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Structural flexibility *versus* rigidity of the aromatic unit of DNA ligands: binding of aza- and azoniastilbene derivatives to duplex and quadruplex DNA[†]

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The known azastilbene (E)-1,2-di(quinolin-3-yl)ethane (2a) and the novel azoniastilbene derivatives (E)-2-(2-(naphthalen-2-yl)vinyl)quinolizinium (**2b**) and (*E*)-3,3'-(ethane-1,2-diyl)bis(1-methylquinolinin-1-ium) (2c) were synthesized. Their interactions with duplex and quadruplex DNA (G4-DNA) were studied by photometric, fluorimetric, polarimetric and flow-LD analysis, and by thermal DNA denaturation studies, as well as by ¹H-NMR spectroscopy. The main goal of this study was a comparison of these conformationally flexible compounds with the known G4-DNA-binding diazoniadibenzo[b,k] chrysenes, that have a comparable π -system extent, but a rigid structure. We have observed that the aza- and azoniastilbene derivatives 2a-c, i.e. compounds with almost the same spatial dimensions and steric demand, bind to DNA with an affinity and selectivity that depends significantly on the number of positive charges. Whereas the charge neutral derivative 2a binds unspecifically to the DNA backbone of duplex DNA, the ionic compounds 2b and 2c are typical DNA intercalators. Notably, the bis-quinolinium derivative 2c binds to G4-DNA with moderate affinity ($K_{\rm b}$ = 4.8 \times 10⁵ M⁻¹) and also stabilizes the G4-DNA towards thermal denaturation ($\Delta T_{\rm m}$ = 11 °C at ligand–DNA ratio = 5.0). Strikingly, the corresponding rigid counterpart, 4a,12adiazonia-8,16-dimethyldibenzo[b,k]chrysene, stabilizes the G4-DNA to an even greater extent under identical conditions ($\Delta T_{\rm m}$ = 27 °C). These results indicate that the increased flexibility of a G4-DNA ligand does not necessarily lead to stronger interactions with the G4-DNA as compared with rigid ligands that have essentially the same size and π system extent.

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Introduction

Among the non-canonical DNA forms, the quadruplex DNA (G4-DNA), that is formed in G-rich DNA sequences upon stacking of at least two guanine quartets, is currently attracting most attention.¹ Thus, several G-rich DNA sequences with the propensity to fold into quadruplex structures have been identified in genomic nucleic acids,² for example in telomeric DNA and some promoter regions of oncogenes.³ Moreover, it has been shown that some biologically relevant processes are directly related to quadruplex-DNA formation, such as the suppression of gene expression,⁴ or the induction of the cellular response to DNA damage.⁵ As a result of the essential biologi-

cal functionality of quadruplex DNA, the association of an exogenous ligand with G4-DNA structures may have a significant effect on the biological function of G-rich DNA sequences, either by simply blocking the binding site of enzymes, which leads to their inhibition, or by increasing the thermodynamic stability and the lifetime of the G4-DNA.^{1,6} In the latter case, enzyme inhibition may also occur when the enzyme requires the unwound form of the particular DNA sequence. Based on this principle, numerous G4-DNA-targeting molecules have been developed that may affect the biological activity of the DNA.⁷ Along these lines, traditional DNA intercalators, *i.e.* cationic, planar, polycyclic (het)arenes, figure as a promising basis for the development of G4-DNA ligands. Thus, it has been shown that such intercalators have the propensity for a terminal π -stacking at the ends of G4-DNA structures.^{1,7} Such as intercalation of a ligand between base pairs in duplex DNA,⁸ the terminal π -stacking is driven by dispersion interactions between the aromatic ligand and the guanine quartet and by an additional hydrophobic effect as the lipophilic ligand migrates from the aqueous solution into the hydrophobic binding site.9 Because of the thermodynamically



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unfavorable process of the unwinding and disassembly of the quadruplex structure, planar aromatic ligands do usually not intercalate into G4-DNA. In this context, highly selective aromatic ligands have been developed that bind to the terminal end of the quadruplex due to their extended π -system, but whose structure does no longer allow a thermodynamically favorable intercalation into duplex DNA.^{1a,b,7} In this context, we have shown that polycyclic azoniahetarenes, for example the diazoniadibenzo [b,k] chrysenes (1a-c), bind to G4-DNA even in the absence of additional functional side chains, thus enabling the assessment of the intrinsic ligand properties.¹⁰ The binding modes of 1a-c were studied in detail and all experimental data indicate the terminal π -stacking of the diazoniachrysenes to quadruplex DNA as the major binding mode. Thus, the size and topology of the π -systems of the cationic hetarenes 1a-c enable sufficient overlap with the G-quartet mainly based on attractive π -stacking interactions.¹⁰ It should be noted, however, that this class of quadruplex ligands consists of highly rigid compounds that have essentially no conformational flexibility. At the same time, some of the most promising and efficient G4-DNA ligands, such as e.g. the bis(quinolinium)pyridine dicarboxamides,¹¹ contain structural elements that provide a significant degree of conformational freedom, as the aromatic units are not completely annelated but connected by flexible linkers. It may be assumed that this structural flexibility facilitates the access of the ligand as well as the fit of the aromatic scaffold to the steric demand of the binding site. Along these lines, the influence of the structural flexibility of G4-DNA ligands has been shown recently by a comparison of structurally resembling flexible and rigid bis(quinolinium)-bisindole dicarboxamide derivatives.¹² In these ligands, the bisindole core unit, that is involved in the terminal π stacking to G4-DNA, is either connected in a flexible biaryl structure or kept rigid in a completely fused structure. It has been shown that these flexible derivatives have a slightly higher affinity to G4-DNA than the rigid ones because of the better ability of the flexible ligands to adapt to the G4-DNA structure. It should be noted, however, that even the rigid ligands in this study still contained flexible elements as the two quinolinium units were attached to the bisindole core through amide bonds that allow torsional movement. Before this background, we proposed that ligands with a similarly extended π -system as the dibenzochrysene derivatives 1a-c, but with an increased conformational flexibility may have an increased affinity to G4-DNA. We identified the azaand azoniastilbene derivatives 2a-c as a promising starting point to check this proposal because they have a similarly extended π -system as **1a–c**, but more conformational flexibility due to the possible rotation about the aryl-alkene single bond. We proposed that these compounds bind to DNA as it has been shown already that pyridinium-based azoniastilbenes bind to DNA.13 And more recently, it has been demonstrated that substituted styryl-quinolinium derivatives bind to G4-DNA.¹⁴ Herein, we present the synthesis of stilbene derivatives 2a-c and the investigation of their DNA-binding properties. For better comparison with respect to size and shape, we also

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synthesized and investigated the dimethyldiazoniadibenzochrysene **1d** in this study, because this derivative has methyl substituents at positions that correspond to the ones in the dimethyl-bis(quinolinium) derivative **2c**.



Results

Synthesis

The (*E*)-1,2-di(quinolin-3-yl)ethane (2a) was prepared according to published procedure¹⁵ and subsequently quaternized by reaction with methyl iodide at high pressure^{10a} to give the (*E*)-3,3'-(ethane-1,2-diyl)bis(1-methyl quinolinin-1-ium) (2c) in 70% yield (Scheme 1). The (*E*)-2-(2-(naphthalen-2-yl)vinyl) quinolizinium (2b) was synthesized in 62% yield by the basecatalyzed Knoevenagel-type reaction of 2-methylquinolizinium (3)^{10b} with 2-naphthaldehyde (4) (Scheme 1).^{10b,c} The dibenzochrysene derivative 1d was synthesized starting from the reaction of 1,5-di(bromomethyl)naphthalene (5)¹¹ with 2-(2methyl-(1,3)-dioxolan-2-yl)pyridine (6)¹³ and subsequent ion metathesis to give bis(pyridiniummethyl)naphthalene 7. The latter was treated with polyphosphoric acid (PPA) at 150 °C to give the cyclodehydration product 1d in 75% yield (Scheme 2).

Absorption and emission properties

The absorption and emission spectra of the aza- and azoniastilbene derivatives **2a–c** were recorded in DMSO, MeCN, MeOH, water, and BPE buffer solution (Fig. 1, Table S1 in ESI†). The corresponding shifts and the band structures do not depend strongly on the solvent properties. Thus, the longwavelength absorption maxima are lying in a rather small range of 325–333 nm (**2a**), 379–396 nm (**2b**) and 366–369 nm (**2c**), respectively. Likewise, most of the emission bands of each compound cover the same wavelength range with small Stokes shifts and low to moderate emission quantum yields (**2a**: $\lambda_{fI} =$ 397–420 nm, $\Phi_{fI} = 0.2-0.5$; **2b**: $\lambda_{fI} = 491-499$ nm, $\Phi_{fI} = 0.2$; **2c**:



Scheme 1 Synthesis of stilbene derivatives 2b and 2c.



Scheme 2 Synthesis of 4a,12a-diazonia-8,16-dimethyldibenzo[*b*,*k*] chrysene (**1d**).

 $\lambda_{\rm fl} = 454-462$ nm $\Phi_{\rm fl} = <0.01-0.3$). As the only exception, the emission spectra of derivative **2a** in water and MeOH deviate from the ones in the other solvents; namely the bands are significantly broader with a very pronounced red-shifted shoulder (Fig. 1A), presumably indicating aggregation.

Spectrometric titrations with ct DNA

The interactions of the stilbene derivatives 2a-c with doublestranded calf thymus (ct) DNA were monitored by photometric and fluorimetric titrations (Fig. 2). The absorption of derivative 2a decreased during titration with no significant shift of the absorption maximum at 329 nm (Fig. 2A1). In the case of ligands 2b and 2c, the absorption maxima at 382 nm (2b) and 366 nm (2c) also decreased on addition of up to 2.8 (2b) and 6.1 (2c) molar equivalents of ct DNA, respectively, without shift of the maxima (Fig. 2A2 and A3). On further addition of DNA the absorption increased with a bathochromic shift of approx. 7 nm in each case. It should be noted that samples of 2b were occasionally contaminated with traces of the photodimer whose formation is indicated by a weak absorption at 333 nm. In general, the characteristic emission of the stilbene derivatives 2a-c was quenched on addition of ct DNA, which was accompanied in some cases by slight shifts of the emission maxima (Fig. 2B1-B3). In the case of ligand 2b, a very small fluorescence light-up effect was observed in the first titration steps (molar equivalent <1) before the quenching occurs. The data from the photometric or fluorimetric titrations were plotted as binding isotherms and analyzed based on the



Fig. 2 Photometric (A) and fluorimetric titration (B); **2a**: $\lambda_{ex} = 384$ nm, **2b**: $\lambda_{ex} = 342$ nm, **2c**: $\lambda_{ex} = 383$ nm of ligands **2a** (1), **2b** (2) and **2c** (3) (*c* = 20 μ M) with ct DNA (*c* = 1.0 mM in base pairs) in BPE buffer (pH = 7.0; **2a**-b: with 5% v/v DMSO); *T* = 20 °C. The arrows indicate the development of the absorption or emission bands during titration. Inset: Plot of absorption Abs/Abs₀ and relative emission *I*/*I*₀, resp., *versus c*_{DNA}.

theoretical binding model (*cf.* ESI[†]).¹⁶ The resulting binding constants are $6.2 \times 10^4 \text{ M}^{-1}$ (2a), $5.1 \times 10^4 \text{ M}^{-1}$ (2b), and $2.0 \times 10^5 \text{ M}^{-1}$ (2c).

Polarimetric titrations with ct DNA

To gain further insight into the DNA-binding modes the interactions between the ligands $2\mathbf{a}-\mathbf{c}$ with ct DNA were examined by circular dichroism (CD) and flow linear dichroism (LD) spectroscopy (Fig. 3). Upon addition of DNA the compound $2\mathbf{a}$ developed a significant positive induced CD (ICD) band at 344 nm and negative ICD bands at 417 nm and 307 nm, but the latter was only observed at ligand–DNA-ratio (LDR) = 0.5 and 1.0. At the same time, the characteristic positive CD band of the DNA at 277 nm fluctuated slightly with increasing LDR values. In addition, *flow*-LD measurements revealed a developing positive signal in the absorption range of the ligand $2\mathbf{a}$



Fig. 1 Absorption (dashed lines; $c = 25 \mu$ M) and emission spectra [continuous lines; $2a: \lambda_{ex} = 334$ nm, 2b: 390 nm, 2c: 320 nm of compounds 2a: (A), 2b (B), and 2c (C) in DMSO (red), MeCN (blue), water (green 2a: with 5% DMSO), MeOH (black), and phosphate buffer (orange, 2a: with 5% DMSO)].



Fig. 3 CD (A) and *flow*-LD (B) spectra of mixtures of ligands 2a-c and ct DNA in BPE buffer (pH = 7.00; 2a,b: with 5% v/v DMSO) and at LDR 0.0 (black), 0.1 (red), 0.2 (blue), 0.5 (magenta) and 1.0 (green); $c_{DNA} = 20 \ \mu$ M; $T = 20 \ ^{\circ}$ C. Arrows indicate the development of CD and LD bands with increasing LDR value.

with a maximum at 361 nm (Fig. 3B1). The addition of DNA to a solution of compound **2b** induced the formation of positive ICD bands at 386 nm and 340 nm with relatively high intensity at large LDR. The complementary LD-spectroscopic experiments showed a weak negative LD band at 403 and 369 nm whose intensity increased slightly with increasing LDR. The addition of DNA to compound **2c** led to the formation of relatively weak positive and negative ICD bands at 332 nm and 405 nm (Fig. 3A3). The *flow*-LD measurements showed a developing negative LD band in the absorption range of the ligand with a maximum at 372 nm. Notably, the intensity of the characteristic negative band of the DNA at 260 nm increased significantly with increasing LDR values (Fig. 3B3).

Fluorescence-monitored quadruplex-DNA melting

The interactions of the ligands 1d and 2a-c with quadruplex DNA were investigated with representative quadruplexforming oligonucleotide sequences. Firstly, the established FRET melting assay¹⁷ was employed to assess the stabilization of the quadruplex structure upon association with the ligands. With this method, the propensity of a ligand to stabilize quadruplex-DNA towards unfolding was determined by monitoring the temperature-dependent emission of the dye-labeled quadruplex-forming oligonucleotide F21T, fluod(G₃TTA)₃G₃-tamra (fluo: fluorescein; TAMRA: tetramethylrhodamine), because only in the quadruplex form a Förster resonance energy transfer (FRET) between the two dyes is possible. The quadruplex-forming oligonucleotide was chosen for first principal studies, as it is among the most commonly used substrates for this well-established assay.¹⁷ The FRET melting experiments revealed that the ligand 2a does not stabilize the G4-DNA F21T, even at high LDR = 5.0 (Table 1), while the ligands 1d, 2b and 2c stabilize the G4-DNA F21T towards unfolding, as clearly indicated by the increase of the melting temperature, $\Delta T_{\rm m}$, of 27 °C (1d), 3 °C (2b) and 11 °C (2c) at LDR = 5.0 (Table 1). To assess whether this stabilization of G4-DNA is a general feature of the ligands 2b and 2c the same experiments were also performed with the representative quadruplex-forming oligonucleotides **FmycT** [fluo-d(TGAG₃TG₃TAG₃TG₃TA₂)-tamra], **FkrasT** [fluo $d(AG_3CG_2TGTG_3A_2GAG_2A)$ -tamra] and Fa2T [fluo $d(ACAG_4TGTG_4)_2$ -tamra] (Table 1), all of which have been used already with this assay.^{7,18} In all cases, the quadruplex structure is also stabilized to significantly more extent by ligand 2c as compared with 2b. Nevertheless, it was also observed that the degree of G4-DNA stabilization upon binding of ligand 2c is not the same for the different oligonucleotides. Hence, the extent of stabilization decreases in the order F21T ($\Delta T_{\rm m}$ = 11 °C), FkrasT ($\Delta T_{\rm m}$ = 9.9 °C), FmycT $(\Delta T_{\rm m} = 6.0 \text{ °C})$, and Fa2T $(\Delta T_{\rm m} = 3.1 \text{ °C};$ all values at LDR = 5). To further assess the selectivity of the quadruplex stabilization by 2c, the melting experiments were also performed in the presence of excess of the duplex-DNA forming oligonucleotide ds26.17 Under these conditions, the ligand 2c shows a thermal stabilization of the F21T by 7.5 °C at LDR = 5.0 (Table 1).

Table 1 Shift of melting temperature, ΔT_{m} , of the G4-DNA F21T, FmycT, FkrasT and Fa2T in the presence of the ligands 1d and 2a-c

LDR	$\Delta T_{ m m}^{\ a}/{ m oC}$									
	1d F21T	$2a^b$	2b	2c	2b Fa2T	2 c	2b FmycT	2c	2b FkrasT	2 c
1.3	+9.3	-0.3	+0.7	+4.3 (2.9)	-0.2	<0.1	0.5	2.4	1.3	4.6
2.5	+21.6	+0.2	+1.6	+7.6(5.0)	0.1	-0.2	-0.3	2.9	1.3	7.2
5.0	+27.1	-0.4	+3.3	+10.6(7.5)	0.3	3.1	0.3	6.0	2.2	9.9

^{*a*} $c_{\text{DNA}} = 0.2$ μM in Na-cacodylate buffer (pH = 7.2); estimated error: ±0.5 °C. Determined fluorimetrically based on the temperature-dependent change of FRET in **F21T**, **FmycT**, **FkrasT** and **Fa2T**. ^{*b*} With 1% v/v DMSO. ΔT_{m} values in parentheses determined in the presence of **ds26** d(CA₂TCG₂ATCGA₂T₂CGATC₂GAT₂G) (*c* = 3.0 μM).

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Spectrometric titrations with quadruplex-DNA

The interactions of the ligands **1d**, **2b** and **2c** with the quadruplex-forming oligonucleotides **22AG** $dA(G_3TTA)_3G_3$ and **a2** $d(ACAG_4TGTG_4)_2$ were investigated by photometric and fluorimetric titrations (Fig. 4 and 5). Upon the addition of **22AG** or **a2**, the absorption maxima of **2b** (381 nm) and **2c** (366 nm)



Fig. 4 Photometric titration of **2b** (1) and **2c** (2) ($c = 20 \mu$ M) with **22AG** (A) and **a2** (B) ($c_{\text{DNA}} = 200 \mu$ M) in K-phosphate buffer (pH = 7.0, 5% v/v DMSO), T = 20 °C. The arrows indicate the development of the absorption bands during the titration. Inset: Plot of absorption Abs./Abs.₀ versus $c_{\text{DNA}}/c_{\text{ligand}}$.



Fig. 5 Fluorimetric titration of 1d (1), 2b (2) and 2c (3) ($c = 20 \ \mu$ M) with 22AG (A) and a2 (B) ($c_{DNA} = 200 \ \mu$ M) in K-phosphate buffer (pH = 7.0, 5% v/v DMSO), $T = 20 \ ^{\circ}C$; 1d: $\lambda_{ex} = 423 \ nm$ (22AG); 2b: $\lambda_{ex} = 390 \ nm$ (22AG and a2); 2c: $\lambda_{ex} = 385 \ nm$ (22AG) and 389 nm (a2). The arrows indicate the development of the emission bands during titration. Inset: Plot of relative emission intensity $I/I_0 \ versus \ c_{DNA}/c_{ligand}$.

decreased with the development of red-shifted absorption maxima at 401 nm (22AG) and 406 nm (a2) for 2b (Fig. 4A1 and B1), and 379 nm (22AG) and 382 nm (a2) for 2c (Fig. 4A2 and B2). In both titrations of the ligand 2c an isosbestic point was observed.

At the same time, the emission of derivatives **1d**, **2b** and **2c** is significantly quenched upon binding to **22AG** and **a2** (Fig. 5). The analysis of the resulting binding isotherms from the fluorimetric titrations of the ligands **1d**, **2b** and **2c** revealed binding constants of $1.5 \times 10^6 \text{ M}^{-1}$ (**1d**), $2.1 \times 10^5 \text{ M}^{-1}$ (**2b**) and $4.8 \times 10^5 \text{ M}^{-1}$ (**2c**) with **22AG** and $5.8 \times 10^5 \text{ M}^{-1}$ (**2b**) and $4.0 \times 10^5 \text{ M}^{-1}$ (**2c**) with **a2**.

¹H-NMR spectroscopic studies

Titrations of the diazoniastilbene 2c to the quadruplexforming oligonucleotide Tel6 d(T2AG3) was monitored by ¹H-NMR spectroscopy (Fig. 6). This hexanucleotide forms an equilibrium between monomeric and a terminally π -stacked dimeric G4-DNA [d(T₂AG₃)]₄ in aqueous solution^{16,19} Although Tel6 is only a very simplified quadruplex model as it does not contain the loop structures, such as e.g. 22AG, this oligonucleotide or variations thereof are often employed for the NMR spectroscopic detection of terminally stacking G4-DNA ligands,²⁰ because the interpretation of the NMR data is straightforward. It should be noted that due to limited solubility at the employed concentration range only up to 1.5 molar equivalents of the ligand 2c could be added. The titration of ligand 2c to Tel6 led to significant shifts of the ¹H NMR signals of the G4-DNA Tel6 (Fig. 6). Thus, NMR signals of the guanine imino protons of the dimeric quadruplex broadened and shifted to high-field by 0.10-0.12 ppm on addition of up to 0.4 molar equivalents of 2c and eventually disappeared during the course of titration. At the same time, the imino protons of the monomeric quadruplex were shifted by 0.27-0.32 ppm to higher field. Except for the proton signals of A3H2 and T1H6, most of the bands of the aromatic protons in the range of 6-8 ppm remained relatively sharp up to 0.8 molar equivalents of 2c (cf. ESI, Fig. S2[†]). In addition, the



11.6 11.5 11.4 11.3 11.2 11.1 11.0 10.9 10.8 10.7 10.6 10.5 10.4 10.3 10.2 *δ/* ppm

Fig. 6 The ¹H NMR spectra (600 MHz) of the imino protons of **Tel6** (2 mM in bases) in K-phosphate buffer ($H_2O: D_2O = 9: 1$; 95 mM, pH 7.0; T = 25 °C) with increasing amount of **2c**; * = monomeric quadruplex.

¹H-NMR signals of ligand **2c** also developed and shifted during the titration. As a general trend, the signals of the protons H2, H4, H6, and H7 were shifted to lower field by up to 0.4 ppm upon association of the ligand with the DNA, whereas the proton H8 is slightly shifted to higher field and H5 does not experience a significant shift (*cf.* ESI, Fig. S2[†]).

Discussion

The results from the spectrometric DNA titrations with ct DNA clearly indicate the binding interactions between duplex DNA and the aza- and azoniastilbene derivatives 2a-c; however, with significantly different affinity and binding mode. Thus, the association of the charge neutral derivative 2a with ct DNA is rather weak, as shown by the lack of a shift of absorption band during the photometric DNA titration and by the inefficient fluorescence quenching by DNA. Moreover, the positive LD signal of the DNA-bound ligand indicates groove binding. As the development and band structure of the ICD bands does not reveal a clear trend and depends strongly on the ligand concentration, it is assumed that this ligand forms only loosely bound aggregates along the DNA grooves whose actual structure depends on the ligand-DNA ratio. This assumption is supported by the observation that the development of absorption bands of 2a during titration is not characteristic of a DNA binder, but rather indicates the random association of the hardly soluble compound along the DNA backbone, as shown by the plain decrease of absorption band with no significant shift. Therefore the determined binding constant is rather an aggregation constant of the ligand. This undirectional binding mode of this ligand to DNA is explained by the lack of a positive charge, which is usually required for high affinity of DNA ligands. In contrast, the ligands 2b and 2c exhibit the characteristic spectroscopic features of DNA intercalators, specifically polycyclic azoniahetarenes,^{10c,21} upon complex formation²² namely a hypochromic effect and red shift of the absorption band, fluorescence quenching, the development of a weak positive or negative ICD band, typical binding constants (2b: $5.1 \times 10^4 \text{ M}^{-1}$, 2c: $2.0 \times 10^5 \text{ M}^{-1}$) and mostly indicative of the binding mode - a negative LD band in the ligand absorption range. It is tempting to conclude that the ligands 2b and 2c have different alignments in the intercalation site as the ICD signals develop with different phase. However, the sign of the ICD signals depends on the relative orientation of the transition dipole moments of the ligand and the DNA base pairs. Considering dipole moments of the ligands that are aligned along the long molecular axis in 2b and along the short molecular axis in 2c, as deduced from the substitution pattern, both ligands intercalate into DNA with the long molecule axis perpendicular to the long axis of the binding site.

In addition, it was demonstrated that the ligands 2b and 2c bind to quadruplex DNA also, as shown exemplarily with the two representative DNA forms 22AG and a2 as well as with the dye labeled quadruplex-forming oligonucleotides

F21T, FmycT, FkrasT and Fa2T. With regard to the association with quadruplex DNA, the azo- and azoniastilbene derivatives 2a-c show a similar trend of binding constants as with duplex DNA. It should be noted that the binding constants of the ligands with ct DNA and quadruplex DNA appear to be the same (e.g. for 2c: $K_{\rm B}^{\rm ctDNA} = 2.0 \times 10^5 \, {\rm M}^{-1}$ $K_{\rm B}^{22{\rm AG}} = 4.8 \times 10^5 \,{\rm M}^{-1}$), however; they were determined in solutions with different ionic strength (10 mM BPE buffer versus 95 mM K-phosphate buffer). Therefore, they cannot be directly compared because it is known that the binding constants, especially the ones of cationic ligands, decrease with increasing ionic strength. In fact, a control experiment showed that 2c has a significantly smaller binding constant with ct DNA of $K_{\rm B} = 6.7 \times 10^4 \, {\rm M}^{-1}$ at higher ionic strength (cf. ESI[†]). In general, with the increasing number of positive charges in the molecule the binding interaction with the DNA is getting stronger; however, this effect is more pronounced in the case of quadruplex DNA 22AG. Thus, the uncharged derivative 2a does not have a stabilizing effect on quadruplex DNA which indicates a very weak binding interaction. In contrast, the positively charged stilbene derivatives 2b and 2c induce a significant stabilization of the quadruplex DNA F21T, FmycT, FkrasT and Fa2T toward unfolding, and this effect is much stronger in the case of the dicationic derivative 2c than with the monocationic one. This difference may be explained by the effect of the positive charge, namely attractive Coulomb interactions with the phosphate backbone as well as thermodynamically favorable release of counter ions from the grooves and their subsequent solvation in the aqueous medium.⁹ The ligands 2b and 2c also bind to the ILPR DNA a2 as indicated by the spectrometric titrations and binding constants that are in the same range as the ones observed with 22AG. However, the stabilization of the ILPR quadruplex toward unfolding is not very pronounced according to the relatively low shifts of melting temperature of Fa2T upon ligand binding. This difference between binding constants and $\Delta T_{\rm m}$ values may be explained by the different buffer solutions that are used in each experiment.¹⁸ Moreover, even the rather moderate increase of the DNA melting temperature of Fa2T is indicative of ligand binding, because similar results were obtained with the well-established quadruplex ligands thiazole orange ($\Delta T_{\rm m} = 3.1$ °C), porphyrine **TMPyP4** ($\Delta T_{\rm m}$ = 6.3 °C) and coralyne ($\Delta T_{\rm m}$ = 1.8 °C) under identical conditions.¹⁸

In the case of ligand 2c, the association with the quadruplex-forming oligonucleotide **Tel6** as simple model was further confirmed by NMR-spectroscopic analysis. The broadening and significant upfield-shift of the imino protons of the guanine residues of the quadruplex usually indicate a terminal π -stacking of the ligand. Although it may be tempting to assume intercalation, this mode of binding is usually not favorable in quadruplex structures.²³ Moreover, the significant shifts of the ligand protons during complex formation may be explained by the π -stacking to a terminal quartet and the positioning of the ligand protons in the anisotropic cones of the guanine quartet.

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The initial goal of this study was the comparison of the DNA-binding properties of the stilbene derivatives 2a-c with the ones of the structurally resembling, quadruplex-binding dibenzochrysenes 1a-c. Specifically, it should be tested whether the increased structural flexibility of the stilbenes while maintaining a similar longitudinal extension of the π -system – increases the affinity of the former ligands to DNA. Indeed, it was observed that the structurally flexible diazoniastilbene 2c has a stabilizing effect on quadruplex DNA F21T $(\Delta T_{\rm m} = 11 \text{ °C}; \text{ LDR} = 5.0);$ but the diazoniabenzochrysene derivatives 1a-c have been shown to induce an even larger increase of the melting temperature of quadruplex DNA F21T under almost identical conditions ($\Delta T_{\rm m}$ = 6–19 °C, LDR = 5.0).^{10b} In the case of $1a^{10b}$ and 2c (Table 1), the thermal stabilization of the quadruplex is only marginally influenced by the presence of duplex DNA ds26, indicating a high selectivity of these ligands for quadruplex DNA relative to duplex. Nevertheless, this selectivity appears to be slightly more pronounced for the rigid diazoniadibenzochrysene 1a because the decrease of the melting temperature is smaller ($\Delta \Delta T_{\rm m}$ = -2.5 °C, LDR = 5.0) than the one observed with 2c ($\Delta\Delta T_{\rm m}$ = -3.1 °C). Moreover, the binding constant of 2c with quadruplex DNA 22AG is about half as large as the ones of the ligands **1a-c** (**2c**: $4.8 \times 10^5 \text{ M}^{-1}$ versus **1a-c**: $2.5-3 \times 10^6 \text{ M}^{-1 \ 10b}$). These observations imply that the gain in flexibility in ligand 2c as compared with the structurally rigid dibenzochrysenes 1a-c does not compensate the loss of overall π -surface. Thus, these results show in a direct comparison of two ligands with resembling extent and shape of the π system that at least under equilibrium conditions the π -stacking or dispersion interactions of a ligand contribute more to the affinity of a ligand to terminal binding sites of the quadruplex structure than its flexibility, although the latter would allow the ligand to adjust its conformation within the binding site in an induced-fit process. Although it may be obvious from literature data^{1,7} that both rigid and flexible ligands can bind to quadruplex DNA with reasonable affinity and selectivity, to the best of our knowledge there is only one direct and explicit comparison reported between two types of ligands with closely resembling ligand structures that mainly differ in terms of rigidity of the π system.¹² However, it should be emphasized that the latter study is not directly comparable with the results presented herein, because even the "rigid" ligands contain flexible substituents that may influence the overall binding affinity to G4-DNA. Complementary to those findings, our results could be helpful for ligand design, because we present the counterintuitive observation that the affinity and selectivity of a given flexible ligand may be even increased by the introduction of more rigidity. This small but significant effect may be explained by thermodynamic factors, because the rigid ligand does not loose conformational freedom, thus causing no additional entropic penalty, upon transfer from the solution to the constrained binding site.

Notably, the differences of the ligand-induced shifts of quadruplex melting temperatures on addition of **2b** and **2c** $(\Delta \Delta T_{\rm m} = 7 \text{ °C} \text{ (F21T)} \text{ and } \Delta \Delta T_{\rm m} = 3 \text{ °C} \text{ (Fa2T)} \text{ at LDR} = 5)$

appear to be too large to be solely caused by the different charges and may need further attention. Considering the known, but still rather unexplored "methyl effect" of DNA ligands, the increased affinity of 2c may also be caused by its methyl substituents, i.e. supported by additional dispersion interactions between the methyl groups and the hydrophobic binding site.²⁴ To assess whether this methyl effect also affects the quadruplex-stabilizing properties of the diazoniadibenzo [b,k]chrysene scaffold and to have a better comparison with 2c, the dimethyl-substituted derivative 1d was also investigated in this study as it resembles the methyl-substitution pattern of 2c. Moreover, it was assumed that due to its close structural resemblance with the parent compounds 1a-c the derivative **1d** also binds to quadruplex DNA through terminal π stacking. In fact, the methyl-substituted derivative 1d also induces a much better stabilization of the quadruplex DNA F21T toward thermal unfolding as the parent compounds 1a-c ($\Delta T_{\rm m}$ = 27 °C; LDR = 5.0), although with slightly smaller binding constant. Unfortunately, the bad solubility of some 1d-DNA complexes hampered its complete investigation. But at least the obtained results are in agreement with the ones with ligand 2c, which indicates that a methyl effect may operate in quadruplex ligands, presumably based on attractive dispersion interactions of the methyl substituents with the hydrophobic binding site. Nevertheless, this assumption has to be verified in a systematic study with a larger series of methyl-substituted ligand derivatives.

Conclusions

We have shown that aza- and azoniastilbene derivatives 2a-c, i.e. compounds with almost the same spatial dimensions and steric demand, bind to DNA with an affinity and selectivity that depends significantly on the number of positive charges. Whereas the charge neutral derivative 2a only binds nonspecifically to the DNA backbone of duplex DNA, the ionic compounds 2b and 2c are typical DNA intercalators. Most notably, the bis-quinolinium derivative 2c binds to quadruplex DNA with moderate affinity and also induces a pronounced stabilization of the quadruplex DNA towards thermal denaturation, presumably caused by an additional methyl effect. In contrast to the proposed properties of the stilbene derivatives 2b and 2c, these ligands have a significantly weaker stabilizing effect on quadruplex DNA than the dibenzochrysene derivatives 1a-d. From this observation we carefully conclude that the increased flexibility of a quadruplex-DNA ligand does not lead to stronger interactions with the quadruplex DNA as compared with rigid ligands that have essentially the same size and extent of π -system. Certainly, this finding has to be further substantiated with a larger series of ligands and quadruplex forms; however, the present study already reveals that structural rigidity and lack of conformational freedom, as in the diazoniadibenzochrysene series, is not a disadvantage with respect to quadruplex-DNA binding.

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Experimental

Equipment

Melting points were determined with a BÜCHI 545 (Büchi, Flawil, CH) and are uncorrected. NMR spectra were recorded on a Bruker AV 400 (¹H: 400 MHz and ¹³C: 100 MHz) or on a Varian VNMR-S 600 (¹H: 600 MHz, ¹³C: 150 MHz) at 20 °C; chemical shifts are given in ppm (δ) values relative to tetramethylsilane (TMS) as internal standard ($\delta = 0.0$). Combustion analyses were carried out by Mr Rochus Breuer (Organic Chemistry I, University of Siegen). Mass spectra were recorded on a Finnigan LCQ deca (driving voltage: 6 kV, impingement gas: argon, capillary temperature: 200 °C, auxiliary gas: nitrogen). Photometric titrations were recorded with a Varian Cary 100 Bio. A Varian Cary Eclipse spectrometer was used for the spectrofluorimetric analyses. The CD and *flow*-LD measurements were performed on a Chirascan spectrometer (Applied Photophysics).

Materials

Commercially purchased reagents were used without further purification. Chemicals were obtained from Alfa Aesar GmbH & Co. KG, Karlsruhe, Germany (N-bromosuccinimide, 3-bromoquinoline, ethenyltriethylsilane), Acros Organics [n-butyllithium solution (2.5 M in hexane), 2-butenoic acid, 4-pyridinecarbonitrile]. 1,5-di(bromomethyl)naphthalene $(5)^{11}$ 2-(2methyl-(1,3)-dioxolan-2-yl)pyridine (6),¹³ 2-methylquinolizinium bromide $(3)^{25}$ and (E)-1,2-di(quinolin-3-yl)ethane $(2a)^{15}$ were prepared according to literature protocols. The oligonucleotides fluo-d(G₃T₂AG₃T₂AG₃T₂AG₃)-tamra (F21T, fluo = fluoresceine; tamra = tetramethylrhodamine), FmycT [fluo-d(TGAG₃TG₃TAG₃TG₃TA₂)-tamra], FkrasT [fluo-d (AG₃CG₂TGTG₃A₂GAG₂A)-tamra], Fa2T fluo-d(ACAG₄TGTG₄)₂tamra, $d(AG_3T_2AG_3T_2AG_3T_2AG_3)$ (22AG), a2 $d(ACAG_4TGTG_4)_2$ and $d(T_2AG_3)$ (Tel6) were purchased from Metabion or Biomers and ct DNA was purchased from Merck and used without any further purification. DNA solutions in buffer were prepared according to published procedures;²⁶ BPE buffer (pH 7.0): 6.0 mM Na₂HPO₄, 2.0 mM NaH₂PO₄, 1.0 mM Na₂EDTA; Na-cacodylate buffer (pH 7.2-7.3): 10 mM Na(CH₃)₂AsO₂·3 H₂O, 10 mM KCl, 90 mM LiCl; K-phosphate buffer (pH 7.0): 25 mM K₂HPO₄, 70 mM KCl.

Methods

The spectrophotometric, spectrofluorimetric and CD-spectroscopic titrations with DNA, and the thermal DNA denaturation studies were performed according to reported protocols (*cf.* ESI†).^{10*a,b*} To ensure a sufficient solubility of the ligands during the titrations, all experiments were performed with 5% v/v DMSO as cosolvent. Binding constants of ligand–DNA complexes were determined by fitting of the experimental data from the photometric and fluorimetric DNA titrations to the theoretical model according to the published procedure (*cf.* ESI†).^{10*a*,27}

Synthesis of 1,5-bis{[1-(2-methyl-1,3-dioxolan-2 yl)pyridinium]methyl}naphtalenebis(tetrafluoroborate) (7). A solution

of 1,5-di(bromomethyl)naphthalene (5)¹¹ (0.75 g, 2.39 mmol) and 2-(2-methyl-(1,3)-dioxolan-2-yl)pyridine $(6)^{13}$ (1.09 g, 6.60 mmol) in DMSO (15 mL) were stirred at r.t. for 10 d. The vellow brown solution was added into EtOAc (100 mL). The precipitated crude solid was filtered and washed with EtOAc and Et₂O. The solid was recrystalized from MeOH (0.5 mL, with added HBF₄) to obtain product 7 (600 mg, 0.92 mmol, 36%); mp = 252–254 °C (dec.). – ¹H NMR (400 MHz, DMSO- d_6): $\delta = 1.84$ (6 H, s, CH₃), 3.73–3.76 (4 H, m, CH₂), 4.02–4.08 (4 H, m, CH₂), 6.25 (4 H, s, CH₂N⁺), 7.43 (1 H, d, ${}^{3}J$ = 7 Hz, CH), 7.63 $(2 \text{ H}, \text{ dd}, {}^{3}J = 8 \text{ Hz}, \text{CH}), 7.99 (2 \text{ H}, \text{ d}, {}^{3}J = 9 \text{ Hz}, \text{CH}), 8.20 (2 \text{ H}, \text{ d})$ ddd, ${}^{3}J = 8$ Hz, ${}^{3}J = 6$ Hz, ${}^{4}J = 1$ Hz, CH), 8.39 (2 H, d, ${}^{3}J = 8$ Hz, CH), 8.74 (2 H, dd, ${}^{3}J = 9$ Hz, ${}^{3}J = 8$ Hz, CH), 9.05 (2 H, d, ${}^{3}J =$ 6 Hz, CH). - ¹³C-NMR (DMSO-*d*₆, 100 MHz): 26.0, 61.1, 65.3, 106.1, 109.1, 125.5, 125.7, 126.9, 128.3, 128.9, 132.2, 133.9, 147.6, 148.8, 156.3. – El. anal. for $C_{30}H_{32}N_2B_2F_8O_4 \times H_2O_3$ (676.21) calcd (%): C 53.29, H 5.07, N 4.14, found: C 52.93; H 4.13; N 4.21.

Synthesis of 4a,12a-diazonia-8,16-dimethyldibenzo[b,k]chrysenebis(tetrafluoroborate) (1d). The bispyridinium 7 (295 mg, 0.45 mmol) was stirred in PPA (3.06 g) at 150 °C for 24 h. The mixture was cooled to 100 °C and saturated aqueous solution of NaBF₄ (10 mL) was added. After cooling slowly to r.t. the aquesous solution was extracted with MeNO2. The organic layer was separated, dried with Na2SO4, and the solvent was evaporated to obtain the product 1d as yellow needles (179 mg, 0.34 mmol, 75%); mp >300 °C. – ¹H NMR (400 MHz, DMF- d_7): δ = 3.49 (6 H, s, CH₃), 8.34 (2 H, dd, ³J = 7 Hz, CH), 8.49 (2 H, dd, ${}^{3}J$ = 8 Hz, CH), 9.14 (2 H, d, ${}^{3}J$ = 9 Hz, CH), 9.17 (2 H, d, ${}^{3}J$ = 9.4 Hz, CH), 9.69 (2 H, d, ${}^{3}J$ = 9 Hz, CH), 9.77 (2 H, d, ${}^{3}J$ = 7 Hz, CH), 11.62 (2 H, s, CH). - ¹³C-NMR (DMF-*d*₇, 100 MHz): $\delta = 13.4 (CH_3), 122.8 (Cq), 123.0 (CH), 124.1 (CH), 125.5 (CH),$ 128.2 (CH), 128.8 (Cq), 132.4 (Cq), 132.9 (CH), 133.9 (Cq), 135.8 (CH). - El. anal. for C₂₆H₂₀N₂B₂F₈·2 H₂O (563.33) calcd (%): C 54.78, H 4.24, N 4.91; found C 55.37, H 4.01, N 5.44.

Synthesis of (E)-2-(2-(naphthalene-2-yl)vinyl)quinolizinium bromide (2b). A solution of 2-methylquinolizinium bromide $(3)^{25}$ (448 mg, 2.00 mmol), 2-naphthaldehyde (4) (469 mg, 3.00 mmol) and piperidine (0.20 mL) in MeOH (5 mL) was stirred under reflux for 5 h. The reaction mixture was cooled to r.t. and filtered. The filter cake was washed with cold MeOH and then with Et₂O. The obtained orange-colored solid was recrystalized from MeOH to give the product 2b (450 mg, 1.24 mmol, 62%) as orange crystals (note: due to its photoreactivity this compound should be handled in the dark); mp = 272–274 °C (dec.). ¹H NMR (600 MHz, DMSO- d_6): δ = 7.55–7.60 $(2 \text{ H}, \text{ m}, 1'-\text{H}, 2'-\text{H}), 7.70 (1 \text{ H}, \text{d}, {}^{3}J = 16 \text{ Hz}, 4'-\text{H}), 7.92-8.04$ (5 H, m, 3-H, 9-H, 7'-H, 8'-H, 9'-H), 8.12 (1 H, d, ${}^{3}J$ = 16 Hz, 5'-H), 8.19 (1 H, s, 10'-H), 8.31 (1 H, t, ³J = 8 Hz, 8-H), 8.43–8.50 $(2 \text{ H}, \text{ m}, 7\text{-H}, 6'\text{-H}), 8.60 (1 \text{ H}, \text{ s}, 1\text{-H}), 9.26 (1 \text{ H}, \text{ d}, {}^{3}J = 8 \text{ Hz},$ 4-H), 9.33 (1 H, d, ${}^{3}J$ = 7 Hz, 6-H). - ${}^{13}C$ NMR (150 MHz, DMSO- d_6): δ = 120.3, 122.8, 123.0, 123.6, 124.3, 126.9, 126.9, 127.3, 127.7, 128.4, 128.7, 129.1, 133.0, 133.1, 133.5, 136.6, 136.9, 138.4, 142.8, 144.9. – MS (ESI+): *m/z* (rel. intensity) = 282 (100) $[M^+]$. – El. anal. for C₂₁H₁₆BrN × H₂O (368.28) calcd (%): C 66.33, H 4.77, N 3.68, found: C 66.41; H 4.71; N 3.64.

Synthesis of (E)-3,3'-(ethane-1,2-diyl)bis(1-methylquinolin-1ium) (2c). In a 200 mL sealed tube, a mixture of $2a^{15}$ (141 mg, 0.50 mmol) in MeI (5.0 mL) was stirred at 140 °C for 5 h. The reaction mixture was cooled to r.t. and Et₂O (30 mL) was added. The yellow precipitate was filtered and washed with Et₂O. The remaining solid was dissolved in a small amount of MeOH (100 ml) and the solid was passed through a bromidesaturated ion-exchange column (DOWEX $@1 \times 8$) three times. The solvent was evaporated and the solid was re-crystalized from MeOH/MeCN to obtain product 2c as a vellow solid (note: due to its photoreactivity this compound was handled in the dark); mp = 292–294 °C. ¹H NMR (600 MHz, DMSO- d_6): δ = 4.71 (6 H, s, $2 \times CH_3$), 7.94 (2 H, s, 1'-H, 2'-H), 8.11 (2 H, t, ${}^{3}J =$ 8 Hz, 6-H, 6"-H), 8.28-8.32 (2 H, m, 7-H, 7"-H), 8.53 (2 H, d, ${}^{3}J = 8$ Hz, 5-H, 5"-H), 8.55 (2 H, d, ${}^{3}J = 9$ Hz, 8-H, 8"-H), 9.40 (2 H, s, 4-H, 4"-H), 9.92 (2 H, s, 2-H, 2"-H). - ¹³C NMR (150 MHz, DMSO- d_6): $\delta = 45.8$, 119.3, 127.8, 129.1, 129.9, 130.5, 130.6, 135.5, 137.5, 142.9, 149.2. - El. anal. for C₂₂H₂₀Br₂·0.5 H₂O (508.25) calcd (%): C 51.99, H 4.76, N 5.51, found: C 52.28; H 4.78; N 5.55.

Conflicts of interest

There are no conflicts to declare.

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