Cationic side-chains control DNA/RNA binding properties and antiproliferative activity of dicationic dibenzotetraaza[14]annulene derivatives.

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Abstract

Studied dicationic dibenzotetraaza[14]annulene derivatives intercalate into synthetic double stranded DNA and RNA, while their positively charged side-chains additionally interact within the minor groove of polynucleotides, contributing to the overall affinity of compounds and controlling pronounced A-T(U) over G-C sequence preference as well as stronger thermal stabilization of ds-DNA than ds-RNA. Furthermore, all compounds showed moderate to high antiproliferative activity against five human tumour cell lines, whereby clear correlation between structure of the side-chain and cytotoxic activity was observed.

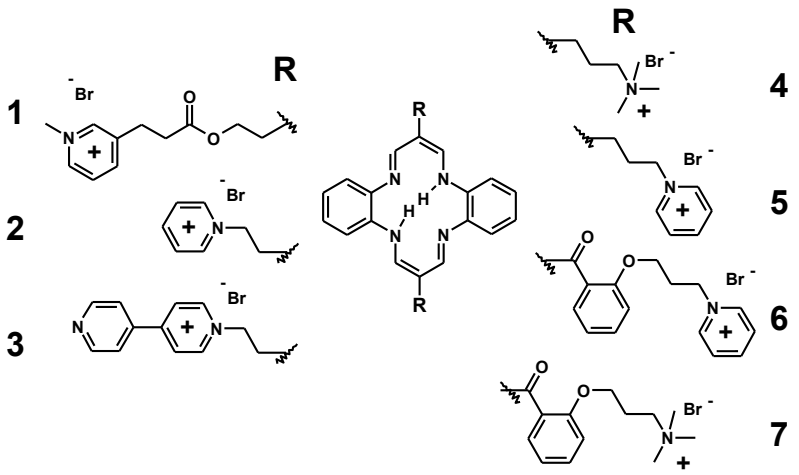
Keywords: Dibenzotetraaza[14]annulene; DNA and RNA polynucleotides; spectrophotometric titrations; antiproliferative activity, tumour cell lines

1. Introduction

Nucleic acids, particularly DNA, but also on a growing scale RNA, are prime targets for several major categories of drugs in the areas of infections and cancer.[1] Although general patterns of recognition are now appreciated, subtle structural features important to the DNA binding affinity or selectivity and the ensuing effects are still being unravelled.[2] In spite of the advances, the de novo design of sequence-selective DNA binding agents is not yet straightforward, and the derivation of therapeutic compounds (e.g., antitumor drugs) remains an even more complex task. Therefore, search for a new lead small molecule either from the natural sources or based on up-till-now untapped small organic molecules is of the utmost

interest, whereby application of numerous methods and approaches is necessary for accurate determination of binding modes and affinity.[3]

Cationic porphyrins are promising and intensively studied class of DNA-binding molecules with potential applications in biology and medicine, in particular, as potent anti-viral and antitumor therapeutic agents.[4] As DNA binding ligands, porphyrins are quite unusual; they may associate with DNA in three distinct binding modes, which include intercalation, groove binding, and outside binding with self-stacking along the DNA helix.[5] However, surprisingly little is known about DNA binding properties and biological activity of close analogues of porphyrins - dibenzotetraaza[14]annulenes (DBTAA), although the structure, rather simple synthesis and modification procedures, along with metal cation binding ability clearly reveal the biological potential of DBTAA derivatives. Therefore, recently we have reported on synthesis, crystal structures, DNA/RNA binding and antiproliferative activity of a series of bis-cationic DBTAA derivatives (**1**-**3**).[6,7] Antiproliferative effect of **1**-**3** on human tumor and normal cell lines was in a good agreement with the strength of observed interactions of **1**-**3** with DNA/RNA, whereby **2** revealed the most interesting properties. Based on the structure of **2**, new series of compounds was prepared (**4**-**7**), with the idea that fine tuning of the length, rigidity and positive charge exposure of cationic substituents attached to DBTAA core could lead to novel DNA/RNA binding properties and eventually to increased antiproliferative activity. Very recent preliminary results revealed improved DNA binding properties of **4**-**7**[8] in comparison with first generation of cationic DBTAA derivatives **1**-**3**.[7] Here we present detailed study of **4**-**7** interactions with synthetic DNA and RNA as well as the screening of their antiproliferative activity.



Scheme 1. Cationic, DNA/RNA active DBTAA derivatives: the first (**1**-**3**)[7] and second (**4**-**7**)[8] generation.

2. Results and Discussion

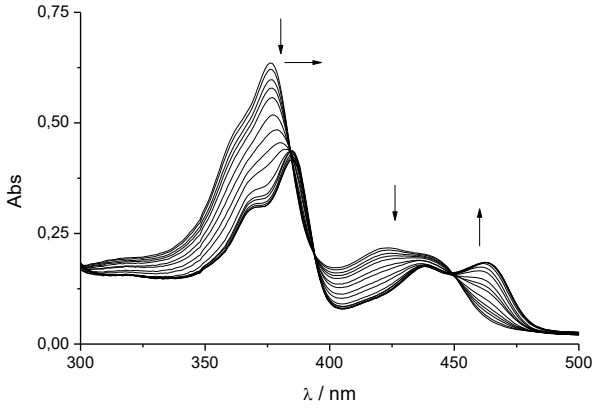
*2.1. Spectrophotometric titrations of **4** - **7** with ds-DNA and ds-RNA in aqueous medium*

Compounds **4**, **5**, **6** and **7** were previously characterized in aqueous medium by several spectrophotometric methods and showed to be stable in biologically relevant conditions.[8] Moreover, preliminary experiments showed that **4**, **5**, **6**, **7** interact strongly with ds-DNA.[8] Here presented studies with synthetic ds-DNA and ds-RNA sequences revealed that addition of any ds- polynucleotide resulted in strong batochromic and hypochromic effects of UV/Vis spectra of studied compounds (Figure 1, spectral changes are summarized in Table 1), which are in general not significantly dependent on the basepair composition of the polynucleotide and do not distinguish between DNA and RNA. It is noteworthy that isosbestic points are observed in the most UV/Vis titrations in the region where only the studied compounds absorb light (λ>300 nm), pointing to the formation of one dominant type of complex.

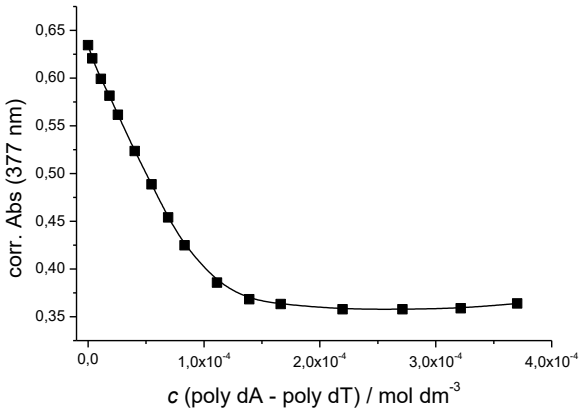
Table 1. Spectroscopic changes of the UV/Vis spectra of **4 - 7** observed in titrations with ds-polynucleotides (pH = 7.0, buffer sodium cacodylate, I = 0.05 mol dm^{-3}).

	poly dA - poly dT			poly A - poly U			poly G - poly C		
compound	[b]H / %	[a] $\Delta\lambda$ / nm		[b]H / %	[a] $\Delta\lambda$ / nm		[b]H / %	[a] $\Delta\lambda$ / nm	
		$\Delta\lambda_1$	$\Delta\lambda_2$		$\Delta\lambda_1$	$\Delta\lambda_2$		$\Delta\lambda_1$	$\Delta\lambda_2$
4	44	8	15	42	10	14	31	2	10
5	27	6	5	37	4	6	40	5	4
6	26	4	-	21	11	-	39	-10	-
7	29	6	-	23	13	-	43	-10	-

[a]$\Delta\lambda$ = λ(**4**, **5, 6** and **7**) - λ(complex);Absorbance maxima λ_1 ($4_{\lambda 377\ nm}$, $5_{\lambda 385\ nm}$, $6_{\lambda 346\ nm}$, $7_{\lambda 344\ nm}$) λ_2 = ($4_{\lambda 424\ nm}$, $5_{\lambda 438\ nm}$); [b]Hypochromic effect calculated by Scatchard for **4, 5, 6** and **7**; H=(Abs(**4**, **5, 6** and **7**) - Abs(complex)) / Abs(**4**, **5, 6** and **7**)) x 100;



a)



b)

Figure 1. a) Changes in UV/Vis spectrum of **4** (c= 1.53 × 10^{-5} mol dm^{-3}) upon titration with poly dA – poly dT; b) Agreement between UV/Vis titration data (λ_{max} = 377 nm) of **4** with poly dA – poly dT (■) and calculated data by non-linear fitting to Scatchard equation (▬), pH=7, sodium cacodylate buffer, I = 0.05 mol dm^{-3}.

The binding constants *Ks* and ratios $n_{[\text{bound compound}]/\ [\text{DNA/RNA}]}$ obtained by processing of UV/Vis titration data with Scatchard equation[9] are summarized in Table 2. In general, **4 - 7** showed similar affinity toward ds- DNA and ds-RNA. The **4** and **5** bind to poly A - poly U somewhat stronger than to poly G - poly C and in line with that is somewhat higher affinity of these compounds toward poly dA - poly dT than toward ct-DNA which contains significant percentage of dG-dC basepairs. The **6** and **7** also show pronounced poly A - poly U over poly G - poly C preference, which is not mirrored to the dA - poly dT over ct-DNA preference. In addition, **6** binds significantly stronger to ct-DNA, poly dA - poly dT and poly A - poly U in comparison to other studied compounds.

Table 2. Binding constants (log*Ks*)[a,b] and ratios *n* ([bound compound]/ [polynucleotide phosphate]) calculated from the UV/Vis titrations of **4, 5, 6, 7** with ds- polynucleotides at pH = 7.0 (buffer sodium cacodylate, $I = 0.05$ mol dm^{-3}).

	poly dA - poly dT		poly A - poly U		poly G - poly C	
	log*Ks*	*n*	log*Ks*	*n*	log*Ks*	*n*
4	6.41	0.15	6.36	0.17	5.32	0.10
5	6.58	0.14	6.88	0.27	4.85	0.41
6	7.21	0.24	7.85	0.27	5.48	0.25
7	6.52	0.22	6.59	0.32	5.24	0.30

[a] Accuracy of $n \pm 10$ - 30%, consequently log*Ks* values vary in the same order of magnitude, [b]Titration data were processed according to the Scatchard equation.[9].

2.2. Thermal denaturation experiments

It is well known that upon heating ds- helices of polynucleotides at well-defined temperature (Tm value) dissociate into two single stranded polynucleotides. Non-covalent binding of small molecules to ds-polynucleotides usually has certain effect on the thermal stability of helices thus giving different T_m values. Difference between ΔT_m value of free polynucleotide and complex with small molecule (ΔT_m value) is important factor in characterisation of small molecule / ds-polynucleotide interactions.

The addition of any of studied compounds strongly stabilised double helices of both, DNA and RNA (Figure 2, Table 3). A more detailed study of thermal stabilisation effects revealed strongly nonlinear dependence of ΔT_m values on the ratio ***r***, suggesting saturation of binding sites at $\boldsymbol{r} = 0.2 - 0.3$, which again is in good accord with calculated values of Scatchard ratio ***n*** (Table 2). All compounds stabilised significantly stronger poly dA - poly dT than its RNA analogue poly A - poly U. Intriguingly, thermal stabilisation of ct-DNA by **4 - 7** is 2-10 times weaker than stabilisation of poly dA - poly dT, and for compounds **4, 5** and **7** it is even weaker than stabilisation of poly A - poly U. Such poly dA - poly dT over ct-DNA preference agrees roughly with correlations between corresponding binding constants (Table 2), and again points toward weaker binding of all studied compounds to G-C sequences.

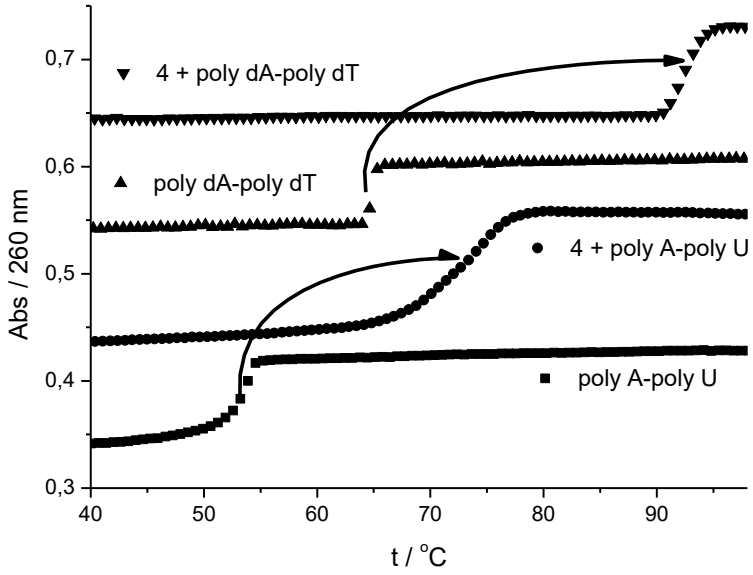


Figure 2. Thermal denaturation of poly dA - poly dT and poly A - poly U upon addition of **4**. Ratio $r_{[compound] / [polynucleotide]}$ = 0.3, pH = 7.0 (buffer sodium cacodylate, I = 0.05 mol dm^{-3}).

Table 3. The [a]ΔT_m values (°C) of studied ds- polynucleotides upon addition of of **4 - 7** at pH = 7.0 (buffer sodium cacodylate, I = 0.05 mol dm^{-3}), $r_{[compound] / [polynucleotide]}$ = 0.3.

	[c]ct-DNA	poly dA - poly dT	poly A - poly U
4	6.6	27.6	20.5
5	3.1	[b]>36	11.5
6	12.8	28.1	12.2
7	10.2	23.5	12.2

[a] Error in ΔT_m: ± 0.5°C;[10] [b]ΔT_m not possible to calculate since T_m is over 100°C; [c] Published results.[6]

2.3. Ethidium bromide displacement experiments

As an alternative method for comparison of ability of studied molecules to compete for binding with classical intercalator already bound to DNA/RNA,[11] we have performed ethidium bromide (**EB**) displacement assays (Figure 3). Compounds **4** – **7** do not interact with **EB** under experimental conditions used. According to DC_{50} values (Figure 3A) **4** - **7** reveal comparable

affinity toward poly dA – poly dT, while DC_{50} values on Figure 3B suggest that **6** and **7** bind somewhat stronger to poly A – poly U than **4** and **5**.

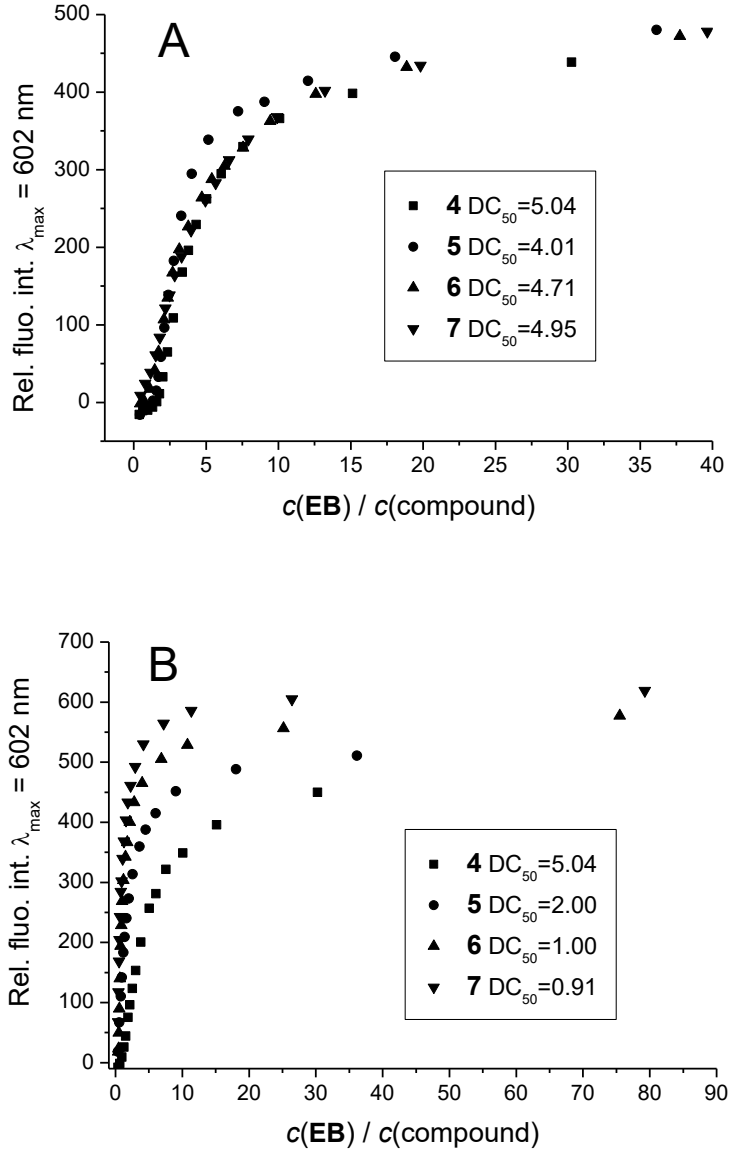


Figure 3. Ethidium bromide (**EB**) displacement assay: poly dA – poly dT (**A**); poly A – poly U (**B**). To polynucleotide solution (c= 5 × 10^{-5} mol dm^{-3}) ethidium bromide (c= 1.5 × 10^{-5} mol dm^{-3}) was added (***r*** ([**EB**]/ [polynucleotide]=0.3), and quenching of the **EB**/ polynucleotide complex fluorescence emission (λ_{ex}=520 nm, λ_{em}=602 nm) was monitored as function of c(**EB**)/c(compound). The given DC_{50} values present the ratio c(**EB**)/c(compound) = [Int(**EB**/polynucleotide) – Int($\mathbf{EB}_{free}$)] / 2, where Int(**EB**/ polynucleotide) is fluorescence intensity of **EB**/ polynucleotide complex and Int($\mathbf{EB}_{free}$) is fluorescence intensity of the free ethidium bromide before polynucleotide is added.

2.4. Circular dichroism (CD) experiments

So far, non-covalent interactions at 25 ^{0}C were studied by monitoring the spectroscopic properties of a studied compound upon the addition of the polynucleotides. In order to get insight into the changes of polynucleotide properties induced by small molecule binding, we have chosen CD spectroscopy as a highly sensitive method toward conformational changes in the secondary structure of polynucleotides.[12] In addition, achiral small molecules can eventually acquire induced CD spectrum (ICD) upon binding to polynucleotides, which could give useful information about modes of interaction.[12] It should be noted that compounds **4** - **7** do not possess intrinsic CD spectrum.

The addition of **4** - **7** resulted in a decrease of CD spectra of DNA/RNA polynucleotides (Figure 4). Additionally, a strong induced CD (ICD) band in the range λ=300 - 500 nm appeared. Since UV/vis spectra of all studied compounds in the corresponding range are attributed to the absorption of DBTAA moiety and compounds do not exhibit intrinsic CD spectra, the observed ICD bands can also be attributed to DBTAA moiety. Additionally, isoelliptic points in the ICD band range (λ=300 - 500 nm) observed for all combinations of compounds and studied DNA/RNA strongly suggest one dominant interaction mode.[13] It is interesting to note that ICD spectra of **4** - **7** upon mixing with DNA (Figure 4) are of similar shape as those induced by addition of RNA, differing only in intensity and resolution of maxima and minima (Figure 4). Such resemblance between DNA and RNA induced CD spectra of all compounds is suggesting analogous orientation of DBTAA moiety with respect to DNA/RNA chiral axis and thus most likely same mode of binding.[12,14]

Strongly pronounced non-linear dependence of changes in CD spectra on the ratio ***r*** is pointing toward saturation of dominant binding sites at about r = 0.2 – 0.3. These ***r*** values are again in a good agreement with the ratios ***n*** obtained in UV/vis titrations (Table 2) as well as with the non-linear dependence of ΔT_m values on the ratio ***r*** in thermal melting experiments.

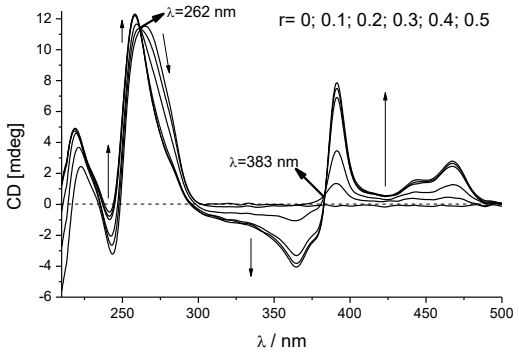
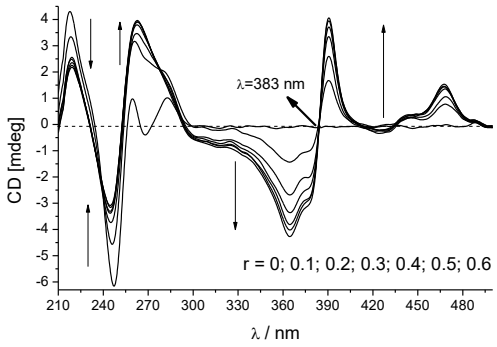
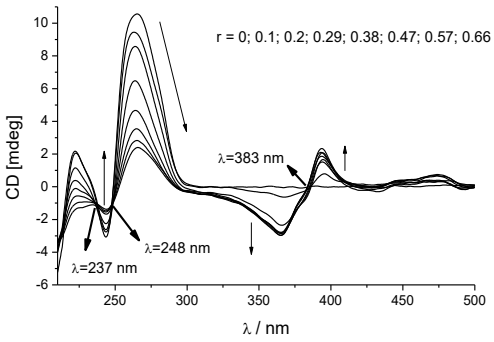
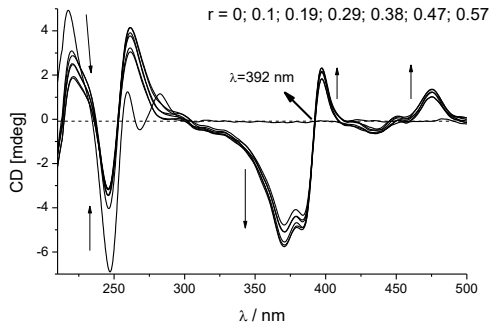
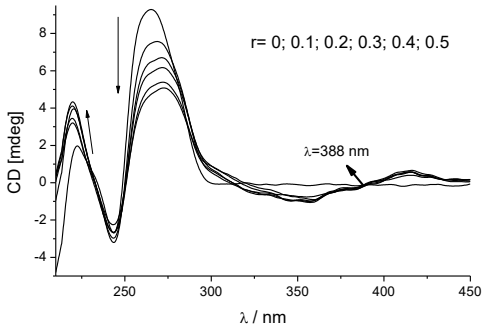
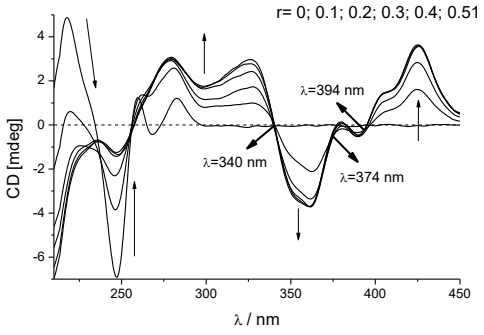
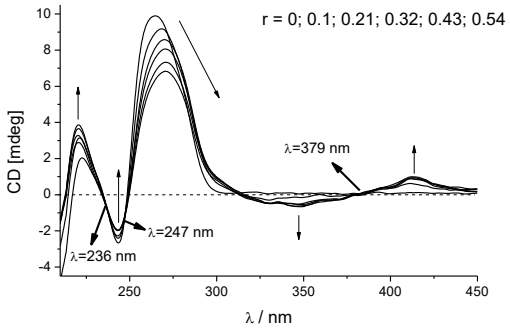
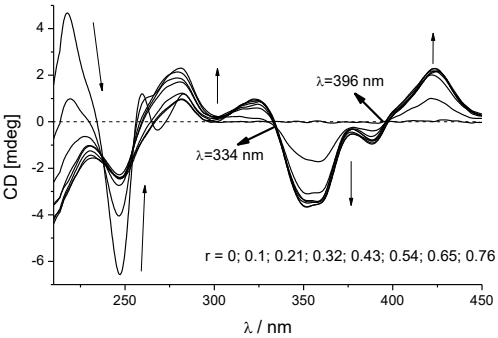
	poly A – poly U	poly dA – poly dT
4		
5		
6		
7		

Figure 4. CD titration of polynucleotides (c = 3.0 × 10^{-5} mol dm^{-3}) with **4, 5, 6** and **7** at molar ratios ***r*** = [compound] / [polynucleotide] (pH = 7.0, buffer sodium cacodylate, I = 0.05 mol dm^{-3}).

Intriguingly, ICD spectra of all compounds and polynucleotides are partially negative (300-380 nm) and partially positive (380 – 500 nm). According to the previously reported experiments and theoretical studies an intercalated chromophore centered near the helix axis of double stranded polynucleotide should exhibit negative induced CD for all long-wavelength transitions polarized parallel to the long axis of the base-pair pocket, while transitions perpendicular to this direction, but still in the plane of the nucleobases (i.e., parallel to the pseudo-dyad axis), should give positive CD.[15,16] Similar negative/positive ICD spectra were observed upon intercalation of 2,7-diazapyrene and its cations into various ds-DNA.[17] We can speculate that bulky side-chains of **5** and **7** are not likely to allow threading intercalation[18] with one of the side-chains positioned in the minor groove and the other in the major groove of double helix. In addition, fast equilibrium upon each mixing of DNA/RNA with compounds (less than 2 min) does not match threading intercalator association rates, which are characteristically much slower.[18] Other possible orientation, with both side-chains positioned in the same polynucleotide groove, would dictate orientation of the in-plane symmetry axis of DBTAA moiety connecting these two side-chains parallel to the long axis of the base-pair pocket and therefore would give negative ICD band (300-380 nm), while transition within DBTAA moiety perpendicular to this direction, but still in the plane of the nucleobases, should give positive ICD band (380-500 nm). However, to provide accurate evidence for the proposed orientation of DBTAA moiety within intercalation binding site, additional LD and theoretical studies are needed,[15,16] whose complexity exceeds the scopes of this work.

2.5. Viscometry measurements

The increase in DNA contour length that accompany an intercalative mode of binding is most conveniently monitored by measuring the viscosity of sonicated rodlike fragments of DNA as a function of ligand binding ratio, r. Cohen an Eisenberg have deduced that the relative increase in contour length in the presence of bound drug is approximated by the cube root of the ratio of the intrinsic viscosity of the DNA-drug complex to that of the free DNA (equation in Supplementary material).[19] Classical monointercalators like ethidium bromide, proflavine and 9-aminoacridine have values of helix extension parameter (viscosity index), α of about 0.8-0.9 while extension parameters of bisintercalators are usually in the range 1.5-1.9. On the other hand, molecules like groove binders which do not insert between base pairs, in general do not elongate DNA double helix and therefore yield no or very small viscosity increase.

Viscometry experiments (Supplementary Material) performed with ct-DNA yielded values of $\alpha = 0.93 \pm 0.03$ (**4**); 1.08 ± 0.04 (**6**); 0.96 ± 0.1 (**7**), which agree well with the value obtained for ethidium bromide (α(**EB**) = 0.84 ± 0.05). Experiment with **5** was hampered by precipitation upon first addition of compound to ct-DNA. Obtained values strongly support intercalation of studied compounds into ds-DNA as the dominant binding mode.[20]

2.6. Discussion of DNA/RNA binding studies

According to the results of all applied methods, studied **4, 5, 6** and **7** strongly bind to both, ds-DNA and ds-RNA, most likely by the same mode of interaction. Strong hypochromic and batochromic effect in UV/vis titrations (Table 1), high affinity (Table 2), strong thermal stabilisation of both, DNA and RNA (Table 3), similar ICD spectra upon DNA and RNA addition, strongly support intercalation as a dominant binding mode for all studied compounds and polynucleotides.[20]

Binding constants (Table 2) and thermal denaturation studies (Table 3) point toward significantly stronger interactions of **4 - 7** with A-T(U) sequences in comparison to G-C sequences. Most of classical intercalators do not differ between A-T(U) sequences and G-C sequences or in some cases show weak preference toward G-C sequences. One of the major differences between A-T(U) and G-C sequences is amino group of guanine protruding into the minor groove of double helix and sterically hindering non-covalent interactions of small molecules like e.g. minor groove binders. Most likely interactions of positively charged side-chains of studied compounds within the minor groove are analogously affected, thus resulting in observed A-T(U) over G-C preference. Furthermore, stronger thermal stabilization of poly dA-poly dT in comparison to RNA analogue (poly A – poly U) can be attributed to the much narrower and hydrophobic minor groove of the ds-DNA in respect to ds-RNA.[21] That observation points out that side chains of **4, 5, 6** and **7** form additional binding interactions only within minor groove of ds-DNA and moreover these interactions are suppressed by the presence of G-C basepairs (therefore G-C basepair containing ct-DNA was stabilized less than RNA poly A – poly U).

*2.7. Evaluation of the antiproliferative effect of **4, 5, 6** and 7 in vitro*

Many of the currently used antitumor drugs base their activity on the intercalation or minor groove binding to cellular DNA. Since **4 - 7** intercalate into DNA and form additional binding interactions by side-chains within minor groove of ds-DNA, we investigated their effects on

proliferation of different human tumor cell lines. The obtained results (Table 4) clearly point to the correlation between antiproliferative activity and a structure of a side-chain attached to DBTAA moiety. Namely, **4** and **7** characterized by aliphatic, flexible side-chains are by far less active against **HCT 116**, **SW 620**, **H 460** cell lines than **5** and **6** possessing aromatic, sterically demanding side-chains. The exception is selectivity of **4** and **7** toward **PC-3**, **MCF-7** and a non-tumor cell line **HaCaT**, which is quite intriguing. On the other hand, **5** and **6** exhibited similar activity toward all cell lines. Different characteristics of aromatic positive charge vs. aliphatic positive charge (e.g. hydrophobicity-related cellular uptake, or different intercellular targets/pathways) can be responsible for observed biological effects. Namely, the act of intercalation *per se* induces local structural changes (e.g. unwinding of the double helix and lengthening of the DNA strand) to the DNA, which leads to the inhibition of transcription and replication.[22] However, the presence of a cationic substituent on the molecule may increase DNA residence time which, in turn, may increase the (geno)toxicity of that compound. If the intercalation brings an electrophilic center in proximity to the DNA, a covalent bond (DNA adduct) may form. Such intercalating compounds are usually the most genotoxic. Still, in mammalian cells the stabilization of DNA double strand breaks arising as a consequence of DNA topoisomerase II (topo II) poisoning, usually accounts for the clinical antitumor activity of intercalating drugs such as doxorubicin (DOX) and m-amsacrine. Minor changes to the compound structures can result in reduction or complete loss of antitumor activity and/or genotoxicity thus indicating the complexities of chemical/DNA/topo II ternary interactions.[23]

Table 4. In vitro inhibition of compounds **4 - 7** on the growth of tumor cells.

Compd	IC_{50} (μM)[a]					
	PC-3	**HCT 116**	**SW 620**	**MCF-7**	**H 460**	**HaCaT**
4	19±16	≥100	≥100	6±5	≥100	≥ 100
5	13±2	29±4	3±1	4±3	5±3	1 ± 0.5
6	5±0.8	10±2	15±7	4±2	9±1	15 ± 8
7	35±6	71±5	39±14	12±9	32±5	90 ± 0.1

[a] IC_{50}; the concentration that causes a 50% reduction of the cell growth.

Many clinically useful drugs exert their cytotoxic effects through poisoning of either topo I or topo II. Topoisomerase-active drugs either inhibit the ability of the enzymes to initially cleave

DNA (catalytic inhibitors) or stabilise the fragile and normally transient 'cleavable complexes' they form by preventing strand religation (poisons). No overall structure-activity relationships are discernible for this property, again but small structural changes within a particular series appear to markedly alter the relative activities of analogues towards the two enzymes. This observation supports the 'drug stacking' model of interaction, where inhibitors with a 'deep intercalation mode' are responsible for topo I-mediated cleavage and those with an 'outside binding mode' are responsible for topo II-mediated cleavage.[24] Although additional experiments (e.g. inhibition of topoisomerases, cell cycle perturbation studies, etc.) are necessary for the accurate elucidation of differences in biological activity of **4, 5, 6** and **7**, it is evident that that different cationic side-chain markedly influence the activity (cytotoxicity) of these compounds, whereby **5** and **6** (characterised by sterically hindered positive charge on side chain) are obviously more nonselectively cytotoxic (probably inducing DNA strand breaks through topoisomerase poisoning), while **4** and **7** (exposed positive charges on side chains) are selective and thus represent interesting lead molecules.

3. Conclusions

Although here studied cationic DBTAA derivatives structurally resemble to the analogous dicationic porphyrins, several differences are observed, like absence of any significant self-stacking of DBTAA derivatives in aqueous medium.[6,8] Moreover, DBTAA derivatives form only one dominant type of complex with DNA/RNA at conditions of an excess of polynucleotide, whereby each DBTAA molecule is bound independently to DNA/RNA binding site, at variance to porphyrins which often agglomerate along polynucleotides and form more different complexes.[25,26] Compounds **4 - 7** most likely intercalate into ds-DNA and ds-RNA, and their positively charged side-chains additionally interact within the minor groove of polynucleotides, contributing to the overall affinity of compounds and controlling pronounced A-T(U) over G-C sequence preference as well as stronger thermal stabilization of ds-DNA than ds-RNA. Furthermore, all compounds showed moderate to high antiproliferative activity against the human tumour cell lines, whereby clear correlation between structure of the side-chain and cytotoxic activity was observed. Such an impact of positively charged DBTAA side-chains on the interactions with DNA/RNA as well as on the antiproliferative activity offers an intriguing and simple synthetic approach to the modulation and fine tuning of DBTAA-derivatives DNA/RNA binding properties and biological activity. For instance, an introduction of a number of DNA/RNA active substituents to the DBTAA side-chains is in progress.

4. Materials and Methods

4.1. Spectroscopic experiments

The electronic absorption spectra were obtained on Varian Cary 100 Bio spectrometer, CD spectra on JASCO J815 spectrophotometer and fluorescence spectra on the Varian Eclipse fluorimeter, all in quartz cuvettes (1 cm). The spectroscopic studies were performed in aqueous buffer solution (pH = 7, sodium cacodylate buffer, I=0.05 mol dm^{-3}). Under the experimental conditions absorbance of **4, 5, 6, 7** was proportional to their concentrations. Polynucleotides were purchased as noted: poly A – poly U, poly G – poly C, poly dA - poly dT, (Sigma), calf thymus (*ct*)-DNA (Aldrich). Polynucleotides were dissolved sodium cacodylate buffer, I = 0.05 mol dm^{-3}, pH=7. Calf thymus (*ct*-) DNA was additionally sonicated and filtered through a 0.45 μm filter.[27,28] Polynucleotide concentration was determined spectroscopically[28] as the concentration of phosphates. Spectroscopic titrations were performed by adding portions of polynucleotide solution into the solution of the studied compound.

Obtained data were corrected for dilution. Titration data were processed by Scatchard equation.[9] Values for K_s and n given in Table 2 all have satisfactory correlation coefficients (>0.999). Thermal melting curves for DNA, RNA and their complexes with studied compounds were determined as previously described[28,29] by following the absorption change at 260 nm as a function of temperature. Absorbance of the ligands was subtracted from every curve, and the absorbance scale was normalized. The Tm values are the midpoints of the transition curves, determined from the maximum of the first derivative and checked graphically by the tangent method.[28] ΔTm values were calculated subtracting Tm of the free nucleic acid from Tm of the complex. Every ΔTm value here reported was the average of at least two measurements, the error in ΔTm is ± 0.5 °C.

Ethidium bromide (**EB**) displacement assay: to polynucleotide solution (c = 2 × 10^{-5} mol dm^{-3}) ethidium bromide (c = 5 × 10^{-6} mol dm^{-3}) was added ($\boldsymbol{r}$ ([**EB**]/ [polynucleotide] = 0.4), and quenching of the **EB**/ polynucleotide complex fluorescence emission (λ_{ex} = 520 nm, λ_{em} = 601 nm) was monitored as function of c(**EB**)/c(compound). The given IC$_{50}$ values present the ratio c(**EB**)/c(compound) = [Int(**EB**/ polynucleotide) – Int(**EB**$_{free}$)] / 2, where Int(**EB**/ polynucleotide) is fluorescence intensity of **EB**/ polynucleotide complex and Int(**EB**$_{free}$) is fluorescence intensity of the free ethidium bromide before polynucleotide is added.

Viscometry measurements were conducted with an Ubbelohde viscometer system AVS 350 (Schott). The temperature was maintained at 25 ± 0.1 °C. Aliquots of drug stock solutions were

added to 5.5 ml of × 10^{-4} mol dm^{-3} ct-DNA solution in sodium cacodylate buffer, I = 0.05 mol dm^{-3}, pH=7, with a compound to DNA phosphate ratio r less than 0.2. Dilution never exceeded 4% and was corrected for in the calculations. The flow times were measured at least five times optically with a deviation of ± 0.2 s. The viscosity index α was obtained from the flow times at varying r according to the following equation: [30]

$$L/L_0 = [(t_r - t_0) / (t_{DNA} - t_0)]^{1/3} = 1 + \alpha * r$$

whereby t_0, t_{DNA} and t_r denote the flow times of buffer, free DNA and DNA complex at reagent / phosphate ratio r , respectively; L/L_0 is the relative DNA lengthening. The L/L_0 to r -plot was fitted to a straight line that gave slope α. The error in α is ≤ 0.1.

4.2. Biological assays

Antiproliferative assays

The experiments were carried out on 5 human cell lines, which are derived from 5 cancer types. The following cell lines were used: PC-3 (prostatic carcinoma), MCF-7 (breast carcinoma), SW 620 and HCT 116 (colon carcinoma), H 460 (lung carcinoma) and HaCaT (immortalized human skin keratinocytes). The cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C. The growth inhibition activity was assessed according to the slightly modified procedure performed at the National Cancer Institute, Developmental Therapeutics Program.[31,32] The cells were inoculated onto standard 96-well microtiter plates on day 0. The cell concentrations were adjusted according to the cell population doubling time (PDT). Test agents were then added in five consecutive 10-fold dilutions (10^{-8} to 10^{-4} mol/l) and incubated for further 72 hours. Working dilutions were freshly prepared on the day of testing. After 72 hours of incubation, the cell growth rate was evaluated by performing the MTT assay, as previously described.[31] Each test point was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound are calculated from dose-response curves using linear regression analysis.

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