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Tetraplex DNA specific ligands based on the fluorenone-carboxamide scaffold

Stefano Alcaro,[a,*] Anna Artese,[a] James N. Iley,[b] Rosanna Maccari,[c] Sotiris Missailidis,[b] Francesco Ortuso,[a] Rosaria Ottanà,[c] Patricia Ragazzon[b] and Maria Gabriella Vigorita[c]

[a]*Dipartimento di Scienze Farmacobiologiche Università degli Studi "Magna Græcia" di Catanzaro, Complesso Ninì Barbieri, 88021 Roccelletta di Borgia (CZ), Italy*
[b]*Chemistry Department, The Open University, Walton Hall, Milton Keynes MK7 6AA, UK*
[c]*Dipartimento Farmaco-Chimico, Facoltà di Farmacia, Università di Messina, V.le SS. Annunziata, 98168 Messina, Italy*

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Abstract—A series of fluorenone-carboxamide compounds was analyzed with regard to DNA binding properties by UV spectroscopy and competition dialysis methods. The morpholino derivative **10** provided interesting results in terms of affinity and specificity toward the DNA G-tetraplex structures. Interactions against this target were evaluated by a comparative molecular modeling study in agreement with the experimental data, proposing a model for the rational design of new agents with potent and selective DNA tetraplex binding properties.

Synthetic derivatives possessing IFN-inducing activity have been reported in the literature as agents that, due to their ability to intercalate DNA, could induce interferon production that, in turn, might stimulate antiviral and/or cytostatic effects in the cell.[1,2] Among them, tilorone, (2,7-bis[2-(diethylamino)ethoxy]-9*H*-fluoren-9-one), was the first low molecular weight IFN-inducer orally effective in vivo against some DNA and RNA viruses.[3,4] These considerations prompted us to design and synthesize hybrid agents[5] between the antiviral tilorone[6] and the antitumor agent daunomycin,[7] two well-known intercalators, to generate novel agents with potential antiviral and antitumor activity, based on their DNA binding properties.

DNA binding agents have for many years formed the basis of cancer chemotherapy, with various agents based on anthracycline or acridine scaffolds currently in the market, exemplified by agents such as daunorubicin.[7] However, DNA has been recognized as being far more polymorphic than originally assumed and can form left-handed and parallel stranded duplexes, hairpins with ordered loops, triplexes, and quadruplexes,[8] which have been shown to play an important role in the biology of the cell. Such higher order DNA structures have provided novel targets for drug design of compounds with improved specificity. Thus, a plethora of molecules have been synthesized to selectively target tetraplex DNA (over duplex DNA).[9,10] Many drugs known to intercalate duplex DNA have been tested and subsequently synthetically modified to interact with a greater selectivity towards tetraplex DNA. These include, in the first instance, anthraquinones,[11] porphyrins[12], and ethidium bromide derivatives,[13] whereas other classes of compounds include the fluorenones,[14] heteroaromatic tetracyclic systems[15], and quinolines.[16] Our own studies on acridine compounds have resulted in molecules with high affinity for higher order DNA structures. A trisubstituted acridine has been recently described as the most potent of this new generation of telomerase inhibitor (EC_{50} in the TRAP assay 0.06 μM) yet disclosed;[17] the difluorodimethyl-quinoacridinium methosulfate salt[18] has an EC_{50} of 0.33 μM.[19] Both compounds have the potential therapeutic advantage of low overall cytotoxicity. Finally, the diethylquinoacridinium iodide pentacyclic salt was found to have an unusually high affinity

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* Corresponding author. Tel.: +39 0961 3694197; fax: +39 0961 391490; e-mail: alcaro@unicz.it

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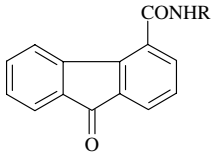
for triplex DNA compared to its second generation lead compounds.[20]

To investigate the relationship between the biological activity and the DNA binding properties of a series of compounds (Fig. 1), we have characterized those using spectroscopic techniques for their binding to Salmon Testes (ST) DNA and potentially higher order DNA forms.

In an attempt to further design molecules with higher affinity and selectivity for DNA and particularly higher isoforms, such as the tetraplex telomeric sequence, we have explored the interactions of a series of fluorenone-carboxamides with such sequences, using a broad range of biophysical and spectroscopic techniques. Structure–activity relationships on the fluorenone framework have offered us some insight into the characteristics needed for binding, whereas our binding results have led us to some conclusions on the efficacy of these compounds as DNA binders, pointing to novel lead structures in anti-cancer drug design that could be used in the development of a second generation of antitumor agents.

The series of active reagents (Fig. 1) were investigated for their ability to bind to double stranded DNA using spectrophotometric approaches, including UV spectrophotometry, ethidium bromide displacement, and thermal denaturation. In the UV spectrophotometric study, the compounds were titrated with ST DNA and the changes in their spectrum at 302 nm were monitored. All compounds had their main electronic transition in the region of 260 nm, with a shoulder at around 300 nm, peaking at 302 nm, and a much weaker peak at around 330 nm. The 260 nm region was unsuitable for monitoring for known interference with the DNA absorbance. The 330 nm peak was also unsuitable due to the weakness at the concentrations tested. The 302 nm peak was thus selected for monitoring during the titration. The DNA has a minimal contribution in this area, which was subtracted from the spectrum of the complex to result in the changes of the drug spectrum alone. Changes at the 302 nm peak were monitored upon addition of DNA aliquots and their results plotted in Origin (Fig. 2) using the formula shown in Supplementary Information.[21]



Compound	R	Compound	R
1	$-(CH_2)_2OH$	**6**	$-(CH_2)_2N(CH_3)_2$
2	$-(CH_2)_3OH$	**7**	$-(CH_2)_3N(CH_3)_2$
3	$-CH_2CH(OH)CH_3$	**8**	$-(CH_2)_2N(CH_2CH_3)_2$
4	$-(CH_2)_4OH$	**9**	$-(CH_2)_2$-Morph
5	$-(CH_2)_2O(CH_2)_2OH$	**10**	$-(CH_2)_3$-Morph

Figure 1. Chemical structures of compounds **1**–**10**.

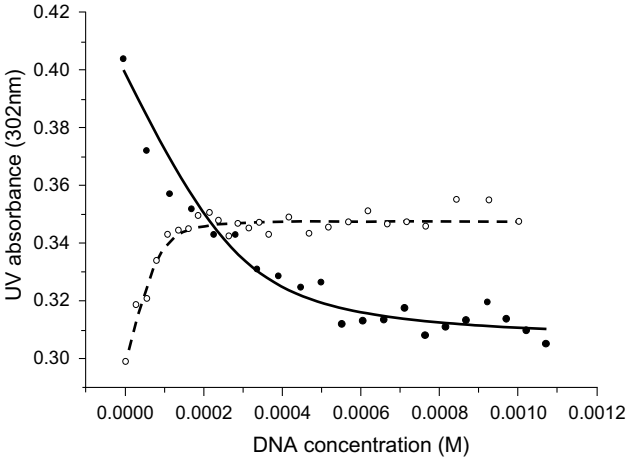


Figure 2. UV binding titration profiles of compound **10** (solid lines and filled dots) versus compound **9** (dashed line and empty dots) measured with different ST DNA concentrations at 302 nm.

All measurement were performed in triplicate at a concentration of 100μM. Prior to the binding titration, the compounds were found to obey the Beer–Lambert law at a concentration of 200 mM. The binding affinities of the compounds for ST DNA varied widely depending on their functional groups, with K_a values ranging from zero (no binding) to $10^5\ M^{-1}$ observed for **9** (relatively strong affinity for DNA) and with the majority of the drugs in the $10^4\ M^{-1}$ range (see Table 1).

The UV data appear to confirm the cytotoxicity data previously described,[5] with the compounds showing no binding to DNA also having no cytotoxicity against the Wish cell lines. The intriguing feature is compound **9**, which presents a different profile from that observed in the other reactive compounds, including **10**, which is structurally very similar. All reactive compounds present a hypochromism upon binding to the DNA, with the exception of **9** that shows hyperchromism. Furthermore, the association constant for this compound is significantly higher (by an order of magnitude) from the other compounds. This was confirmed at a range of concentrations and in triplicate measurements (Fig. 2).

Thermal denaturation experiments were performed to test the ability of the drugs to stabilize the DNA double helix upon binding. The experiments were performed at

Table 1. Binding measurements by UV spectroscopy and thermal denaturation

Compound	K_a (M^{-1})	$\Delta T_{m_{DNA}}$
1	—	1.9
2	$8.4 \cdot 10^3$	3.1
3	—	2.1
4	—	−0.8
5	—	0.2
6	$3.3 \cdot 10^3$	0.2
7	$3.1 \cdot 10^4$	2.1
8	$7.8 \cdot 10^4$	2.1
9	$1.6 \cdot 10^5$	1.2
10	$2.3 \cdot 10^4$	−0.8

a ratio of DNA/drug equal to 5:1 (in base pairs). ST DNA melts with a single transition (from double to single stranded) at ~70 °C. Many DNA interacting agents, and particularly known intercalators such as acridines and anthracyclines, have the ability to stabilize DNA. However, our compounds did not show any significant stabilization effect on the DNA double helix, with only minor changes in the melting temperature of the ST DNA upon addition of the drug (see Table 1).

The effects of our fluorenone-carboxamide compounds on the fluorescence of the ethidium–DNA complex have also been studied. The quenching values for each compound were determined using native ST DNA. Compound **3** gave the largest quenching of fluorescence when using ST DNA at pH 7.4. The total ethidium–DNA quenching of fluorescence induced by the drugs was variable from compound to compound and ranged between 60% and 93% (data not shown).

According to Morgan et al.[22] the ethidium displacement assay employing an excess of DNA (see Supplementary Information) can give information on the binding site size for a drug, and is therefore a method for distinguishing simple intercalating agents (which have an effective site size of two base-pairs) from agents which occlude a large number of base pairs. Agents which have a large site size for DNA binding require correspondingly smaller concentrations to saturate the sites.

The number of base pairs per drug molecule was found for all compounds to be different than two. Thus, the ethidium displacement data suggest that these compounds have a large site size for DNA binding. Consequently, a non-intercalating binding mode must be also present, rather than a strict intercalation of the drug into the double-helix. This is in accordance with previously reported results.[5]

To investigate the specificity of the above compounds for various DNA isoforms, including higher order DNA structures such as triplex and quadruplex formations, we have used competition dialysis experiments using a range of polynucleotides: ST DNA, poly[dG]–poly[dC], poly[dG–dC]–poly[dG–dC], poly[dA]–poly[dT], poly[dA–dT]–poly[dA–dT], triplex forming poly-nucleotide poly[dT]*poly[dA] poly[dT] (pyrimidine triplex), the d[$G_3A_4G_3$]*d[$G_3A_4G_3$]d[$C_3T_4C_3$] (purine triplex) oligonucleotide, and the tetraplex forming sequence d[AG_3 (T_2AG_3)$_3$]. The experiments were subsequently conducted according to the procedure of Ren and Chaires[23] (see Fig. 3).

The results indicate that the compounds **1**–**10** have DNA binding activity, with varying affinities for the individual DNA isoforms. However, most of them do not present pronounced specificities for individual DNA forms, but appear to be rather non-specific binders with similar affinities to all tested DNA sequences. The only exception is represented by compound **10**, revealing a consistent preference for the tetraplex form. Despite the close molecular similarity due to the presence of the morpholino moiety in the side chain, **9** exhibited much less selective binding than compound **10**.

The molecular modeling study started from previously reported conformational analysis of these compounds.[5]

The goal of the theoretical work was to identify the binding differences of the most selective tetraplex compound **10** versus the chemically closest derivative of this series **9**. Docking studies were performed using as target the crystallographic model of the telomeric DNA sequence d[$AG_3(T_2AG_3)_3$].[24]

In the pre-treatment of this model, (see Supplementary Information modeling section) the energy minimization without the K^+ counter-ions and water molecules induced some modifications in the original crystallographic model (Fig. 4). The conformational effects of the energy minimization influenced mainly the loop regions, with differences in terms of root mean square (RMS) deviation between the two models equal to 2.92 Å. So, we included both X-ray and optimized conformations in our docking experiments.

In order to take into account the conformational variability of the ligands, an energy cutting criterion was applied to select most stable conformers (see Supplementary Information). Respectively, 16 and 21 conformers for compounds **9** and **10** were included into the analysis. The different selection is in agreement with the higher level of flexibility of **10** versus **9**, due to the additional methylene in its side chain (Fig. 1).

The docking experiments were carried out following our 'quasi-flexible' MOLINE method,[25] recently successfully applied to other ligand-macromolecule case studies. A 1:1 stochiometry was considered for the molecular recognition of both telomeric–ligand complexes. In the first step, rigid systematic search and clustering operation were performed, generating more than 2 million configurations in either case. In the second step, the clustering and the rigid optimization procedures dramatically reduced these numbers to 3283 and 4383, respectively, for complexes with **9** and **10**. Finally the full energy minimization of the most stable ones yielded 344 and 455 configurations. Their analysis was carried out first by comparing interaction free energies and type of contacts between the ligands and the telomeric structure, second by the RMS deviation of the DNA versus the crystallographic tetraplex conformation (Table 2).

The effects of the ligand in the recognition of the telomeric tetraplex DNA are different. **10** resulted in more tight interaction than **9**, with a drop in both free energy and enthalpy of complexation to lower than −2.4 kcal/mol. The analysis of energy minimum complex configurations, seven in both cases, revealed a slight RMS reduction of **10** with respect to **9**.

A possible 2:1 stoichiometry of the ligand–DNA complexes was investigated by the statistical thermodynamic analysis of the top and bottom recognition sites of the telomeric receptor. The energy minimized configurations with weighted Boltzmann population at 300 °C higher than 0.1% were clustered on the basis of the recognition site, to evaluate each binding probability according to

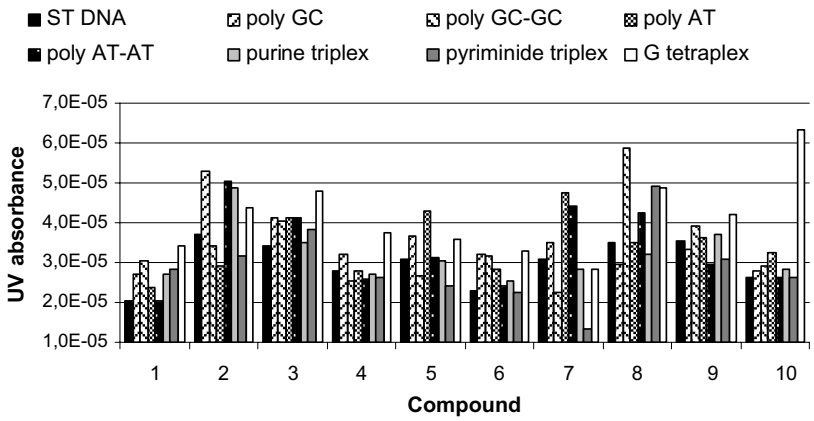


Figure 3. Competition dialysis data using various DNA base compositions and structural isoforms expressed as UV absorbance.

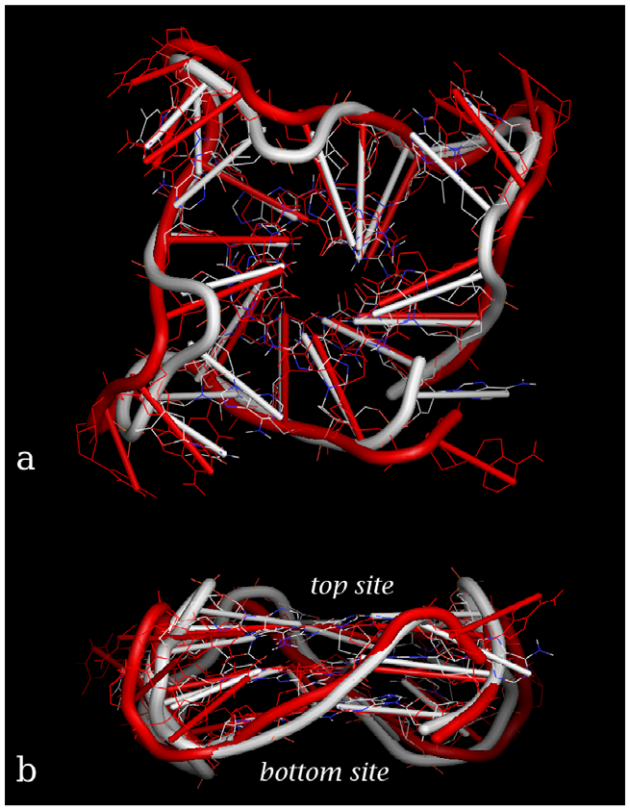


Figure 4. (a) top view and (b) side view of telomeric DNA sequence d[$AG_3(T_2AG_3)_3$] in the crystallographic model 1KF1 (red ribbon polytube model) superimposed after energy minimization (white ribbon polytube model). The two putative recognition areas are indicated as top and bottom sites.

Table 2. Thermodynamic data of the recognition process computed at 300 °C

Complex	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol)	RMS (Å)
9	−37.41	−37.16	0.83	3.00
10	−40.12	−39.62	1.67	2.93

The RMS deviation was computed comparing the atomic coordinates of the telomeric structure in most stable complex with respect to the crystallographic model.

the MOLINE method.[25] Compound **9** resulted mainly bound to the top site (>95%) of the telomeric DNA structure. Conversely, compound **10** showed a 72% of top binding preference against a 28% into the bottom site.

In order to evaluate the ionization degree of our compounds, a theoretical method was employed (see Supplementary Information). Three different kind of pK_a were found. Compounds with no tertiary nitrogen atoms **1**–**5** were predicted with extreme pK_a values >14. Compounds **6**–**8** with *N*-methyl/ethyl tertiary nitrogen gave pK_a results higher than 7.8. The morpholino derivatives **9** and **10** showed values around 6 and 7, respectively.

This work has focused on the interactions of a series of fluorenone-carboxamide derivatives with different DNA polymorphs. Ten analogs were investigated for their ability to bind DNA, using experimental and theoretical techniques. Most of them were found to be weak DNA binders, as determined by UV spectroscopy and ethidium bromide displacement assays. The compounds showed a varied range of affinities for the double stranded genomic ST DNA, depending on the side chains on the fluorenone-carboxamide scaffold. Such affinities range from no measurable interaction to affinities in the 10^5 M^{-1} for **9**, the strongest binding molecule. Furthermore, the compounds did not present any significant ability to stabilize the DNA duplex structure, with modest ΔT values.

Competition dialysis studies, using a whole range of DNA polymorphs, revealed unexpected complexity. Some compounds were found to be non-specific, weak DNA binders, well in agreement with the spectroscopic analysis data. However, some exceptions, such as compounds **2** and **8**, should be appreciated as shown in Figure 3. The particularly interesting finding from the competition dialysis experiment was the distinct affinity and specificity of the related compound **10** (see Fig. 1) for the G-tetraplex structure. Furthermore, although compounds **9** and **10** were structurally very similar, they displayed very different DNA binding affinities. The biophysical studies conducted have shown that the DNA binding profiles of the drugs also differ. **9** was found

to have a stronger affinity to duplex DNA, GC rich duplex and triplex sequences when compared with **10**, which only exhibited prominent affinity for the tetraplex structure. This characteristic specificity of the compound for G-quadruplex can be exploited in future studies for the rational drug design of a second generation of molecules with improved selectivity.

The molecular modeling study was employed to understand the binding process of the most selective drug, **10**, for the tetraplex DNA versus its closest analog **9**, both bearing the morpholino moiety as terminal group of the side chains. The interaction energies obtained in the computational work were found in agreement with the competition dialysis observations. Moreover, the stabilization of the telomeric structure in the presence of **10** was evaluated by the RMS reduction of the DNA structure, when compared to the crystallographic conformation.

Both compounds **9** and **10** showed a preferential recognition of the DNA tetraplex from the top side of the guanine-based quadruplex structure (Fig. 5). This feature is particularly displayed by the fluorenone moiety, establishing multiple van der Waals contacts with planar purine groups, that is by π–π stacking interaction. In both complexes, the amide hydrogen of the ligand established hydrogen bonds with one guanine oxygen. Conversely, a consistent difference of the two recognition models was observed in the morpholino moiety. In the compound **9**, only two methylene groups can establish productive van der Waals interaction with the telomeric structure, due to a bad orientation of the six-member ring, likely induced by the short linker (two methylene units only) to the fluorenone ring. In this configuration, this moiety cannot give large contributions to the binding of the ligand and the stabilization of the telomeric structure (Fig. 4a). Opposite is the morpholino recognition of **10** within the same site of interaction onto the telomeric structure. In this case the larger flexibility of the linker (three methylene units) allowed a better accommodation of the terminal heterocyclic moiety in the same site.

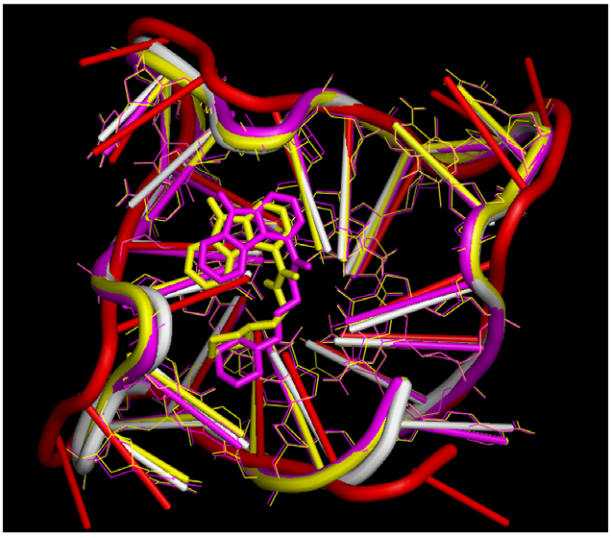


Figure 5. Superimposition of the telomeric DNA sequence d[$AG_3(T_2AG_3)_3$] in the crystallographic structure (white model), after energy minimization (red model), in the energy minimum configurations with **9** (yellow model) and **10** (magenta model).

The analysis of the interaction energy differences between the two ligands indicated the van der Waals as the major contributing term to the binding (about 45%), followed by the electrostatic term (about 30%) and by the solvating term (about 25%).

The binding site population analysis suggested a possible concurrent binding in the top and bottom DNA sites, related to a 2:1 stoichiometry, for compound **10** only.

Regarding the ionization issue, a preliminary theoretical analysis suggested no correlation between the pK_a and the experimental data shown in Figure 3. However, the ionization of these ligands can play an important role in the recognition mechanism, especially against the tetraplex DNA, where the negatively charged phosphate moieties converge in ordered loops around the putative recognition sites. This important issue will be the object of further scientific studies on this class of compounds.

In conclusion, in this manuscript we present the study of a set of compounds based on the fluorenone-carboxamide skeleton as novel DNA binding agents with potential antiviral and antitumor properties. We have identified our compounds as weak DNA binders, with the exception of the morpholino derivative **10**, which resulted in a remarkable selectivity for the quadruplex telomeric sequence. Consistent differential effects on the binding preferences of the morpholino derivatives **9** and **10** were confirmed by molecular modeling, demonstrating a major role in the length of the linker between the aromatic and heterocyclic rings. The results of this work can be useful for the rational design of new telomeric ligands based on this class of compounds.

Acknowledgments

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Supplementary data

All experimental details about drugs, nucleic acids, buffers, triplex helix formation, preparation and confirmation of tetraplex formation, UV titration, calculation of binding parameters, DNA-thermal denaturation studies, competition dialysis and molecular modeling can be found in the online version.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.02.022.

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