *A priori* one can anticipate that it might affect their molecular recognition by ligands. Therefore, we have also reported here the effect of molecular crowding with ficoll as the molecular crowding agent on the association between CHL and Pu27.

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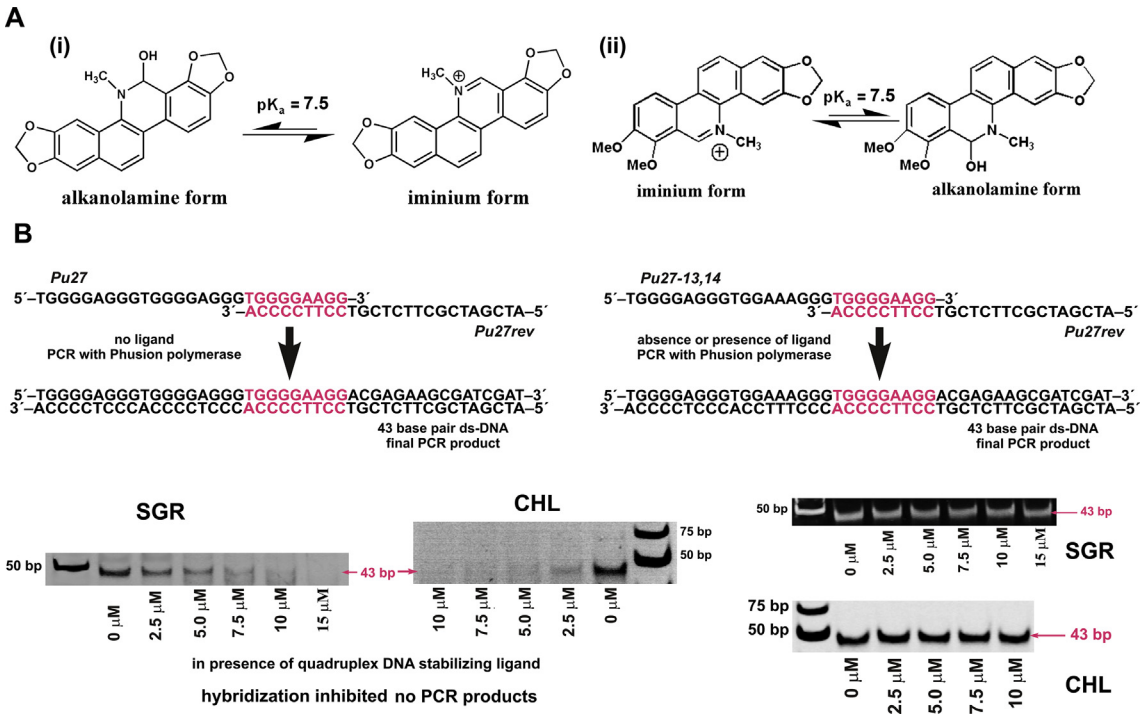

Fig. 1. (A) Chemical structure of (i) sanguinarine (SGR) and (ii) chelerythrine (CHL). The protonated form intercalates into double stranded DNA. (B) Schematic representation of PCR-stop assay. The experiments were run in triplicate with indicated amounts of the molecule.

2. Materials and methods

2.1. Materials and preparation of stock solutions

CHL, SGR, potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, sodium acetate, acetic acid, ficoll 70, acrylamide/bisacrylamide solution and desalted and HPLC purified oligomers (Table T1) were purchased from Sigma Chemical Corporation, USA. Phusion high-fidelity DNA polymerase was obtained from Thermo Scientific. SYBR Green I nucleic acid gel stain was obtained from Invitrogen, USA. Ultra low range DNA ladder was obtained from Fermentas. All buffers were prepared in MilliQ water from Millipore Water System, Millipore, USA and filtered through 0.1 μm filters from Millipore, USA prior to use.

CHL, SGR, quadruplex and duplex DNA were prepared using standard protocol [16,17,25]. The concentrations of the oligomers were determined by absorbance measurement using molar extinction values (ε) as listed in Table T1. To study the effects of molecular crowding on Pu27-CHL interactions, Pu27 was dissolved in 10 mM potassium phosphate buffer, pH 6.8, containing 150 mM KCl in presence of 40% (w/v) ficoll 70. All experiments were carried out in 10 mM potassium phosphate buffer, pH 6.8, containing 150 mM KCl either in the presence or absence of 40% (w/v) ficoll 70.

Table 1
Dissociation constant (determined from absorbance studies and ITC) and stoichiometry for the interaction of chelerythrine and Pu27 in the absence and presence of ficoll in 10 mM potassium phosphate buffer pH 6.8 containing 150 mM KCl.

System	Dissociation constant, K_d (μM) at 25 °C		Stoichiometry
	Absorption	ITC	
Pu27-CHL (dilute)	2.54 ± 0.20	3.58 ± 0.65	1:2 (Job Plot)
Pu27-CHL (MC)[a]	4.11 ± 0.24	ND	1:2

[a] MC-molecular crowding with 40% (w/v) ficoll 70, ND-not determined.

2.2. Methods

2.2.1. PCR-stop assay

PCR-stop assay was performed with a modified protocol of the previous studies [7–9,26]. Pu27, Pu27-13,14 and Pu27rev (Table T1) were used in the current study. The reactions were performed in 1 × PCR buffer, containing 4 μM of each oligonucleotide, 0.16 mM dNTP, 0.04 U phusion high-fidelity DNA polymerase and indicated amount of CHL or SGR. Reaction mixtures were incubated in a Veriti 96 well thermal cycler (Applied BioSystem) with the following cycling conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The amplified products were resolved on 18% non-denaturing polyacrylamide gel in 1 × TBE, followed by staining with SYBR Green I.

2.2.2. Absorption spectroscopy

Absorption spectra were measured in CECIL spectrophotometer. 10 μM of CHL was titrated against Pu27 in absence and presence of 40% (w/v) ficoll 70. The dissociation constant and stoichiometry was determined from the plot of $\Delta A/\Delta A_{max}$ at 316 nm versus quadruplex concentration [16,17,25]. The method of continuous variation (Job Plot) was also employed to determine the binding stoichiometry [27].

2.2.3. Isothermal titration calorimetry

ITC experiments were done in iTC200 Microcalorimeter, Microcal Inc., USA at different temperatures between 10 °C and 35 °C. 25 μM CHL was titrated against Pu27.

25 μM CHL was titrated against Pu27 duplex to determine the association constant for the interaction at 25 °C. To maintain the