Constrained bisantrene derivatives as G-quadruplex binders

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Abstract
We here report the synthesis and evaluation of constrained derivatives of a bisantrene isomer known to bind DNA when structured in G-quadruplex. Enhanced high-resolution mass spectrometry was used to evaluate the binding efficiency and stoichiometry of the compounds towards G-quadruplex DNA using a duplex sequence for comparison. Fluorimetric DNA binding studies supported and confirmed these preliminary observations. One of the newly synthesized compounds showed a high tendency and specificity to bind the structured G-quadruplex DNA.

Keywords: Anthracene, bisantrene, DNA, G-quadruplex, mass spectrometry, fluorimetry

Introduction

G-quadruplex DNA structures play important roles in the indirect inhibition of telomerase in cancerous cells,[1-4] in the regulation of the oncogenetic expression[5-9] and in viral functions.[10-12] The action of a small molecule directed towards these targets could result in contrasting cancerous cells immortalization or modifying the expression of the related genes by stabilizing this peculiar DNA arrangement.[13] The potential implications in cancer and infectious disease have turned these systems into very desirable targets for the development of new therapeutical strategies, stimulating the design and the synthesis of novel G-quadruplex binders. Besides this therapeutic application, these compounds may find application as chemical probes to selectively recognize and bind structured nucleic acid sequences, providing structural insights on the 3D arrangements of DNA or RNA. The binding of the small molecule to G-quadruplex DNA has been shown to preferentially occur through stacking on an external G-tetrad and, generally, binders share a common structural motif based on an aromatic surface and the presence of

protonable side chains. In this research work we focused on the synthesis of derivatives of bisantrene, a known intercalating agent endowed with topoisomerase-II poisoning activity.[14,15] We previously demonstrated how the number and the relative positioning of side chains on the anthracene scaffold modulate cytotoxicity and topoisomerase-II-associated DNA cleavage[16,17] and we later demonstrated how its isomer, Ant1,5 efficiently binds G-quadruplex DNA.[18] Pushing our investigation to the further level, in this research work we aim to provide insights in the influence of small, focused structural constrains modulating the flexibility and rotational properties of the side chains on the G-quadruplex binding efficiency. While designing the constrained derivatives, we inserted small constraining groups, with a perturbing effect on general structure as low as possible: for compound Ant1,5-Ri a methyl group and for Ant1,5-Ro a methylene residue (Figure 1). This operation was meant to compare the effects of different levels of constrain in providing an efficient interaction with nucleic acid. The synthesized compounds were screened for their G-quadruplex binding capability using a mass spectrometry-based assay and fluorimetric titrations. The selectivity for G-quadruplex was also evaluated by comparison to the duplex DNA.

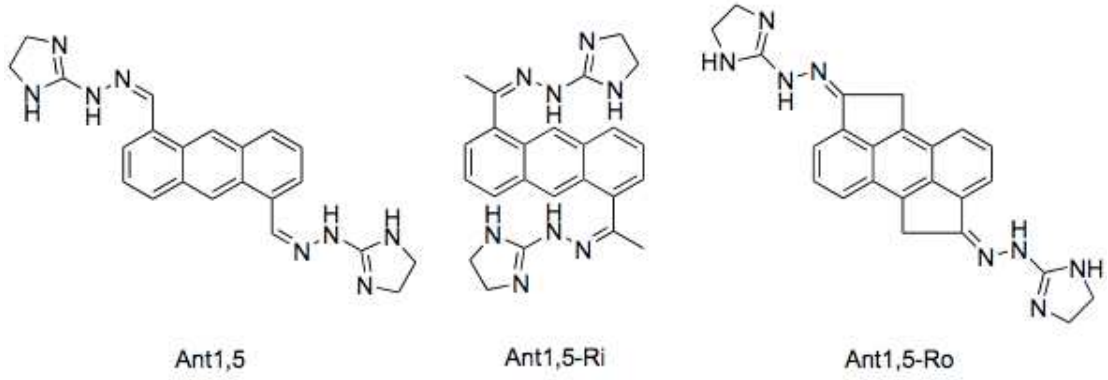


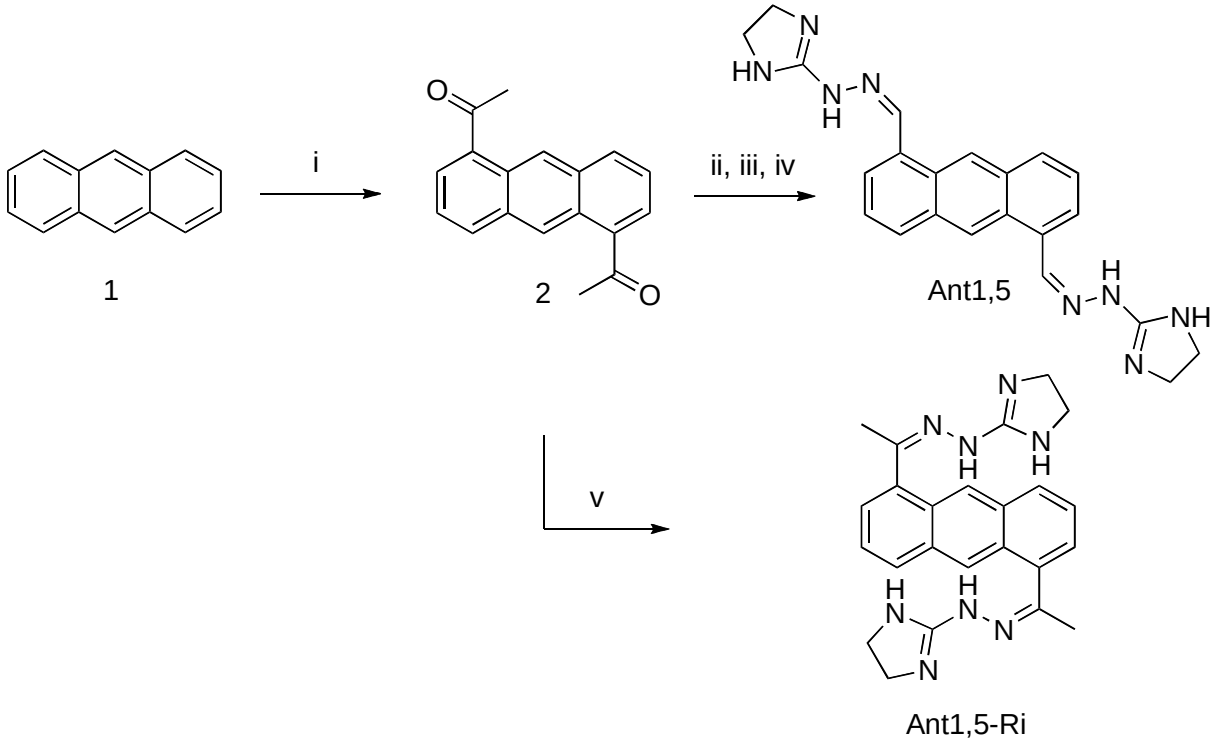
Figure 1. Chemical structure of tested compounds.

Results and Discussion

Synthesis of constrained derivatives

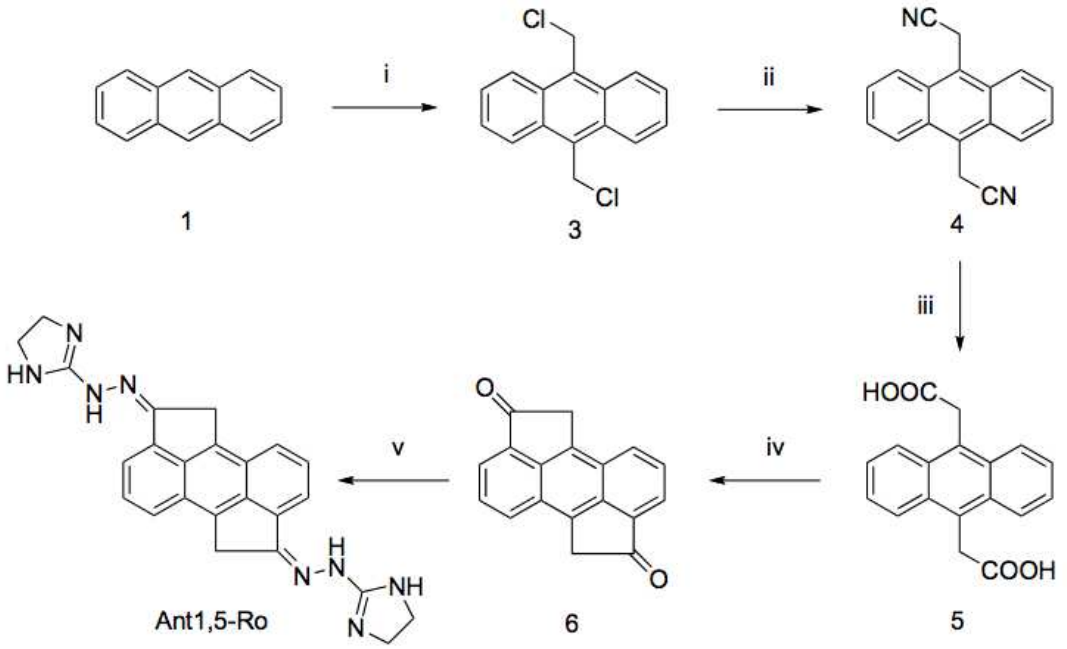
We previously reported the synthesis and characterization of Ant1,5.[18,19] Compounds were obtained by standard solution synthesis following the reported procedures, and the synthetic strategy is summarized in Scheme 1. Briefly, the procedure is based on a Friedel-Crafts acylation of the anthracene with acetyl chloride in the presence of $AlCl_3$ in dichloroethane. Subsequent oxidation with NaOBr (starting from a NaOH 30% solution and Br_2) in ethanol leads to the carboxylic acid that is then treated with dimethylsulfate in DMSO to obtain the methyl ester. After the reduction of the ester to aldehyde with sodium bis(2-methoxyethoxy)aluminumhydride, the final step is the imine formation with 4,5-dihydro-1*H*-imidazol-2-yl-hydrazine in ethanol.

Compound Ant1,5-Ri was directly obtained from 1,5-diacetyl anthracene through imine formation by reacting with 4,5-dihydro-1*H*-imidazol-2-yl-hydrazine in isopropanol in presence of methanesulfonic acid.



Scheme 1. Reagents and conditions: (i) acetyl chloride, $AlCl_3$, dichloroethane; (ii) NaOH 30%, Br_2, ethanol; (iii) dimethylsulfate, DMSO then sodium bis(2-methoxyethoxy)aluminumhydride; (iv) 4,5-dihydro-1*H*-imidazol-2-yl-hydrazine, ethanol; (v) 4,5-dihydro-1*H*-imidazol-2-yl-hydrazine, methanesulfonic acid, iPrOH.

Scheme 2 describes the synthesis of compound Ant1,5-Ro. The synthesis was designed integrating and optimizing some procedures previously reported by Ryu *et al.*[20] and Mohebbi *et al.*[21] for intermediates 3-6. The imine formation with 4,5-dihydro-1*H*-imidazol-2-yl-hydrazine in isopropanol in presence of methanesulfonic acid gave the final product Ant1,5-Ro.



Scheme 2. Reagents and conditions: (i) $ZnCl_2$, paraformaldehyde, conc. HCl, dioxane; (ii) NaCN, DMSO; (iii) p-toluenesulfonic acid, propanol then LiOH, THF/water; (iv) $SOCl_2$, $AlCl_3$, dichloroethane; (v) 4,5-dihydro-1*H*-imidazol-2-yl-hydrazine, methanesulfonic acid, iPrOH.

Intermediates and final compounds were purified by preparative flash chromatography or crystallization and were fully characterized by ^{1}H-NMR, ^{13}C-NMR, high resolution mass spectrometry and tandem mass spectrometry while purity was assessed by HPLC (see *Supplementary Material* for spectra).

Mass spectrometry (MS) binding assay

An electrospray ionization-mass spectrometry (ESI-MS) experiment was designed to evaluate the efficiency and stoichiometry of the binding to G-quadruplex DNA.[22-24] The assay was performed using a guanine-rich DNA sequence that is accepted as a model of human telomeric G-quadruplex showing the peculiar TTAGGG repeat.[18] This sequence, from here on called "GQ" (5' - AGG GTT AGG GTT AGG GTT AGG GT - 3', average mass 7270.774 Da), was incubated with the samples according to the conditions reported in the experimental part and in the *Supplementary Material*. ESI mass analysis in negative ionization mode showed that ammonium adducts to the DNA strand were present, with a good abundance of the $(GQ + 2NH_4^+)^-$ ion, diagnostic of the presence of G-quadruplexes structure (see *Supplementary Material* for spectra). Considering the structure of the analyzed sequences, a G-quadruplex coming from the self-arrangement of the monomeric strand should be structured as three planar G-quartets stacked one over the other; there are in fact three guanines in every repeat while the other bases are supposed to form the loops; based on this, a G-quadruplex made by three G-quartets should be structured

around two ammonia ions. The detectable $(GQ + 1NH_4^+)^-$ and $(GQ)^-$ ions in the mass analysis, can be assumed as non-G-quadruplex structured DNA sequences resulting from non-specific interactions between the ammonium ion and the nucleic acid or from an ammonium ion loss during the mass experiment. The ESI-MS analysis was performed on the GQ sequence using a 5:1 compound to DNA ratio with a final concentration of 5 µM in oligonucleotide in 150 mM ammonium acetate.

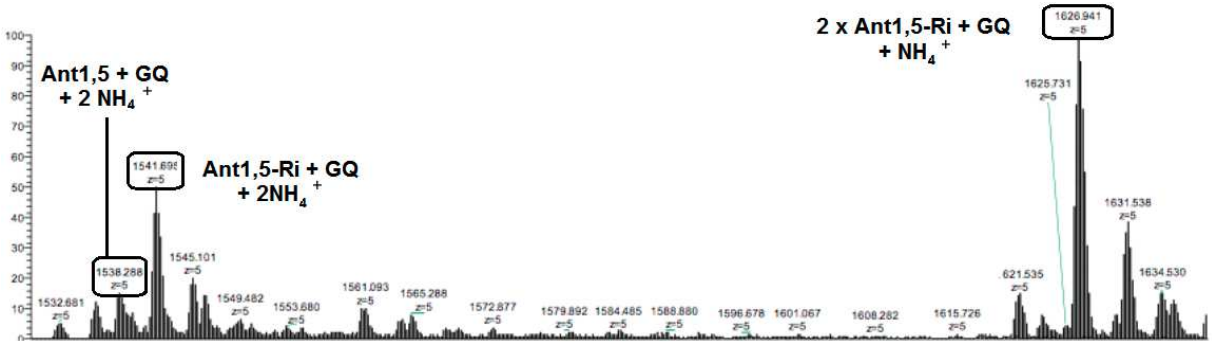


Figure 3. ESI-MS binding experiments for compounds Ant1,5, Ant1,5-Ri and Ant1,5-Ro with the telomeric 5' - AGG GTT AGG GTT AGG GTT AGG GT - 3' DNA sequence.

Compounds were tested singularly towards GQ DNA and then a more comprehensive experiment was designed in order to evaluate at the same time the competitive binding capability of the three molecules; the three molecules were mixed together with the GQ DNA sequence before the analysis and the resulting mixture was then analyzed (see *Supplementary Material* for further details and spectra). The here reported spectrum (Figure 3) shows that both Ant1,5 and Ant1,5-Ri were able to give an interaction with DNA. It appears clearly that Ant1,5-Ri, the slightly constrained derivative, has a greater tendency to bind the structured oligonucleotide. In particular, when considering the 1:1 DNA-ligand adducts, the peak with the highest intensity in the reported range of the -5 charge state is the one corresponding to the complex $(Ant1,5\text{-}Ri + GQ + 2\ NH_4^+)^-$, suggesting that the compound is bound to a structured quadruplex. This evident tendency of binding DNA is also confirmed by the presence of an intense peak corresponding to the 2:1 complex with DNA $(2\ x\ Ant1,5\text{-}Ri + GQ + 1\ NH_4^+)^-$, likely due to an aspecific interaction with the nucleic acid or to ammonium displacements after binding. The completely constrained derivative Ant1,5-Ro did not show any detectable interaction with the nucleic acid in these conditions.

The tendency of the compounds of forming a complex or, in general, binding the G-quadruplex structured nucleic acid is commonly expressed in terms of equilibrium association constant (K)[25] and/or binding affinity (BA),[26] as long as relative intensities (I) in a mass spectrum are assumed to be proportional to the concentrations of the injected solution.

$$BA = I_{(DNA\text{-}stabilizer\ complex)} / (I_{(DNA\text{-}stabilizer\ complex)} + I_{(DNA)})^{26}$$

According to the reported mass spectrum, Ant1,5-Ri shows a binding affinity towards G-quadruplex DNA realistically exceeding the one of lead compound Ant-1,5, even considering that the presence of the above discussed 2:1 complex was encountered.

Mass spectrometry (MS) competition assay

Based on these preliminary observations, competition electrospray ionization mass spectrometry (ESI-MS) experiments[27-29] allowed to establish relative affinity of Ant1,5-Ri towards the GQ quadruplex and QM duplex (QMup: 5'-GTG AGA TAC CGA CAG AAG-3', average mass 5581.714 Da annealed with QMdown: 5'-CTT CTG TCG GTA TCT CAC-3', average mass=5416.574 Da) sequences. Equimolar amounts of GQ and QM duplex were incubated with an excess of Ant1,5-Ri. Since ESI-MS transfers intact biomolecules complexes in the gas phase and signal intensity is proportional to the abundance of each species in solution, the ratio between m/z signals of each bound species and the corresponding bound and free DNA (Figure 4) was calculated (Table 1).

Ant1,5-Ri binds GQ forming mono- and di-adduct detected as intense signal while the signal of the ant 1,5-Ri bound to duplex is much lower (Figure 4). Interesting no binding to the single stranded QM up was detected suggesting the absence of aspecific binding to the DNA. The presence of QM up in the sample is likely due to an excess compared to the complementary QM down rather than from the dissociation because QM down is not detected.

This analysis clearly shows that Ant1,5-Ri preferentially binds the quadruplex sequence over the duplex sequence. Therefore we confirmed a general, even though not absolute, selectivity towards the G quadruplexes.

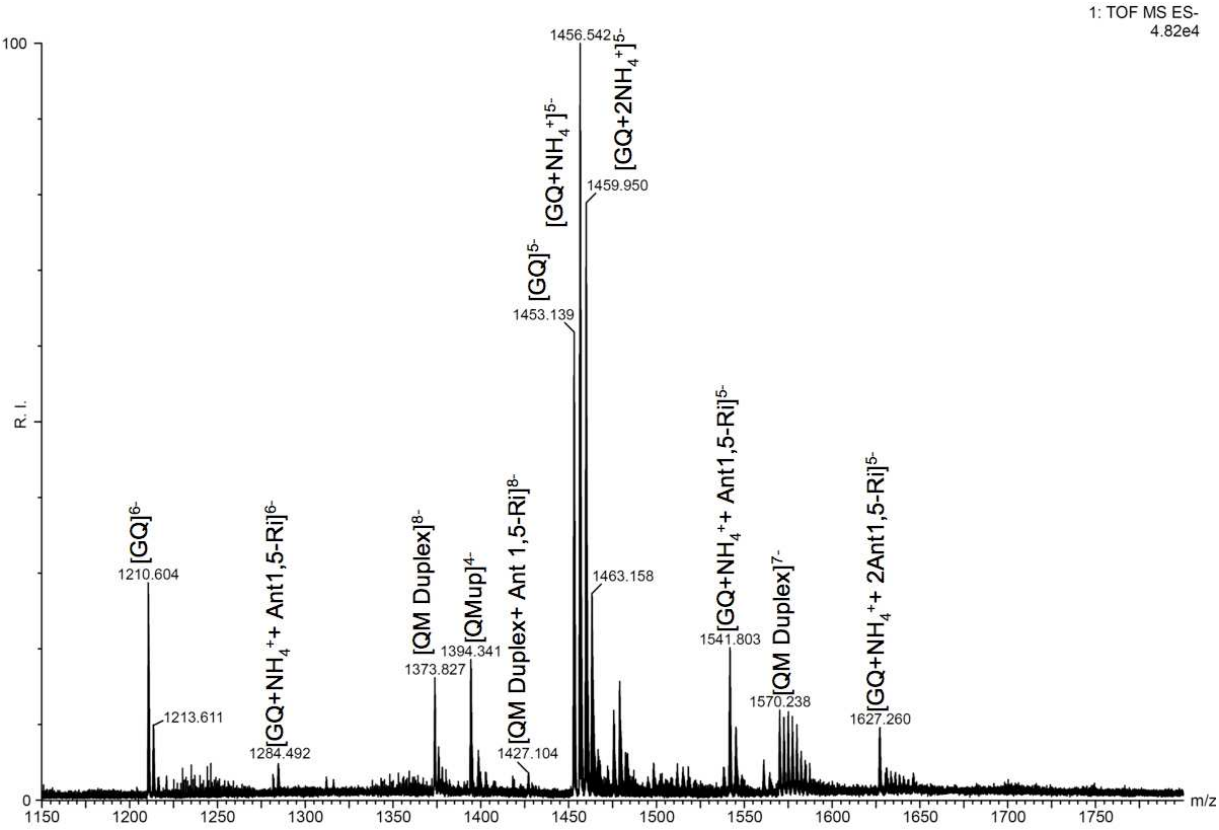


Figure 4. ESI/MS competition assay representative spectrum. Equimolar amounts of GQ and QM duplex were incubated with an excess of Ant 1,5-Ri and analyzed by ESI/MS. The identity of *m/z* signals is shown above each signal. RI stands for relative intensity.

Table 1. Relative binding affinity of Ant1,5-Ri for the GQ G4-folded and QM duplex folded oligonucleotides

Oligos	Binding affinity
GQm	16.1
Qm duplex	9.1
Qm up	/

Fluorimetric DNA binding studies

On the basis of the above results, the G-quadruplex binding capability of Ant1,5-Ri was further examinated using fluorimetric titrations. The same experiment was also performed on the QMup-QMdown duplex sequence for comparison (Figure 5). The titrations supported the observations from mass spectrometric measures, showing a remarkable difference in DNA recognition by the

compound. Data were analyzed according to Job plot and Scatchard analysis as previously reported.[18] Ant1,5-Ri showed a remarkably stronger interaction with GQ (K_a = 2,28 ± 0,32 x 10^{-6} M^{-1}) than with the QMup-QMdown duplex (K_a = 0,21 ± 0,06 x 10^{-6} M^{-1}), confirming a notable selectivity of this compound towards G-quadruplex.

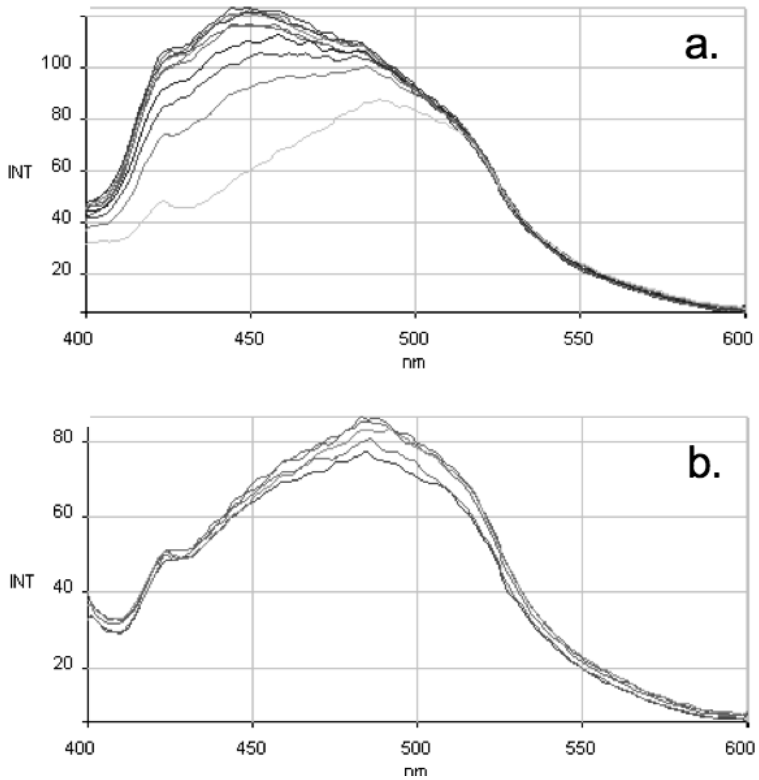


Figure 5. Fluorimetric titration for Ant1,5-Ri with GQ (a) and QMup-QMdown duplex (b).

Docking studies

While it is generally accepted that the π-π stacking interaction to an external G-tetrad represents the preferential binding motif to G-quadruplex DNA, other complex structures are possible. Indeed, ionic interaction or specific\non-specific binding to the bases not involved in the formation of the G-quartets have been described.[30] For this experiment, the structure of a telomeric G-quadruplex sequence annealed in a K^+ buffer and solved by X-ray (PDB ID: 3T5E), was used to dock the compounds following previously reported procedures.[26,31]

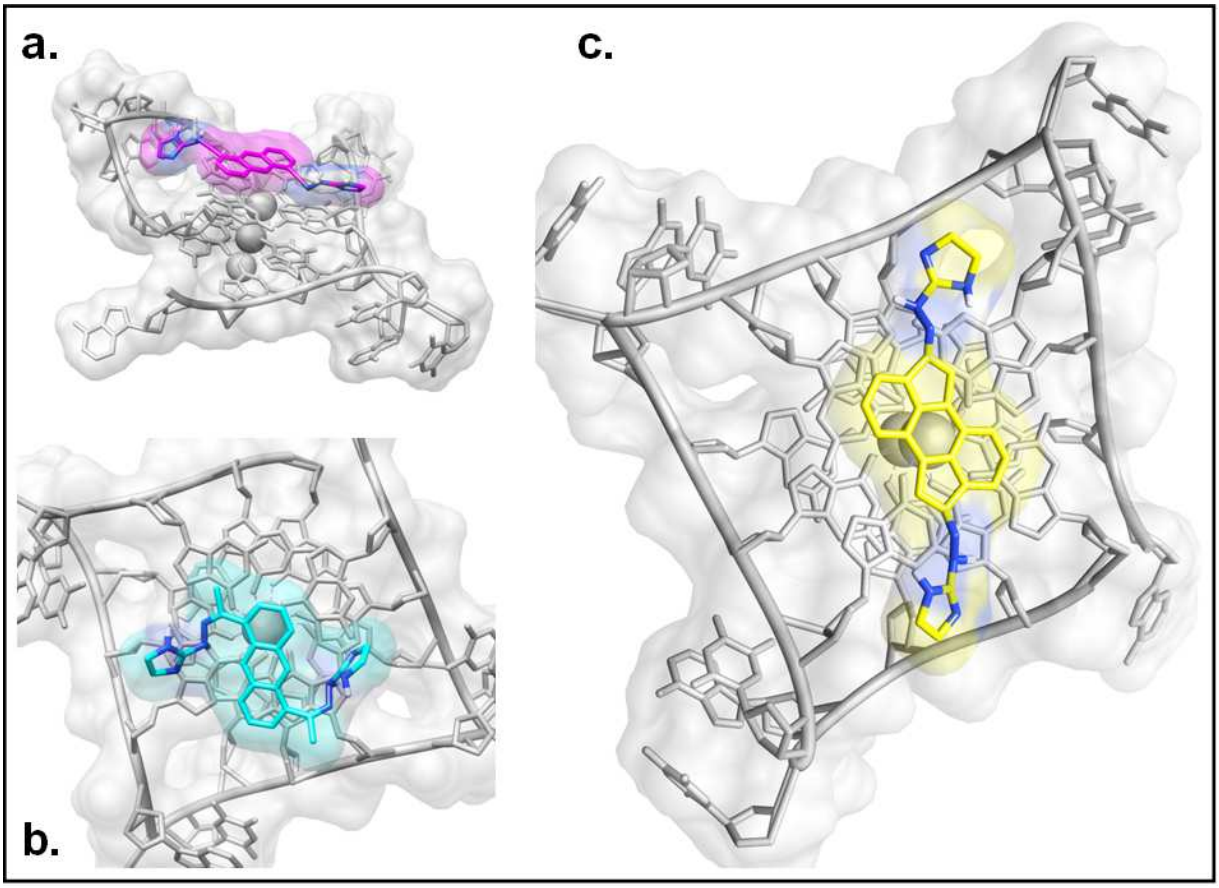


Figure 6. Possible interaction motif for the synthesized compounds.

Ant1,5 (a), Ant1,5-Ri (b) and Ant1,5-Ro (c) were docked and based on the obtained models, the three compounds share the π-π stacking with an external quartet as preferential binding motif (Figure 6).

Conclusions

In this work we described the rational design and a facile synthetic approach for the preparation of constrained analogues of the reported efficient G-quadruplex stabilizing agent Ant1,5. The binding efficiency towards human telomeric G-quadruplex was evaluated using enhanced mass spectrometry-based experiments. In addition to this, the potential binding motif of the lead compound and the newly synthesized derivatives were investigated through docking studies, confirming the π-π interaction as a realistic interaction pattern.

Mass spectrometric data shows that Ant1,5-Ri has an interestingly high tendency to bind the G-quadruplex DNA, also in a 2:1 stoichiometry, with a binding affinity exceeding the one measured for the lead compound Ant-1,5. Moreover, Ant1,5-Ri showed a notable degree of

selectivity towards G-quadruplex DNA and this was also supported by fluorimetric DNA binding experiments. Besides, Ant1,5-Ro did not show any detectable interaction with the nucleic acid, suggesting that only slight modifications affecting the overall flexibility of the side chains are allowed in order to preserve the G-quadruplex recognizing capability. This cannot be imputable to the nature of the planar anthracene system or to position, or length, or charge of side chain substituents, but to wrong spatial location of side chain groups. The conformational constrain may impair stacking and electrostatic binding, weakening effective interactions between the ligand and the G-quadruplex structure(s), notwithstanding the presence of the typical ingredients for successful recognition.

Experimental Section

General. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 400 MHz spectrometer; NMR spectra were run using $CDCl_3$ or $(CD_3)_2SO$ as solvent and the solvent peak was used as internal standard. Data analysis was carried out using the TopSpin and iNMR suites. Chemical shifts (δ) are expressed in parts per million relative to tetramethylsilane (ppm), and spin multiplicities are indicated as an s (singlet), d (doublet), dd (double doublet), t (triplet), and m (multiplet) and the *J* values expressed in Hz. Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel plates (Merck 60F254), and spots were visualized with UV light at 254 nm. Flash chromatography was performed using Biotage Isolera, Biotage SNAP and Buchi Sepacore Silica Flash cartridges. High resolution mass spectra (HRMS) were obtained using a Mariner ESI-TOF (Perceptive Biosystems Inc.). Melting points were measured with a Buchi Melting point MP-560 apparatus. Purity profile was assessed by high performance liquid chromatography (HPLC) using a Varian ProStar 210 combined with a Zorbax Eclipse XDB-C18 by Agilent Technologies column. An appropriate ratio of water (A) and acetonitrile (B) was used as mobile phase with an overall flow rate of 1 mL/min; the general method for the analyses is here reported: 0 minutes (90% A-10% B), 15 minutes (10% A-90% B), 20 minutes (10% A-90% B), 21 minutes (90% A-10% B), 25 minutes (90% A-10% B). The purity of all compounds was estimated ≥ 96%, unless otherwise stated. All starting materials not described below, the solvents and the deuterated solvents were purchased from commercial sources, mainly Aldrich and Fluka. All reagents and solvents were used as received from commercial sources without additional purification, unless differently stated.

Synthetic procedures

Ant1,5. We previously reported the synthetic procedure for compound Ant1,5.[18,19]

Anthracene-1,5-diacetyl (2). A three-neck round bottom flask was charged with anthracene (10.00 g, 56 mmol) and $AlCl_3$ (23.00 g, 172 mmol) in 300.0 mL of dichloroethane. Acetyl chloride (11.8 mL, 165 mmol) was carefully added from a dropping funnel to the stirred mixture cooled with an in ice bath. The suspension was stirred at 0°C for two hours, and then for four

hours at room temperature. The brown solid precipitate collected by filtration was suspended in a mixture of ice and conc. HCl. The suspension was stirred for two hours and then filtered again. The dark yellow solid isolated was recrystallized from toluene leading to bright yellow crystals. 8.70 g of the pure 1,5-isomer **2** were isolated while the 1,8-isomer was removed with the washings. Yield: 59%, mp 197 °C. δ_H (400 MHz; $CDCl_3$) 2.85 (6H, s, Me), 7.56 (2H, dd, *J* 8.4 Hz, *J* 7.0 Hz, PhH), 8.12 (2H, dd, *J* 7.0 Hz, *J* 1.0 Hz, PhH), 8.29 (2H, dd, *J* 8.4 Hz, *J* 1.0 Hz, PhH), 9.60 (2H, s, PhH). δ_C (100 MHz; $CDCl_3$) 29.9 (Me), 121.5, 126.6, 129.6, 131.3, 132.8, 133.2, 136.5 (Ar-C), 199.6 (C=O). HRMS (ESI): found 263.0999 ($C_{18}H_{15}O_2$, $[M+1]^+$), calc. 263.0994. Anal. found: C, 82.3; H, 5.4. Calc. for $C_{18}H_{14}O_2$: C, 82.4; H, 5.4%.

Compounds 3,4,5. The synthesis of these intermediates was performed following a procedure previously reported by Ryu *et al.*[20] Spectroscopic analysis of the synthesized compounds was coherent with the one proposed by the authors.

Compound 6. The synthesis of this intermediate was performed following a procedure previously reported by Mohebbi *et al.*[21] Spectroscopic analysis of the prepared compound was coherent with the one proposed by the authors.

1,5-Anthracenediacetyl bis((4,5-dihydro-1*H*-imidazol-2-yl)hydrazone) (Ant1,5-Ri). A round bottom flask was charged with **2** (200 mg, 0.76 mmol) in 38.0 mL of isopropanol with 2.0 mL of methansulfonic acid and 4,5-dihydro-1*H*-imidazol-2-yl hydrazine hydrobromide (410 mg, 2.26 mmol). The mixture was stirred at room temperature for twelve hours and then a yellow solid was collected by filtration. Flash chromatography was performed on this precipitate (isocratic, 70% dichloromethane, 29% methanol and 1% triethylamine) leading to 127 mg of light yellow crystalline product. Yield: 38%, mp 136 °C. δ_H (400 MHz; DMSO) 2.68 (6H, s, Me), 3.79 (8H, s, CH_2), 7.59 (2H, m, NH), 7.66 (2H, dd *J* 7.5 Hz *J* 7.6 Hz, PhH), 8.32 (2H, dd, *J* 7.5 Hz, PhH), 8.71 (2H, dd, *J* 7.6 Hz, PhH), 9.40 Hz (2H, s, PhH), 11.48 (2H, s, NH). δ_C (100 MHz; DMSO) 32.3 (Me), 57.9 (CH2), 113.3, 125.7, 126.6, 127.5, 144.1, 144.6, 147.9 (ArC), 148.8 (C=N), 153.4 (C=N). HRMS (ESI): found 427.2288 ($C_{24}H_{27}N_8$, $[M+1]^+$), calc. 427.2280. Anal. found: C, 67.6; H, 6.0; N, 26.2. Calc. for $C_{24}H_{26}N_8$: C, 67.6; H, 6.1; N, 26.2%.

4,12-bis[2-(2-Imidazolinyl)hydrazono]pentacyclo[8.6.1.22,5.013,17]nonadeca-1,5,7,9(18),10(17),13,15,18-octaene (Ant1,5-Ro). The procedure is the same reported for **Ant1,5-Ri** but 4,5-dihydro-1*H*-imidazol-2-yl hydrazine hydrobromide and **6** were used as starting materials. The desired compound was obtained as a light yellow solid. Yield: 34%, mp 143-145 °C. δ_H (400 MHz; $CDCl_3$) 3.83 (8H, s, PhCH2), 4.26 (4H, s, CH2), 7.82 (4H, m, PhH), 8.14 (2H, d, *J* 6.1 Hz, PhH), 8.30 (2H, d, *J* 6.1 Hz, PhH). δ_C (100 MHz; DMSO) 47.3 (Ar-CH_2), 68.1 (CH_2), 102.1, 111.1, 123.4, 126.7, 137.4, 137.6, 144.4 (Ar-C), 150.4 (C=N), 154.6 (C=N). HRMS (ESI): found 423.0811 ($C_{24}H_{23}N_8$, $[M+1]^+$), calc. 423.0809. Anal. found: C, 68.1; H, 5.1; N, 26.4. Calc. for $C_{24}H_{22}N_8$: C, 68.2; H, 5.2; N, 26.5%.

Mass spectrometry (MS) binding assay.

All DNA substrates were obtained from commercial sources (*i.e.*, Integrated DNA Technologies, Coralville, IO or from Sigma-Aldrich, Milan, Italy). Desalting was performed by ultrafiltration

against 150 mM ammonium acetate (pH adjusted to 7.0) in Millipore (Billiberica, MA) Centricon devices with 3 KDa MWCO.

Samples were analyzed by direct infusion ESI on ThermoFisher Scientific (Waltham, MA) LTQ-Orbitrap Velos mass spectrometer. All analyses were performed in nanoflow mode by using quartz emitters produced in house whith a Sutter Instruments Co. (Novato, CA) P2000 laser pipette puller. Up to 5µL samples were typically loaded onto each emitter by using a gel-loader pipette tip. A stainless steel wire was inserted in the back-end of the emitter to supply an ionizing voltage that ranged between 0.9 and 1.2 kV. Source temperature and desolvation conditions were adjusted by closely monitoring the incidence of ammonium adducts and water clusters. Tandem mass spectrometry experiments involved isolating the precursor ion of interest in the LTQ element of the instrument, activating fragmentation in either the LTQ or the C-trap, and performing fragment detection in the Orbitrap. The instrument was calibrated by using a mixture of 0.5 mg/ml of CsI in 50% methanol, which provided 1-3 ppm mass accuracy. Samples were prepared just before the analysis from opportune dilutions of stock solutions. The final concentration of the oligonucleotide was 3 µM in 150 mM ammonium acetate, with a ratio compound/oligo of 5:1 if a binding study was being performed. Samples were prepared just before the analysis and experiments were recorded in negative ionization mode.

Mass spectrometry (MS) competition assay

Oligonucleotides were heat-denatured and folded in 150 mM ammonium acetate (AA) pH 7 at 4 °C at least 1 week. The oligonucleotides where diluted to final concentration of 10 µM and incubated with the tested compound at ratio DNA:compound ranging from 1:5 to 1:50 overnight at 4 °C. Samples were analyzed by direct infusion electrospray ionization (ESI) on a Xevo G2-XS QTof mass spectrometer (Waters, Manchester, UK). The injection was automatically performed by an Agilent 1290 Infinity HPLC (Agilent Technologies, Santa Clara, CA, US) equipped with an auto sampler; the carrying buffer was AA 100 mM. Up to 5 µL samples were typically injected for each analysis. The electrospray capillary was at 1.8 kV, the source and desolvation temperatures were 45°C and 65°C, respectively, and the sampling cone was at 50 V. All these parameters ensured minimal fragmentation of the DNAs complexes. The instrument was calibrated using a 2 mg/mL solution of sodium iodide in 50% isopropanol. Additionally, the use of the LockSprayTM during the analysis provided a typical <2 ppm mass accuracy. The internal standard LockSprayTM consisted in a solution of leu-enkephalin 1 µg/ml in acetonitrile/water (50:50, v/v) containing 0.1% formic acid. Binding affinities were calculated for each experiment using the reconstructed-ion chromatogram area for each species calculated by MassLynx V4.1: this analysis was made possible by the experimental design that used an HPLC system to inject the samples in the mass spectrometer. The binding affinity was calculated with the following formula: [BA= (ΣDNAb/(ΣDNAf+ ΣDNAb)) x 100], where BA is the binding affinity, DNAb is chromatogram area of bound DNA and DNAf is the chromatogram area of free DNA. DNAb comprises DNA with one or two bound ligands, where present. The more intense signals with charge states 6-, 5- were used for the free and bound GQ DNA, charge state 4- for

QM duplex. The adducts of the other charge states resulted to be too weak to calculate the area and were excluded from the calculation. Free DNA and ammonium adducts were included in the calculation.

Fluorimetric DNA binding studies

Fluorimetric measurements were performed in accordance with a procedure that we reported previously.[18] Titrations were carried out with a PerkinElmer LS30 fluorometer at 25 °C. Binding was evaluated by addition of increasing amounts of folded DNA to a freshly prepared compound solution in 150 mM ammonium acetate. To contain systematic inaccuracies due to experimental errors, the range of bound drug fractions considered for calculations was 0.15–0.85. For GQ sequence, the experimental data were analyzed according to equation 1, which describes a 1:1 binding mode.

$$\frac{\Delta F}{\Delta F_{\max}} = \frac{\{[L_0] + [G_0] + K_d - [([L_0] + [G_0] + K_d)^2 - 4 \times [L_0] \times [G_0]]^{1/2}\}}{(2 \times [L_0])} \quad (1)$$

where L_0 and G_0 are the ligand and G-quadruplex concentration, respectively. For the QMup-QMdown duplex data were evaluated according to the equation of McGhee and Von Hippel[32] for non-cooperative ligand–lattice interactions:

$$\frac{r}{m} = \frac{K_i(1 - nr)^n}{[1 - (n-1)r]^{n-1}} \quad (2)$$

where r is the molar ratio of bound ligand to DNA, m is the free ligand concentration, K_i the intrinsic binding constant, and n the exclusion parameter (close to 2 for the considered sequence).

Docking studies.

The docking procedure was designed, developed and carried out in accordance with other recently reported ligand-G-quadruplex interaction studies.[26]

Briefly, AutoDockTools 1.5.6 was used to establish the Autogrid points and to visualize the docked poses resulting from the Autodock4 analysis.[33] Both ligand and G-quadruplex 3D models were optimized for the experiment removing the co-crystallized ligand, adding polar hydrogens to the structures and computing partial charges. The docking grid maps were spaced at 0.500 Å, and the center of the G-quadruplex structure was set as the grid center. One hundred docking runs were performed.

Acknowledgements

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