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Research Article

Methylene Linker Assisted DNA binding of Vinblastine and Simpler Analogs: Purine-Pyrimidine Specificity of Indole Derivatives

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Abstract: Studies on structural analogs of indole alkaloid Vinblastine sulphate, indole-2-carboxylic acid, indole-3-acetic acid, indole-3-propanoic acid and indole-3-butanoic acid have demonstrated DNA binding profile of these molecules. The mode of binding of these molecules was found to be non-specific, in the minor groove of DNA. Fluorescence studies suggest that the carboxylic acid terminus anchors the small molecule in the minor groove and methylene linker of longer length facilitates a better binding mode for drug molecules as compared to short linker length or no methylene group present between the carboxylic acid terminus and the indole ring. An interesting phenomenon of purine-pyrimidine specificity on DNA binding of these drug candidates was detected as against the DNA base sequence specificity. Such a specificity measure can be further implemented for future drug-DNA complexes, in the cases of non-specific DNA binding. The structural assignment of DNA oligomer [d(GATCCGGATC)$_2$] using nD-NMR techniques was furnished. The complexation of vinblastine-DNA complex was evaluated using NMR methods.

Introduction

Vinblastine (VLB, **I**), an indole alkaloid from *Vinca rosea*, is used as an anticancer agent for the treatment of various types of Leukemia. Vinblastine is effectively administered in clinics for the treatment of Hodgkin's disease, lymphocytic lymphoma, histiocytic lymphoma, advance testicular cancer, advanced breast cancer, Kaposi's sarcoma and Letterer-Siwe disease [1-4]. The anticancer profile of VLB has been attributed to its binding with tubulin protein inside the cancer cell, where it depolymerises the microtubular assembly. Subsequently it arrests the cell division resulting in the cell death. A number of quinoline alkaloids have been demonstrated to possess the RNA binding profile as well [5, 6]. In addition, the structure of Vinblastine also has other structural features like hydrogen bond acceptor/donor atoms providing the DNA binding motif in its structure, which prompted us to investigate its binding with double helical DNA through H-bond formation.

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In order to decipher the DNA binding profile of Vinblastine, it was proposed to study a group of small molecules having H-bond acceptor/donor atoms in the carboxylic acid, ethanoic acid, ethanamide, or propanoic acid groups, which bear a structural similarity to one of the structural halves of VLB i.e. catharanthine **(2)**. A group of small indole derivatives (Fig. 1) were selected viz., indole-3-butanoic acid (**3**), indole-3-propanoic acid (**4**), indole-3-ethanoic acid (**5)** and indole-2-carboxylic acid (**6**). Among them, indole ring is having a carboxylate group present on position **2** or **3**.

Several research groups have demonstrated that $CDPI_3$ DNA-oligomer conjugates containing methylene linker of variable length resulted in the increase of the melting temperature of resulting complexes [7, 8]. This increase in melting temperature indicated the formation of more stable DNA duplexes. The optimum size of the methylene linker length was found to be 5-7 methylene groups for the snug-fit binding of drug in the minor groove DNA. Recent research investigations have shown in conjugated DNA duplex that optimal methylene linker length between the $CDPI_3$ drug and DNA allowed the drug to fold back in the minor groove of the DNA duplex facilitating proper binding through snug-fit [7, 8].

Our previous study [9] on indole derivatives has also underlined the DNA binding characteristics of such molecules. Incorporating methylene linker of an appropriate length, the smaller molecules (**2-6**) were chosen to determine whether the methylene linker length would facilitate their binding with the DNA duplex. The carboxylate terminus is supposedly helping in anchoring these molecules (**3-6**) in the minor groove of DNA. In the present study, we have also investigated the parent molecule, Vinblastine sulfate (**1**) and evaluated its DNA binding profile *vis-à-vis* its simpler structural analogues (**2-6**).

Materials and Methods

The excitation band pass was fixed at 5 nm while different emission band pass wavelengths were used (5 nm, 10 nm & 20 nm). The scan speed of 240 nm/min was kept fixed during all the experiments. Vinblastine sulfate (VLB, **1**), and Indole derivatives were purchased from Sigma-Aldrich Chemicals Co., USA, and used after checking for their purity on HPLC. Catharanthine tartrate was purchased from Hysel India Pvt. Ltd., New Delhi. Calf thymus DNA (sonicated) was also obtained from Sigma-Aldrich. Four AT/GC-specific DNA decamer sequences were purchased from Sigma-Aldrich as desalted base sequences, viz,

DNA-1: 5’-d(GATGGCCATC)$_2$

DNA-2: 5’-d(GATCCGGATC)$_2$
DNA-3: 5’-d(GGCAATTGCC)$_2$
DNA-4: 5’-d(GGCTTAAGCC)$_2$

Stock solutions of Vinblastine (I), simpler analogs of VLB (**3-6**) and DNA were made in 20 mM sodium phosphate buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 1 mM $(Na)_2$ EDTA and 3 mM NaCl) at pH 7.5. Concentrations of VLB (**1**), Catharanthine (**2**) and four DNA decamer sequences (DNA-1 to DNA-4) were determined spectrophotometrically using the molar extinction coefficients, $\varepsilon_{365} = 4647\ M^{-1}cm^{-1}$ for Vinblastine sulphate & $\varepsilon_{281} = 8090\ M^{-1}\ cm^{-1}$ for Catharanthine. For DNA decamer sequences, the molar extinction coefficients used were, $\varepsilon_{260} = 95000$ for DNA-1, $\varepsilon_{260} = 92600$ for DNA-2, $\varepsilon_{260} = 93200$ for DNA-3, $\varepsilon_{260} = 96600$ for DNA-4. The concentration of indole derivatives (**3-6**) were calculated volumetrically. All the solutions were freshly prepared and stored below freezing point duly protected. No change in the optical properties of the drugs (**1-6**) or DNA was observed.

All the compounds obeyed Beer’s law in the concentration range employed in the present study. The DNA decamers were

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annealed slowly in the buffer solution. Initially all the compounds were tested using UV-absorbance spectroscopy and it was found that these compounds (**1-6)** gave an absorption maximum in or around 260 nm, a region where DNA oligomers (DNA-1 to DNA-2) also absorb. Due to this overlap of the absorbance maxima of these compounds, it was found that UV absorbance spectroscopy is not very suitable for evaluating drug-DNA binding in the proposed study. Consequently UV Fluorescence technique was thus chosen for evaluating the drug-DNA binding.

The Fluorescence measurements were recorded on a Hitachi model F4010 spectrofluorimeter (Hitachi Ltd, Tokya, Japan), where a fixed concentration of Vinblastine & other indole derivatives was titrated with increasing concentration of DNA decamers in fluorescence free quartz cuvette of 1 cm path length. Fluorescence titration experiments of Vinblastine and other simpler analogues with calf thymus DNA (CT-DNA) were performed with excitation band pass 5 nm while emission band pass of 20 nm. In case of decamer DNA, the excitation and emission band pass were 5 nm each.

All the proton NMR experiments were carried out at the regional NMR facility at Indian Institute of Technology (IIT), Roorkee on a 500 MHz Bruker AVANCE 500 spectrometer equipped with Siemens workstation with Topspin NMR processing software. The analysis of the NMR spectrum was accomplished on SPARKY software developed at UCSF [10]. NOESY 2D-NMR experiments on DNA-2 [d(GATCCGGATC)$_2$] were conducted using the following experimental parameters: Temperature = 297 K, solvent = D_2O, Mixing time = 300 ms, Initial delay = 1.5 seconds, Number of scans = 48, Total number of experiments in first dimension = 256, FID resolution = 2.93 Hz/point, and Sweep width = 6009 Hz.

1D ^{1}H-NMR titration was conducted to evaluate the VLB-DNA complexation. An NMR titration of DNA decamer and drug molecule (VLB) was furnished at 297K temperature using 20 mM phosphate buffer. DNA sample was taken in an NMR tube and its 1D ^{1}H-NMR spectra was recorded. Aliquots of VLB were then added and a 1D ^{1}H-NMR spectrum was recorded after each addition. The titration was stopped when molar ratio of 2:1 of drug:DNA was achieved.

Results and Discussion

Vinblastine (**1**) has 2 or 4 methylene groups attached to an indole nucleus in the nine-membered ring of catharanthine. This methylene linker provides extra flexibility to the VLB molecule in the event of substrate binding to furnish a stable drug-DNA complex. These methylene linkers are attached to position 2 and 3 of the indole nucleus of catharanthine half of VLB. Current study indicated towards the evidence of the longer methylene linker facilitating the DNA binding through proper fit [11-13]. In the present study, we compared the role of methylene linker *vis-à-vis* Vinblastine and Catharanthine, and learnt about the DNA binding phenomenon of VLB (Fig. 2).

It was found that VLB (**1**) and simpler structural precursors, viz. catharanthine (**2**), indole-3-ethanoic acid (**4**) and indole-2-carboxylic acid (**5**) gave consistent quenching in fluorescence maxima after each addition of DNA. Vinblastine sulphate gave fluorescence maxima at 362 nm when excited at 265 nm. The titration data obtained in fluorescence was fit by double reciprocal method to obtain the DNA binding constant K. As shown in Table 1, the value of DNA binding constant K was found to be in the order of 10^5 to 10^6 per mole for Vinblastine sulphate (**1**) and of 10^4 to 10^5 per mole for indole derivatives (**3), (4)** & (**5**). The present study showed that Vinblastine binds strongly with CT-DNA

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while other simpler analogs also interact with duplex DNA suggesting a possible similarity in their DNA binding motif. Since the simpler molecules employed in this study were all indole derivatives, it can be suggested that their interaction is mainly due to the presence of van-der-Waals forces, electrostatics and hydrogen bonding. The oxygen atoms attached in the molecules possibly facilitate the H-bond formation with H-atoms of the NH_2 group of guanine in the DNA minor groove. Kumar et al. [11-13] have successfully demonstrated this observation in their study on Hoechst-33258-DNA complex using NMR.

The possibility of intercalation facilitated [14, 15] by indole ring moiety in case of small molecules also exists. In view of the absence of any strong positive charge in the ring system, it is quite likely that the intercalation is not the favorable mode of binding. Whereas, previous studies [11-13] have also forwarded the hypothesis that the molecule prefers to sit in the minor groove instead of preferentially intercalating between the DNA base pairs.

The possibility of sequence specificity of DNA binding of Vinblastine molecule was further evaluated using DNA decamer sequences designed to have 4 base pair specific central core. In this study, four DNA decamers were used to assess the base sequence specificity of DNA-binding interaction of Vinblastine. To understand the interaction of Vinblastine with specific DNA sequences**,** the drug was titrated with four different DNA decamers. The DNA binding results obtained (Table 1) in fluorescence titration experiments clearly suggest that Vinblastine interacts with DNA. However, this does not indicate the mode of interaction of the drug.

The structural assignment of DNA-2 was furnished by 1D ^{1}H-NMR & 2D-NOESY. A 2D-NOESY spectrum of DNA decamer 5'-d(GATCCGGATC)$_2$ reveals the sequential NOE connectivities between H6/H8 protons of DNA bases and their corresponding H1' protons (Fig. 3). The corresponding chemical shift values of H1', H2', H2", H3', H4' and H6/H8 protons are given in Table 2. In addition, assessment of Vinblastine-DNA binding was also furnished by employing 1D ^{1}H-NMR titration with DNA-2 (Fig 4). No significant change in the chemical shift values upon each addition of drug aliquots was noticed. However, the broadening of the NMR signals was observed as a result of titration.

Conclusions

A gradual increase in the DNA binding constants of small molecules e.g. indole derivatives (**3-6)** with the increase in the methylene linker chain length, points towards the facilitation of binding, with larger length of the methylene linker in the indole derivatives (**3-6)**. It suggests that the carboxylic acid group anchors the small molecule in the minor groove and the indole ring is accommodated between the DNA base pairs or in the minor groove of DNA duplex. This was further explored using more sensitive method like NMR spectroscopy.

We propose a model of DNA binding of small indole derivatives (**3-6)** with decamer DNA duplexes, which is based on the experimental observation of fluorescence data and docking studies (Fig. 4). According to this model, the DNA binding strength of indole derivatives increases with the increase in the linker chain length attached to the indole ring without increasing the stacking forces. The methylene linker chain length plausibly provides greater flexibility to the indole unit. Thereby a faster exchange of indole ring between the DNA base pairs and in the minor grove is facilitated. We used the four DNA sequences to evaluate the possible DNA sequential preference of the drug candidates.

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In order to understand the forces responsible for DNA binding of indole derivatives, Vinblastine sulfate and Catharanthine, we employed a docking program named DNADock, based on a specialized protocol for DNA minor groove binding [16]. The DNADock program performs rigid body docking calculations and allows the drug to remain in the flexible mode in order to obtain a low energy conformation at a specific site on DNA minor groove. DNADock also takes into account the thermodynamic aspect of drug-DNA binding. Thermodynamic calculations are essential in order to complement the structural data. Contrary to a large family of DNA binding agents, all the compounds employed in this study were considered having no formal charge. Consequently, the docking results showed a large amount of non-electrostatic contribution to the overall binding energy of these molecules. The binding model indicates that there was an H-bonding mechanism which operates in the binding of these indole derivatives with DNA.

Since DNA binding constants are moderately high, they suggest that the electrostatic interaction may not be the only mode possible for the DNA binding of these compounds. It was observed in the modeling that the H-bond donor (OH) group and acceptor (=O) atoms present in the indole derivatives along with the H-bond donor (Guanine 2-NH_2 group) and H-bond acceptor (Cytosine O-atom at position 2) in the minor groove wall of DNA duplex provide sufficient anchoring support for H-bond formation. This H-bond assited DNA binding provides the basis of free movement of indole ring between the DNA base pairs. The greater the chain length, the greater the flexibility in the free movement of the indole ring, thus greater is the drug residence time on the DNA binding site, reflected by higher values of binding constants [17]. Moreover, this flexibility also affords the formation of a few new H-bonds between the drug and DNA atoms further providing stability to the drug-DNA complexes.

Purine-Pyrimidine Specificity pattern: The study was designed to find out the DNA base sequence specific patterns of small molecules with designed DNA oligomers. However, docking results as well as experimental data showed no such DNA base-sequence specific patterns, hence a non-specific DNA binding. On the other hand, the docked structures of Indole derivatives viz., **(3)**, **(4)** & **(5)** showed Purine-Pyrimidine specific patterns which could be important in designing lead compounds for specific sequences. Purine-Pyrimidine specificity has been observed in case of protein-DNA [18] and drug-DNA [19] complexes. The analysis of docked structures was based on a base proximity model in which the DNA bases in the close immediacy of the bound molecule were taken into consideration (Table 1). It should be envisaged in view of the fact, that purines (guanosine and adenosine) and pyrimidines (cytidine and thymidine) separately have similar electron density around them in their group. As a result thereof either purines or both pyrimidines offer the same electron density and electrostatic potential for the purpose of drug binding in case of non-specific DNA binding.

The binding site of indole derivatives and Vinblastine sulfate consisted of 3-base pairs. Out of three DNA decamer sequences, the binding patterns of any two sequences were grouped based on their similarity. The exact match of purines and pyrimidines of the binding site was defined as ‘Same’ pattern. ‘1-Base altered’ pattern was assigned to the groups which contain altered pattern of a base but the overall number of purines and pyrimidines remains same in both the docked structures of each group. It was observed that all 6 drug candidates gave ‘same’ pattern of purine-pyrimidine specific binding site, while others gave ‘1-base altered’ pattern. Among

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them catharanthine (**2**) and indole-3-butanoic acid (**3**) demonstrated 'same' purine-pyrimidine specificity pattern of DNA binding.

Thus, we can herewith propose that purine-pyrimidine specificity patterns can be used as another measure of DNA binding specificity exhibited by a drug. In the case of non-specific DNA binding of drugs, the 'purine-pyrimidine' specificity can be employed as a measure to assess the DNA binding specificity in a broader sense.

Structural Assignment of DNA-2 by NMR

All the ^{1}H-NMR chemical shift values were obtained by deciphering the sequential inter- and intra-nucleotide cross-peaks between base protons and corresponding H1' protons in the finger print region (Fig. 4). Later, other regions of NOE cross-peaks (base proton-H3'; base proton-H4'; and base proton to H2'/H2" protons) were also analyzed. An unambiguous assignment of almost all DNA base protons and sugar protons was furnished and listed in the Table 2. All the 10 intranucletide NOE crosspeaks for base-H3' protons were not resolved. In the base proton-H4' region, however, only 5 intranucleotide NOE cross peaks were assigned for G1, A2, G6, G7, A8 residues of DNA-2 decamer duplex. An overall standard B-DNA structure with 2'-endo conformation of sugar pucker was found for all the nucletide residues and all the residues were having anti-conformation of bases *vis-à-vis* deoxy
ribose sugar, based on the 2D-NMR results.

NMR titration of Vinblastine-DNA Complex

1D ^{1}H-NMR titration of Vinblastine with DNA-2 was performed in order to quantitatively estimate the DNA binding phenomenon of VLB at NMR time scale. The initial results, however, indicated that the drug-DNA complex was not formed at the experimental conditions used in the NMR titration. A little turbidity appeared in the NMR tube, which persisted during the whole titrations. However, all the ^{1}H-NMR spectra of the complexation titrations were recorded. It shows that the complex formation under NMR conditions is faster than the NMR time scale. Though, line broadening of the NMR signals of both drug and the DNA ^{1}H-NMR signals indicates towards the non-specific DNA interaction of VLB.

The VLB-DNA complex had been formed under 10^{-6} M concentration ranges using fluorescence spectroscopy; whereas we prepared 10^{-3} M solutions for NMR measurements. It is quite plausible that the aggregation of NMR ferquencies resulted at this higher concentration. Hence, this could be one of the reasons for not observing the VLB-DNA complex formation, beside other experimental factors like salt concentration, pH, etc. It is therefore concluded that multiple NMR titration experiments are needed in order to achieve precise experimental parameters to obtain drug binding induced chemical shift changes in the DNA decamer proton resonances. Complexation of VLB is being further investigated with other DNA oligomer sequences as well and will be reported elsewhere.

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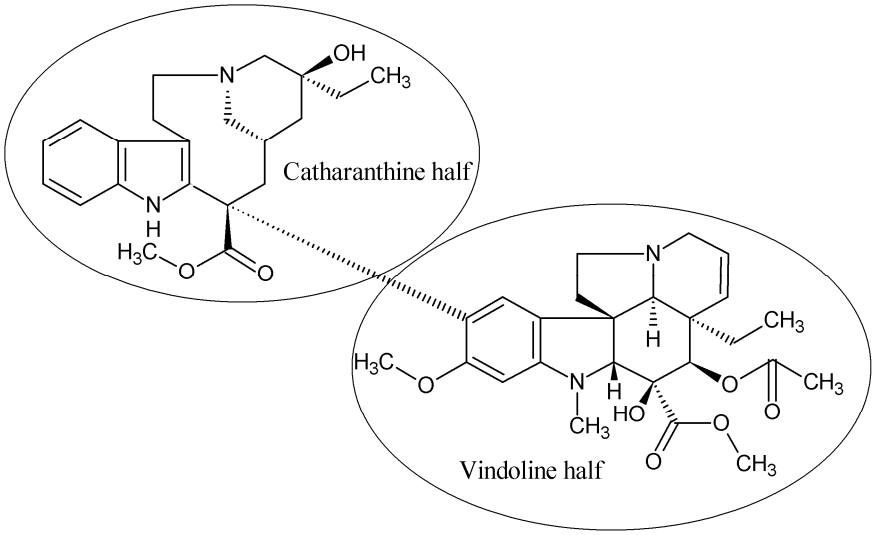
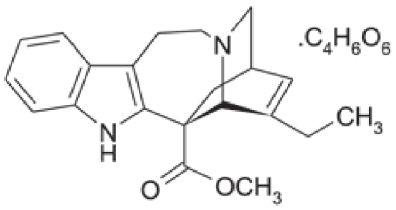
India is gratefully acknowledged. Authors acknowledge the use of DNADock software developed by Prof Jayaram and group at the SCFBIO Facility of Department of Chemistry, Indian Institute of Technology, New Delhi, India.



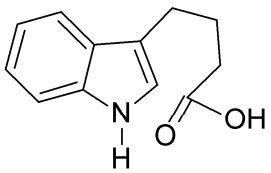
Vinblastine sulfate (VLB)

(1)



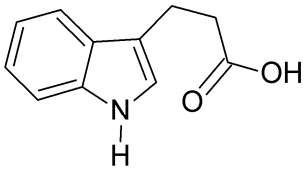
Catharanthine tartarate (CTN)

(2)



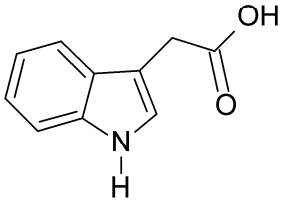
4-(1H-indol-3-yl)butanoic acid

(3)



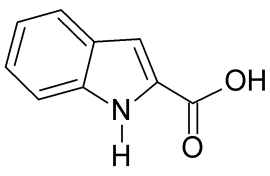
3-(1H-indol-3-yl)propanoic acid

(4)



1H-indol-3-ylethanoic acid

(5)



1H-indol-2-carboxylic acid

(6)

Figure 1: Structure of Vinblastine and its structural analogues **2-6**

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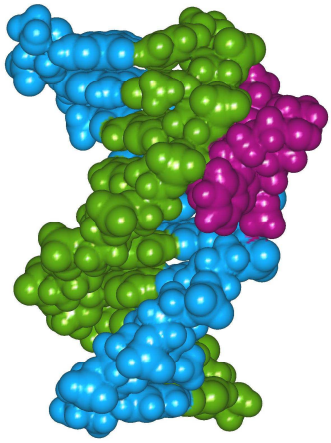

Figure 2: Binding model of Vinblastine sulfate with DNA decamer DNA-1. Vinblastine is shown in magenta.

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Table 1

Drug	Complex	Binding Site	Pu-Py Specificity	Binding Pattern	K_{exp} ($mole^{-1}$)	K_{calc} ($mole^{-1}$)
Vinblastine sulfate (1)	DNA-1	5'-G_4-G_5-C_6	5'-Pu-Pu-Py	Same	5.27×10^4	5.69×10^5
	DNA-2	5'-G_7-A_8-T_9	5'- Pu-Pu-Py		4.28×10^5	8.34×10^6
	DNA-3	5'-T_6-T_7-G_8	5'-Py-Py-Pu	1-Base altered	4.28×10^5	8.34×10^6
	DNA-4	5'-T_5-A_6-A_7	5'- Py-Pu-Pu		5.65×10^5	5.96×10^6
Catharanthine (2)	DNA-2	5'-A_2-T_3-C_4	5'-Pu-Py-Py	Same	5.10×10^4	5.69×10^5
	DNA-3	5'-A_5-T_6-T_7	5'-Pu-Py-Py		1.35×10^5	6.74×10^5
	DNA-1	5'-T_3-G_4-G_5	5'-Py-Pu-Pu	Same	1.95×10^4	6.74×10^5
	DNA-4	5'-T_5-A_6-A_7	5'-Py-Pu-Pu		3.30×10^4	4.82×10^5
Indole-3-butanoic acid (3)	DNA-1	5'-$C_6C_7A_8$	5'-Py-Py-Pu	Same	1.23×10^5	1.26×10^5
	DNA-2	5'-$C_4C_5G_6$	5'-Py-Py-Pu		1.96×10^5	2.10×10^5
	DNA-3	5'-$T_7G_8C_9$	5'-Py-Pu-Py	Same	1.00×10^5	1.76×10^5
	DNA-4	5'-$A_7G_8C_9$	5'-Py-Pu-Py		1.11×10^5	1.76×10^5
Indol-3-propanoic acid (4)	DNA-1	5'-$C_6C_7A_8$	5'-Py-Py-Pu	1-Base Altered	7.33×10^4	7.61×10^4
	DNA-2	5'-$G_7A_8T_9$	5'-Pu-Pu-Py		7.30×10^4	7.61×10^4
	DNA-3	5'-$T_6T_7G_8$	5'-Py-Py-Pu	Same	1.41×10^5	1.49×10^5
	DNA-4	5'-$T_4T_5A_6$	5'-Py-Py-Pu		1.38×10^5	1.06×10^5
Indol-3-ethanoic acid (5)	DNA-1	5'-$C_7A_8T_9$	5'-Py-Pu-Py	1-Base altered	5.81×10^4	5.44×10^4
	DNA-3	5'-$A_4A_5T_6$	5'-Pu-Pu-Py		1.04×10^5	1.06×10^5
	DNA-2	5'-$G_6G_7A_8$	5'-Pu-Pu-Pu	Same	1.11×10^5	1.06×10^5
	DNA-4	5'-$A_6A_7G_8$	5'-Pu-Pu-Pu		6.39×10^4	6.43×10^4
Indole-2-carboxylic acid (6)	DNA-1	5'-$T_3G_4G_5$	5'-Py-Pu-Pu	1-Base altered	1.00×10^5	1.06×10^5
	DNA-4	5'-$A_6A_7G_8$	5'-Pu-Pu-Pu		9.44×10^4	1.49×10^5
	DNA-2	5'-$T_3C_4C_5$	5'-Py-Py-Py	Same	1.19×10^5	1.26×10^5
	DNA-3	5'-$C_3T_4T_5$	5'-Py-Py-Py		1.48×10^5	1.76×10^5

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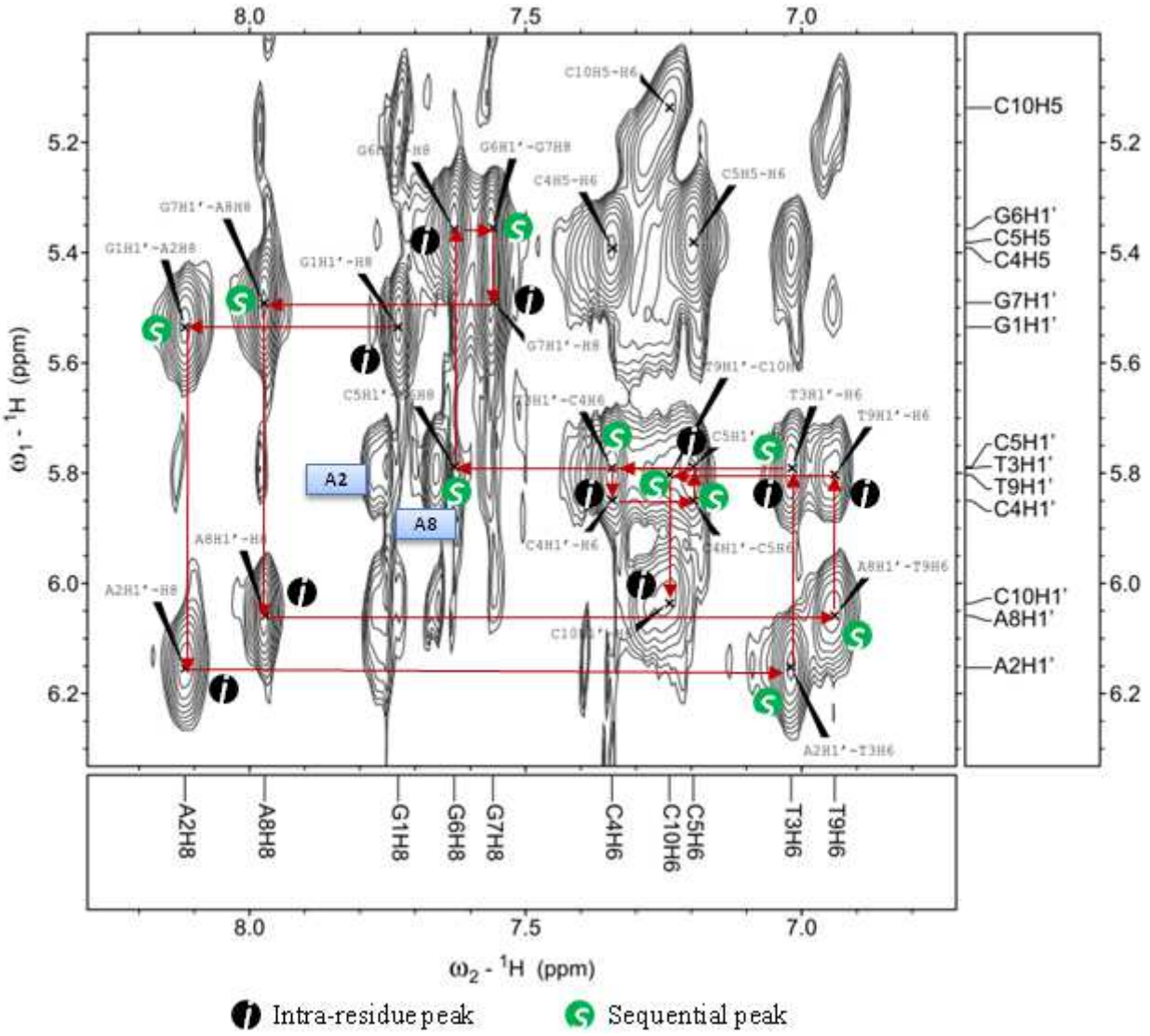

Figure 3: NOESY spectrum of 5'-d($G_1A_2T_3C_4C_5G_6G_7A_8T_9C_{10}$)$_2$ (DNA-2) sequence showing finger print region of the spectrum. A2 signifies A2H2 proton cross peak with T3H1' and A8 signifies A8H2 proton cross peak with T9H1'.

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Table 2

Residue	H8/H6	H5	Me	H1’	H2’	H2”	H3’	H4’
G1	7.73	-	-	5.53	2.46	2.63	n.a.	4.35
A2	8.12	-	-	6.15	2.56	2.81	4.89	4.30
T3	7.02	-	1.18	5.81	1.93	2.31	4.89	n.a.
C4	7.35	5.39	-	5.79	1.94	2.29	n.a.	n.a.
C5	7.19	5.33	-	5.38	1.73	2.11	n.a.	n.a.
G6	7.63	-	-	5.36	2.48	2.53	4.83	4.14
G7	7.56	-	-	5.49	2.43	2.57	4.84	4.21
A8	7.97	-	-	6.05	2.41	2.74	4.83	4.26
T9	6.95	-	1.16	5.79	1.78	2.30	4.81	n.a.
C10	7.25	5.15	-	6.04	2.10	2.10	4.36	n.a.

n.a. Not assigned

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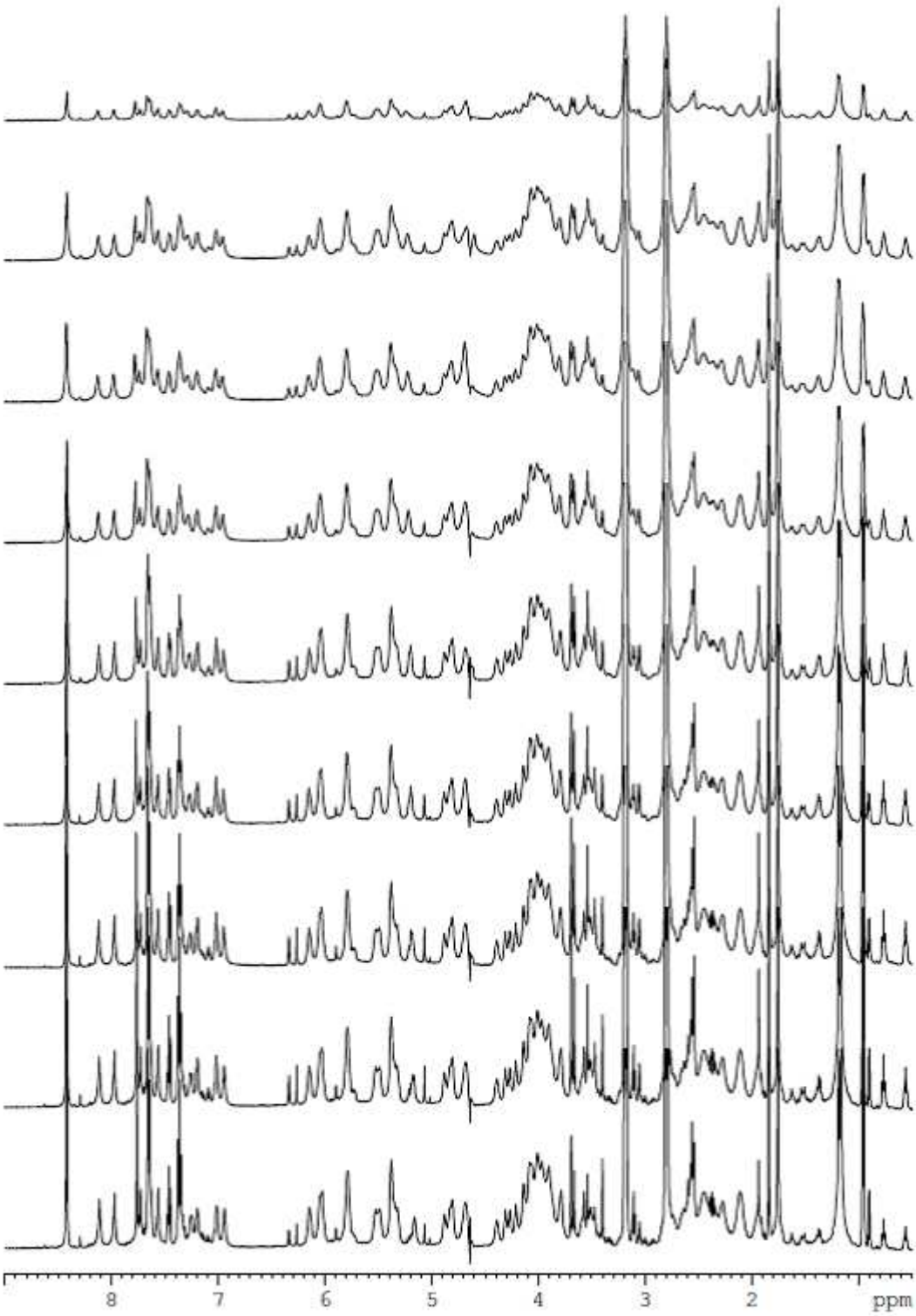

Figure 4: 1D ^{1}H-NMR titration of VLB-DNA-2 complex.

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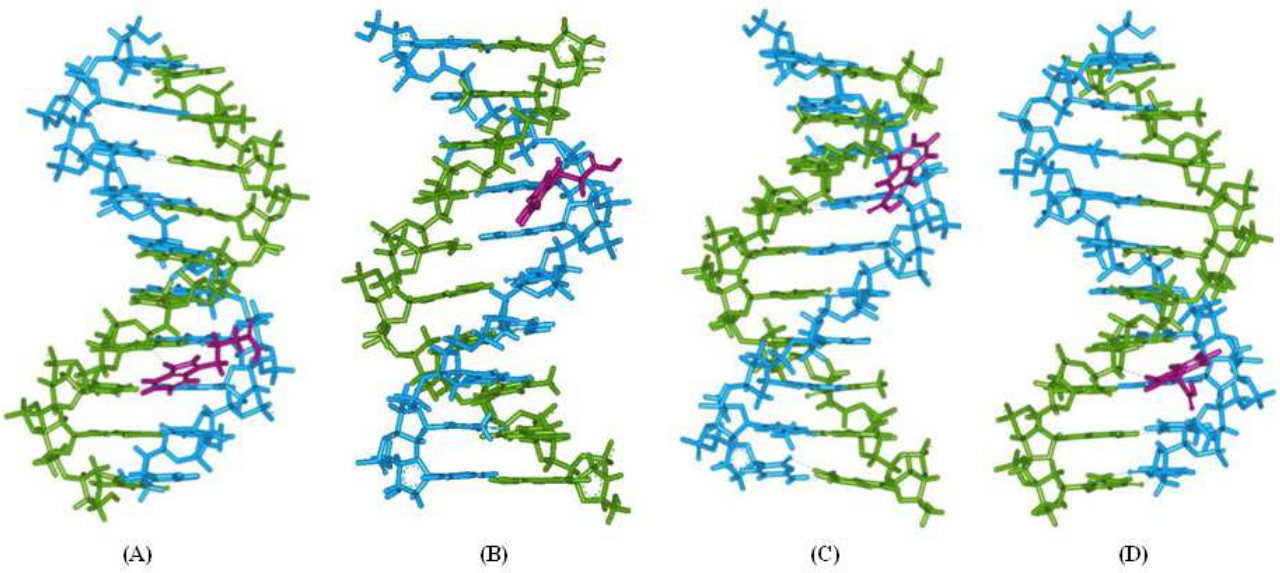

Figure 5: DNA Binding models of Indole derivatives: (A) Indole-3-butanoic acid (**3**), (B) Indole-3-propanoic acid (**4**), (C) Indole-3-ethanoic acid (**5**) and (D) Indole-2-carboxylic acid (**6**) with decamer DNA-1.

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