Recognition of homo-polynucleotides containing adenine by a phenanthridinium bis-uracil conjugate in aqueous media†

Lidija-Marija Tumir,[a] Ivo Piantanida,*[a] Iva Juranović,[b] Zlatko Meić,[b] Sanja Tomić[c] and Mladen Žinić*[a]

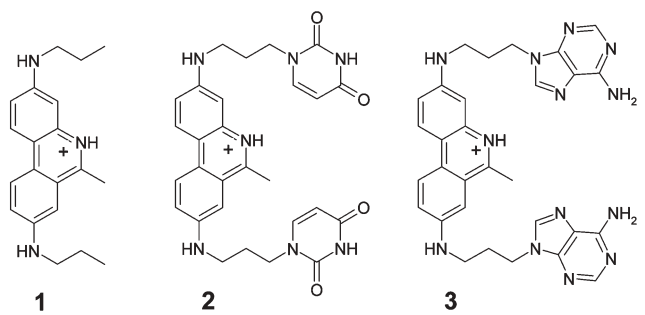
Received (in Cambridge, UK) 13th January 2005, Accepted 9th March 2005
First published as an Advance Article on the web 18th March 2005
DOI: 10.1039/b500617a

Among novel bis-nucleobase–phenanthridinium conjugates bis-uracil analogue 2 stabilized significantly more effective poly-dA–poly-dT and poly-AH$^+$–poly-AH$^+$ than adenine analogue 3 and reference compound 1. For the alternating poly-dAdT–poly-dAdT however, the binding preference is lost, pointing to the importance of specific interactions of uracils of 2 with homopolynucleotides containing consecutive adenines.

Small molecules containing intercalator (for strong binding to DNA/RNA), nucleobase (for recognition of the complementary non-paired base at the DNA/RNA abasic site) and a linker between them have been the subject of intensive research in the last decade.[1] Among the number of different approaches, a series of intercalator–nucleobase conjugates have shown intriguingly different specificity of binding to complementary[2] and non-complementary[3] polynucleotides. These results led us to design and synthesise the novel phenanthridinium bis-nucleobase conjugates shown in Scheme 1.

Such conjugates can be considered as close analogues of the previously studied mono-nucleobase derivatives[2,4] but also introduce two major differences: (i) the presence of additional nucleobase offers possibility of doubled recognition between conjugate and complementary polynucleotide,[5] and (ii) the two linker-nucleobase units tethered to the opposite sites on the longer axis of phenanthridinium should influence intercalation into ds-polynucleotides due to steric reasons. Therefore, the binding studies with the new conjugates and various well defined synthetic polynucleotides may result in observation of novel recognition events and shed more light on the fine interplay of steric (position and number of substituents) and electrostatic (influence of two tethered nucleobases) interactions that lead to recognition of polynucleotides by synthetically modified intercalators. All the presented compounds were synthesized by modified procedures elaborated earlier,[4] have satisfying elemental analyses or mass spectra and their structures were verified by detailed 1D and 2D NMR analysis.[6] Aqueous solutions of **1–3** exhibit p$K_a \approx 6$ attributed to protonation of the phenanthridine nitrogen.[4] Owing to the low solubility of phenanthridine at pH = 7 all further experiments in aqueous media were done at pH = 5, all compounds being in a protonated (phenanthridinium) form. Spectroscopic experiments (UV/Vis, fluorescence, NMR)† clearly point to the intramolecularly stacked conformation of **2** and **3**, the former being much less stable than the latter. Addition of equimolar amounts of studied DNA and RNA resulted in significant bathochromic and hypochromic changes in UV/Vis spectra of **1**, **2** ($\Delta\lambda_{max}$ = 17–22 nm; ΔAbs = 20–30%) characteristic of an intercalation binding mode,[7] while changes observed for **3** were close to the error limits of the method. Precipitation at higher excess of DNA/RNA hampered collection of enough data points for accurate calculation of ratios n ([bound compound]/[polynucleotide]) and stability constants (K_s). Low solubility also prevented NMR study or viscometry experiments.

Results of thermal melting experiments obtained for **2** (Table 1) reveal an outstanding influence of bis-uracil substituents on



Scheme 1 Structures of novel phenanthridinium–bis-nucleobase conjugates.

† Electronic supplementary information (ESI) available: Spectroscopic characterization, experimental data and procedures. See http://www.rsc.org/suppdata/cc/b5/b500617a/

*pianta@irb.hr; zinic@irb.hr. (Ivo Piantanida)

Table 1 ΔT_m Values[a] (°C) of different ds-polynucleotides with **1–3** at pH = 5 (I = 0.027 mol dm^{-3}, sodium citrate buffer)

	r[b]	1	2	3
ct-DNA	0.1	1.5	1.6	1.9
	0.2	4.6	5.0	4.0
	0.3	5.2	6.0	5.1
Poly-dAdT –poly-dAdT	0.1	1.0	0.6	0.5
	0.2	1.2	—	1.1
	0.3	2.0	1.3	2.2
Poly-dA–poly-dT	0.1	5.0	16.0	1.0
	0.2	13.0	18.0	6.0
	0.3	13.0	20.6	6.0
PolyA–polyU[c]	0.1	7.3/1.3[c]	0/6.3[c]	1.4/0.7[c]
	0.2	7.1/0.9[c]	2/12.4[c]	1.5/0.3[c]
	0.3	7.9/1.2[c]	2/15.1[c]	2.5/0.5[c]
PolyAH$^+$–polyAH$^+$[c]	0.1	0.5	3.6	0.5
	0.2	0.5	8.0	0.5
	0.3	0.5	14.0	0.5

[a] Error in ΔT_m: ±0.5 °C. [b] r = [bound compound]/[polynucleotide]. [c] Biphasic transitions: the first transition at T_m = 28.5 °C is attributed to denaturation of polyA–polyU and the second transition at T_m = 80.1 °C is attributed to denaturation of polyAH$^+$–polyAH$^+$ since polyA at pH = 5 is mostly protonated and forms ds-polynucleotide.[3,9]

stabilization of polynucleotides. Stabilization of poly-dA–poly-dT induced by **2** is more than three times stronger than stabilization of ct-DNA and almost double than that found for **1**. Even more pronounced specificity in stabilization is observed for polyAH$^+$–polyAH$^+$; **2** yielded a value (for $r = 0.3$) more than ten times higher compared to values of **1**, **3** or **EB**.[8] The ΔT_m values for **1**, **2** and **3** with polyAH$^+$–polyAH$^+$ are close to the values of the second transition observed in the polyA–polyU melting experiments (Table 1, results in the row PolyA–polyU) due to formation of polyAH$^+$–polyAH$^+$ in the latter system.[9] Such a strong stabilization of the second transition by **2** very likely destabilizes the **2**–polyA–polyU complex yielding values of ΔT_m for the first transition much lower than found for **1**. All compounds (**1–3**) stabilize poly-dA–poly-dT more efficiently than its alternating analogue (poly-dAdT–poly-dAdT). However, this selectivity is most pronounced for **2** (double compared to **1** and triple compared to **3**). Apparently, for the stronger binding of bis-uracil conjugate (**2**) at least one poly-adenine strand is neccesary.

RNA preference characteristic for **EB**[8,10] is actually reversed in the case of **1**, whose DNA preference correlates well with most of the other intercalators.[10] DNA over RNA preference is even more pronounced for **3** than for **1**. Stabilization effects found for bis-adenine conjugate **3** are in general much smaller than those found for reference **1**, very likely due to the stable intramolecular stacking interactions between adenines and phenanthridinium. As observed for **EB** and **1**, **3** does not significantly stabilize polyAH$^+$–polyAH$^+$.

Solutions of all studied compounds exhibited a strong fluorescence increase (5–8 times) upon titration with any ds-polynucleotide (including polyAH$^+$–polyAH$^+$). In most cases small hypsochromic shifts of emission maxima were also observed ($\Delta\lambda_{em} \approx 5$ nm). Such changes in emission spectra agree well with those observed for the well-known intercalator ethidium bromide (**EB**) and its derivatives.[11] Taking in account that structures of **1–3** are closely related to that of **EB** it is reasonable to propose the same mechanism of fluorescence increase upon binding to ds-DNA and ds-RNA.[12] After mixing ds-polynucleotides with the studied compounds it was observed in all cases that equilibrium was reached in less than 60 s. In the following 2–3 h, fluorescence and UV/vis spectra of the complexes remained constant. Owing to the use of much lower concentrations of studied compounds and polynucleotides in the fluorimetric titrations no precipitation occurred.[13]

Processing the fluorimetric titration data using the Scatchard equation[14] gave the stability constants (K_s) and ratios n (Table 2).

Table 2 Stability constants ($\log K_s$) and ratios n ([bound compound]/[polynucleotide]) calculated according to fluorimetric titrations[a,b]

	1		**2**		**3**	
	n	$\log K_s$	n	$\log K_s$	n	$\log K_s$
ctDNA	0.1	5.5	0.1	5.4	0.1	5.3
Poly-dAdT –poly-dAdT	0.1	4.3	0.1	5.1	0.1	5.3
Poly-dA–poly-dT	0.1	4.2	0.1	4.6	0.1	5.0
PolyA–polyU	0.1	5.9	0.1	5.0	0.1	5.1
PolyAH$^+$–polyAH$^+$	0.1	5.2	0.1	5.2	0.1	5.1

[a] Fluorimetric titrations were performed at pH = 5 (I = 0.027 mol dm^{-3}, sodium citrate/HCl buffer), λ_{exc} = 465–500 nm, λ_{em} = 530–650 nm. [b] Processing of titration data by means of the Scatchard equation gave values of ratio $n = 0.1 \pm 0.03$, for easier comparison all K_s values were re-calculated for fixed $n = 0.1$.

The only significant difference in affinity toward ds-polynucleotides could be observed for **1**. The lowest affinity of **1** toward poly-dA–poly-dT compared with other studied ds-DNA and ds-RNA is in line with the results obtained for other classical intercalators.[15] Compounds **2** and **3** show comparable affinity toward all ds-polynucleotides, although again the lowest values were obtained for poly-dA–poly-dT. Values of stability constants (K_s) obtained for bis-uracil conjugate **2** did not reveal selectivity toward poly-dA–poly-dT and polyAH$^+$–polyAH$^+$ observed in thermal melting experiments. Similar non-consistency was observed for ethidium bromide (**EB**), whose pronounced RNA *vs.* DNA selectivity in thermal stabilisation experiments[8,10] was not reflected in values of stability constants (K_s).[8] This finding suggests a cooperative effect on stabilisation of the double helix at conditions close to saturation of polynucleotide with the studied compounds (present in thermal melting experiments). In fluorimetric titrations high excess of polynucleotide over the studied compound was used, consequently the binding properties of the compound on isolated and therefore independent binding sites were monitored.

To shed more light on increased stabilization of homopolynucleotides containing consecutive adenines by the bis-uracil conjugate **2**, we performed a molecular modeling experiment. Using the experimental structure of the polynucleotide as a template,[16] a series of complexes of poly-dA–poly-dT with **2** bound in different orientations were built and energy optimized (AMBER force field[17]). The low-energy complex with partial intercalation of phenanthridinium and both uracils located in the same groove was soaked into the box of water and molecular dynamics simulation was performed at room temperature (following equilibration and heating of the system from 0 to 300 K) over a duration of 1 ns. During the simulation partial intercalation of phenanthridinium was preserved while the number and type of hydrogen bonds between the side chains and different parts of DNA fluctuated. In the most frequent pattern one uracil interacts with adjacent adenines, and the other is oriented from DNA while its side chain interacts either with the sugar or phosphate group (Fig. 1).

Besides the unspecific π–π stacking interactions between phenanthridinium and adjacent base pairs, additional stabilization could be achieved by two hydrogen bonds between uracil tethered at the position 3 of phenanthridinium with two consecutive adenines of the DNA tetramer, while simultaneously the secondary amino group at the position 8 of phenanthridinium is forming the hydrogen bond with the sugar 5′-OH oxygen of the polynucleotide backbone (Fig. 1). Such a combination of additive interactions is in agreement with specific stabilization of polynucleotides containing an uninterrupted poly-A or poly-dA sequence by bis-uracil conjugate **2**.

Presented results strongly support classical intercalation as the main binding mode of reference **1** to DNA and RNA. However, for **2** and **3** classical intercalation into ds-polynucleotide is not easy to occur due to the two nucleobases being tethered to the opposite sites of the long axis of phenanthridinium, leaving only few possibilities of binding: (a) threading intercalation with phenanthridinium longer axis orthogonal on the polynucleotide base-pair longer axis; (b) bis-intercalation of tethered nucleobases into the polynucleotide with phenanthridinium as a positively charged linker; (c) partial intercalation of phenanthridinium with both

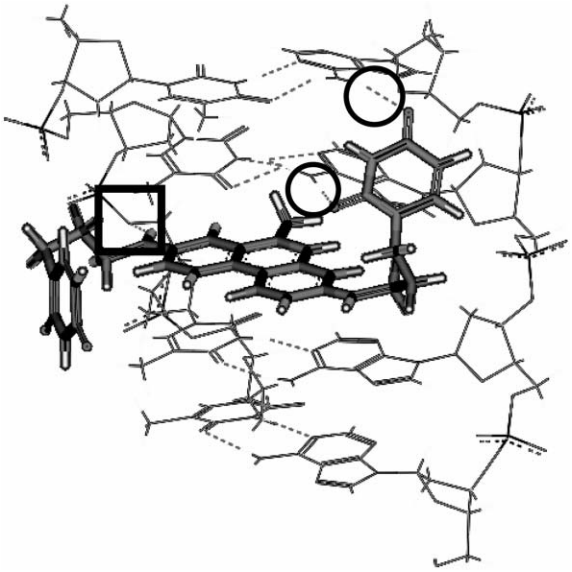


Fig. 1 Optimised structure of the poly-dA–poly-dT tetramer–**2** intercalative complex. Hydrogen bonds between uracil 2,4-carbonyl groups and amino groups of consecutive adenines are indicated by circles and interaction of the phenanthridinium-8-amino group with the sugar 5′-OH oxygen of the DNA backbone is indicated by a square.

tethered nucleobases located in the same groove; (d) minor groove binding of intramoleculary stacked form of conjugate. The possible mode (a) can be excluded by observation of the short incubation times,[18] since in all our UV/Vis and fluorimetric titration experiments equilibrium upon addition of DNA and RNA was reached in less than 60 s. The bis-intercalation of small aromatic units, such as adenines (**3**) and especially uracils (**2**) (binding mode (b)), is not likely to take place.[19] Thus, binding modes (c) and (d) could be considered as the most probable. Compounds **2** and **3** differ in protonation ability at pH = 5. Bis-uracil conjugate **2** can posses only one positive charge on N5 of the phenanthridinium unit, while adenine bases of **3**, since they are involved in π–π aromatic stacking,[20] can be protonated at the N1 position with pK_a = 5–6. Therefore, **3** bound in a complex with DNA/RNA can receive up to three positive charges (one on N5 of phenanthridinium and two on adenines). The obtained results pointed to significantly different binding modes of **2** and **3** to polynucleotides. Taking into account rather strong intramolecular stacking in **3**, strong selectivity in stabilization of ds-DNA compared to ds-RNA (characteristic for classical minor groove binders)[10] and, in addition, the possible multiple positive charge, the minor groove binding of **3** to ds-DNA/RNA seems the most probable binding mode. In contrast to **3**, the bis-uracil conjugate **2** exhibits weak intramolecular stacking, only one positive charge, and almost no DNA/RNA selectivity in thermal stabilization. Molecular modelling experiments for **2** suggest as a dominant binding mode the partial intercalation of phenanthridinium with both tethered uracils located in the same groove and one of them involved in additional hydrogen bonding interactions with adjacent adenines. The results presented here clearly show that **2** is capable of much stronger thermal stabilization of ds-poly-nucleotides with consecutive adenines than of poly-dAdT-poly–dAdT containing alternating adenines and thymines. A selective stabilisation effect observed for **2** is evident only at conditions of saturation of the polynucleotide with the studied compounds (thermal melting experiments) but not at high excess of polynucleotide over **2** (fluorimetric titrations).

Lidija-Marija Tumir,[a] Ivo Piantanida,*[a] Iva Juranović,[b] Zlatko Meić,[b] Sanja Tomić[c] and Mladen Žinić*[a]

[a]*Laboratory of Supramolecular and Nucleoside Chemistry, Department of Chemistry and Biochemistry, Ruđer Bošković Institute, HR 10002 Zagreb, P.O.B. 180, Croatia. E-mail: pianta@irb.hr; zinic@irb.hr.; Fax: +385 1 4680195; Tel: +385 1 4571210*

[b]*Laboratory of Analytical Chemistry, Department of Chemistry, Faculty of Science, Strossmayerov trg 14, HR 10000 Zagreb, Croatia*

[c]*Laboratory for Chemical and Biological Crystallography, Department of Physical Chemistry, Ruđer Bošković Institute, HR 10002 Zagreb, P.O.B. 180, Croatia*

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