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Cooperative 2:1 Binding of a Bisphenothiazine to Duplex DNA

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Drugs based on the phenothiazine scaffold have a wide variety of therapeutic applications. They are used for the treatment of mental diseases, in antihelminthic therapy, and for their antibacterial pathogen inactivation properties. They generally exhibit low toxicity and mutagenicity.[1,2] Phenothiazines are also used as DNA photosensitizers, and their binding mode to double-stranded DNA is highly structure and sequence dependent. For example, methylene blue intercalates in poly(dG), poly(dC), but binds via the minor groove in poly(dA), poly(dT).[3] In the course of a screening affinity of bisphenothiazine ligands for various DNA sequences and structures, we serendipitously found that ligand RP12274 cooperatively forms a 2:1 complex with duplex DNA.

ESI-MS allows the resolving of complex mixtures and determining all stoichiometries that are present simultaneously in the injected sample.[4] In the ESI-MS spectra (Figure 1), the peak of the 2:1 complex (two ligands bound to one duplex) is much larger than the peak of the 1:1 complex for d(CGTAAATTTACG)$_2$ (DK33) and d(CGCGAATTCGCG)$_2$ (DK66). However, only a low intensity peak of 1:1 complex is observed for the duplex d(CGCGGGCCCGCG)$_2$ (DK100). For each mass spectrum, the equilibrium concentrations of free DNA, 1:1, and 2:1 complexes are determined from the corresponding peak areas, and the concentration of free ligand is determined from mass balance equations. The equilibrium binding constants defined in Equations (1) and (2) can therefore be determined in a model-free manner.[5] Mass spectra were recorded from solutions with varying drug concentrations (Figure 1, lower panel), and the binding constants are given in Table 1. For duplexes DK33 and DK66, K_2 is more than 100 times larger than K_1, indicating a cooperative 2:1 binding.

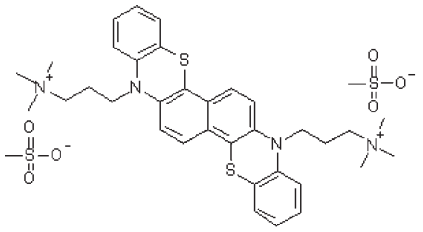
$$K_1 = [1:1]/[\text{ligand}][\text{DNA}] \quad (1)$$

$$K_2 = [2:1]/[\text{ligand}][1:1] \quad (2)$$

Table 1. Binding constants of RP12274 for double-stranded DNA.

	ESI-MS[a]			SPR[b]	
	K_1 [M^{-1}]	K_2 [M^{-1}]		K_1 [M^{-1}]	K_2 [M^{-1}]
DK33	$(2.8\pm0.7)\times10^4$	$(5.5\pm0.9)\times10^6$			
DK66	$(2.1\pm0.4)\times10^4$	$(3.0\pm0.8)\times10^6$	[AATT]	$(1.1\pm0.2)\times10^5$	$(1.1\pm0.8)\times10^8$
DK100	$(1.6\pm0.3)\times10^5$	$(1.7\pm0.8)\times10^5$	[CG]	$(1.2\pm0.3)\times10^5$	–

[a] Mean values and standard deviations are obtained from eight drug/DNA ratios. [b] See Supporting Information materials and methods for the oligonucleotide sequences.



RP12274

Surface plasmon resonance (SPR) experiments were performed as an independent method to determine the binding constants of RP12274 to an AT- and a GC-rich duplex. Two biotin-labeled hairpin oligonucleotide sequences with [CGAATTCG] and [(CG)$_4$] stems were used (Figure 2 and Supporting Information). Experiments were performed in ammonium acetate, to allow comparison with the ESI-MS experiment. With the AT-rich duplex, the maximum RU value obtained at saturation is clearly larger than with the 100% GC duplex (with the same oligonucleotide loading quantity on the chip). The saturation RU for AATT and RP12274 is twice as much as for the minor groove binder netropsin ($RU_{max}=45$) which is known to form only 1:1 complex in the AATT site.[6] As the preferred stoichiometries had been revealed by the ESI-MS spectra, a two-binding constants model [Eq. (4)] was used to obtain the affinity constants from the SPR sensorgrams for [AATT], and a single binding site model was chosen for the [(CG)$_4$] data [Eq. (3)]. When eq. (4) was used for [(CG)$_4$], the K_2 value was close to zero. Again, a significant cooperativity, with K_2 1000 times greater than K_1 is revealed from the SPR measurements (Table 1). Results obtained with the more commonly used HBS buffer showed similar results to those obtained with NH_4OAc (see Figure S1 in the Supporting Information).

The binding mode of the bisphenothiazine RP12274 to duplex DNA cannot be intuitively predicted from the structure. On the one hand, the trimethyl-propanaminium chains likely

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

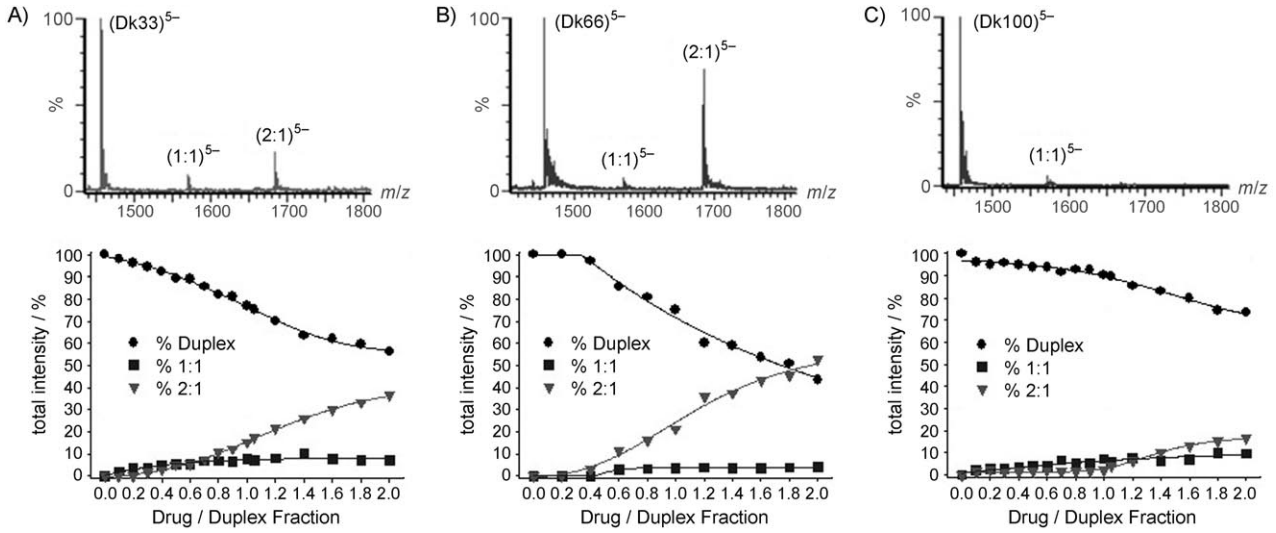

Figure 1. ESI-MS spectra of solutions containing 8 μM RP12274 and 5 μM DNA A) DK33 = $(CGTAAATTTACG)_2$ duplex, B) DK66 = $(CGCGAATTCGCG)_2$ duplex, and C) DK100 = $(CGCGGGCCCGCG)_2$. Graphs of the relative abundance of the free duplex (●), the 1:1 (■), and 2:1 (▼) complexes versus the drug molar fraction added to a 5 μM duplex solution are shown in the lower panel.

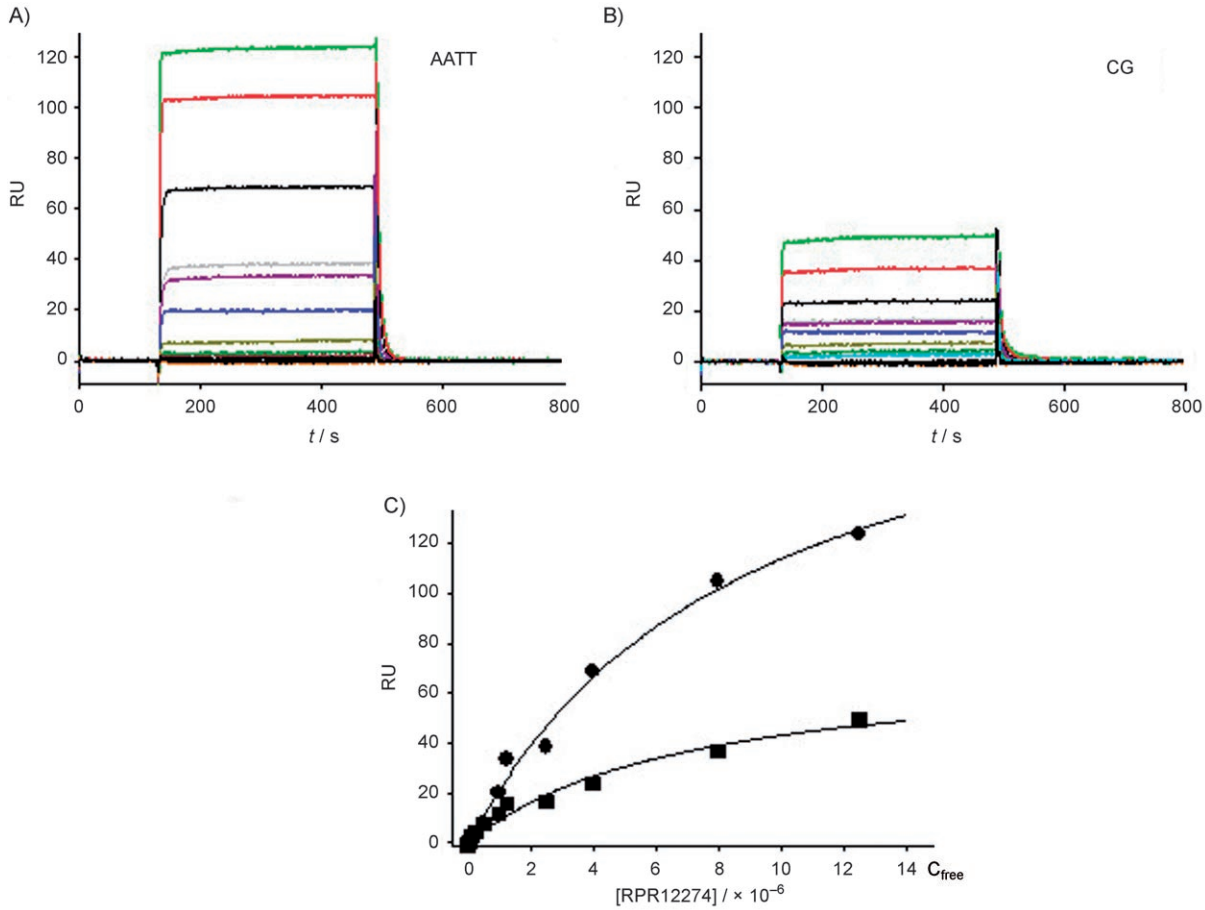

Figure 2. SPR sensorgrams for binding of RPR12274 to A) AATT and B) $[CG]_4$. The concentration of unbound ligand [mol L^{-1}] in the flow solution varies from 7.5 nM for the lowest curve to 12.5 μM for the top curve. C) Binding curves used to determine the equilibrium binding constants for RPR12274 interacting with (●) AATT duplex and (■) CG duplex.

cause steric problems upon intercalation. On the other hand, typical minor groove binders usually have a crescent shape that matches the DNA groove, which is not the case here. We used circular dichroism (CD) spectroscopy to characterize the

binding mode of RP12274 to the three duplexes DNA (Figure S2). With the AT-rich duplexes, a positive CD signal is observed between 291 and 370 nm. Positive CD signal for the ligand is a signature of minor groove binding mode.[7,8] Isoelliptic points are observed indicating the presence of only two species in equilibrium (according to ES-MS, the free DNA, and the 2:1 complex).

Altogether, our experimental data suggest a model involving minor groove binding of two ligands in A/T tracts. We used molecular modeling to test hypothetical structures for the 2:1 complex with RP12274. In a first step, the possible stacked dimer geometries were explored using PM6 level of theory. Figure 3A shows two views of the energy minimized dimer. The two molecules are staggered, and the repulsion between the four ammonium groups is minimized, all positive charges being >9 Å from each other. In the second step, the dimer is docked in the AT-rich region of the minor groove of duplex DK66, and the system is allowed to relax prior to the molecular dynamics. In the resulting complex (Figure 3B), both phenothiazine aromatic rings have extended van der Waals contacts with the minor groove. Ammonium groups interact with phosphates, giving a large electrostatic stabilization of the complex.

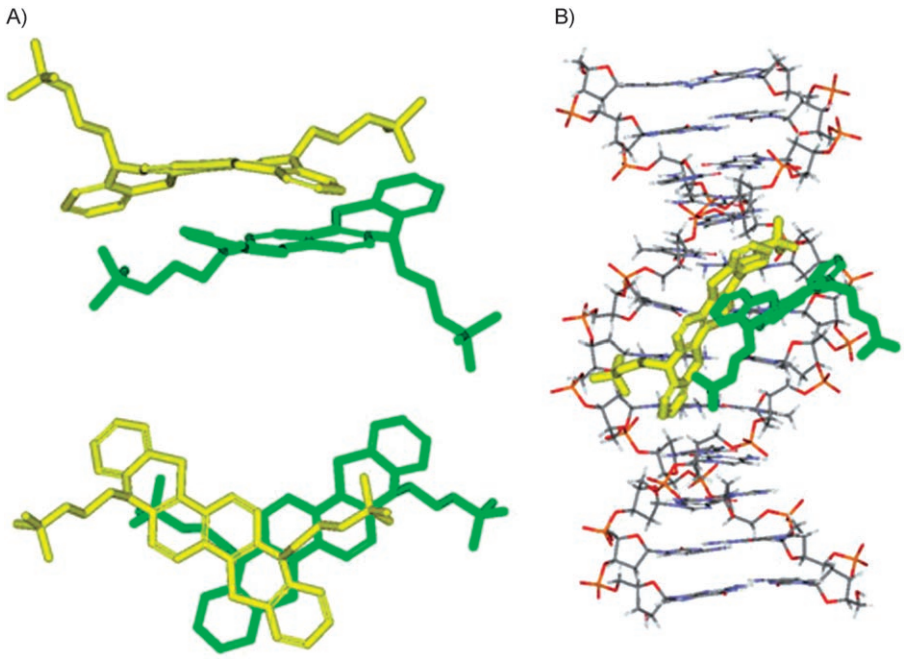

Figure 3. A) Geometry optimization of the dimer: side view and top view. B) Structure of the 2:1 complex between the RP12274 dimer and the duplex (CGCGAATTCGCG)2 obtained after molecular dynamics.

Cationic ligands are more prone to interact with the AT-rich groove as the electrostatic potential is more negative in this region,[9] and this explains the preference of RP12274 dimers for AT sequences. Minor groove binders adopting a preferential 2:1 stoichiometry are typically polyamide monocations that can form head-to-tail dimers, such as distamycin A[10–12] and lexitropsins.[13,14] Dications usually do not form dimers because of charge repulsion, with the notable exception of diamidines DB293 and some of its derivatives which form antiparallel dimers in the minor groove.[15,16,17] RP12274 is dicationic, but its chemical structure is very different from that of DB293. This bisphenothiazine scaffold can therefore be exploited for future design of new minor groove binding agents having photosensitizing properties.

Experimental Section

Electrospray mass spectrometry (ESI-MS): ESI-MS experiments were performed on a Q-TOF Ultima Global (Micromass, now Waters, Manchester, UK) with its standard ESI source. The capillary voltage was set to −2.2 kV, the cone voltage to −35 V, and the RF Lens1 to −60 V. The hexapole collision voltage of 10 V was used for full scan MS. Source block and desolvation temperatures were set to 70 °C and 100 °C, respectively. Spectra were acquired from 1000 to 2000 *m/z* and only a portion of the mass range is shown on Figure 2 for clarity. As the starting concentrations are known, the concentration of the free DNA, the 1:1, and the 2:1 complex at equilibrium are calculated from the relative intensities of the species in the mass spectra as described in reference [5].

Surface plasmon resonance (SPR): SPR measurements were performed with a four-channel BIAcore 2000 optical biosensor system (Biacore Inc.) and streptavidin-coated sensor chips (SA). The sensor chips were first conditioned with three consecutive 1 min injections of NaCl (1 M) in NaOH (50 mM) followed by extensive washing with buffer. The same amount of each oligomer was immobilized on the surface by noncovalent capture (350 response units, or RU). One of the flow cells was left blank as a control. Steady-state binding analysis was performed with multiple injections of RP12274 at different concentrations over the immobilized DNA and the reference surfaces during 6.25 min at a flow rate of 10 μL min^{-1} and at 25 °C. Dissociation with buffer follows each injection. The instrument response (RU) in the steady-state region is proportional to the amount of bound drug and was determined by averaging over a 60 s time span. The values from the steady-state region of the sensorgrams were fitted with either Equation (3) for a single binding site, or Equation (4) for two binding sites, using Sigmaplot software for nonlinear least squares optimization of the binding parameters to obtain the affinity constants.

$$RU = RU_{max} K_1 [drug] \frac{1}{1 + K_1 [drug]} \quad (3)$$

$$RU = RU_{max} K_1 [drug] \frac{1 + 2K_2 [drug]}{1 + K_1 [drug] + K_1 K_2 [drug]^2} \quad (4)$$

Molecular modeling: Molecular modeling was performed using Mopac 2007 (openmopac.net) with PM6 theory to optimize the phenothiazine dimer. The duplex + phenothiazine dimer was prepared and optimized using AMBER99 force field within Hyperchem 7.5 software (Hypercube, Inc.). The starting duplex d(CGCGAATTCGCG)$_2$ was the solution structure deposited in the Protein Data Bank with code 1GIP. RP12274 drug was manually docked in the minor groove and energy minimized in the force field generated by the duplex until an energy gradient of 0.5 kcal mol^{-1} Å^{-1}) was reached (Polak–Riebiere conjugate gradient algorithm). Then the whole complex was immerged in of periodic box (40 × 40 × 50 A) containing 2640 water molecule (TIP3P potential). After relaxation of the water molecules, the whole system was submitted to 0.2 ns unconstrained molecular dynamics. The final structure (Figure 3B) was obtained after removing the water molecules and counterions. When the RP12274 drugs are docked in the major groove, the complex is unstable (several ps) and the drugs goes "out" of the duplex because of the absence of favorable contact.

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