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State-of-the-Art Methodologies for the Discovery and Characterization of DNA G-Quadruplex Binders

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Abstract

Nowadays, the molecular basis of interaction between low molecular weight compounds and biological macromolecules is the subject of numerous investigations aimed at the rational design of molecules with specific therapeutic applications. In the last decades, it as been demonstrated that DNA quadruplexes play a critical role in several biological processes both at telomeric and gene promoting levels thus providing a great stride in the discovery of ligands able to interact with such a biologically relevant DNA conformation. So far, a number of experimental and computational approaches have been successfully employed in order to identify new ligands and to characterize their binding to the DNA. The main focus of this review is the description of these methodologies, placing a particular emphasis on computational methods, isothermal titration calorimetry (ITC), mass spectrometry (MS), nuclear magnetic resonance (NMR), circular dichroism (CD) and fluorescence spectroscopies.

Keywords : G-quadruplex ; isothermal titration calorimetry (ITC) ; fluorescence ; nuclear magnetic resonance (NMR) ; circular dichroism (CD) ; computational methods ; mass spectrometry (MS).

INTRODUCTION

The interest in G-quadruplexes has been conspicuously increased in the last years due to their role in anticancer therapy [1-3]. The stabilization by small molecules of G-quadruplex structures in crucial positions as in telomeric ends or gene promoter regions is a promising strategy to control cancer proliferation [1, 4, 5]. G-quadruplexes form through the stacking of adjacent guanosine-quartets stabilized by Na^+ or K^+ cations and their formation *in vitro* has been investigated mainly by NMR spectroscopy and X-ray crystallography Fig. (1) [6].

Evidence for G-quadruplex formation *in vivo* has been demonstrated in telomeres of ciliates [7] and in c-MYC proto-oncogene promoter [8, 9]. Telomeres are nucleoprotein complexes which preserve the chromosome ends from catastrophic instability and abnormal chromosome segregation. The telomeric DNA overhang comprises tandem repeats of guanine-rich sequences which, during the normal cellular life, after each replication cycle, are gradually shortened until a critical limit causing the cellular senescence and ultimately apoptosis [10].

The enzyme telomerase, a ribonucleoprotein complex with reverse transcriptase activity, synthesizes TTAGGG repeats onto the end of single-stranded overhangs [11]. Telomerase is overexpressed in around 85% of cancers, which favours telomerase elongation and cell immortalization [12]. Inhibition of telomerase with small-molecules selectively inhibits cancer cell growth and suggests that induction of telomere shortening is a promising anticancer strategy [13]. The formation of G-quadruplexes at the end of telomeres inhibits telomerase catalytic activity because the single stranded overhang is essential to start the catalytic cycle [14].

The human telomeric single stranded overhang is protected from higher order aggregation by a DNA binding protein (POT1), which is part of a complex of proteins, denominated shelterin [15], that regulates telomerase activity in cancer cells. This leads to maintaining telomere length [16, 17]. Thereby, both POT1 and G-quadruplex play important roles in regulating telomere length homeostasis [18]. POT1 ablation leads to telomere deprotection and initiates DNA damage-response mediated cell death. Small molecules that compete with POT1 and promote the folding of the single strand into G-quadruplex structures may initiate the damage-response [19-21]. Therefore, G-quadruplex stabilization beyond the function of telomerase inhibitors could be also a DNA damage signal [22].

Furthermore, it has been found that G-quadruplex formation at the 3' end of telomere DNA inhibits its extension by the alternative lengthening of telomere (ALT) mechanism, as well as the unwinding of quadruplexes by helicase [23]. For all these functions G-quadruplexes serve as effective drug targets for controlling telomere function.

The well-defined architecture of G-quadruplexes makes them amenable to usage in a selective manner with respect to DNA double helix sequences. After the discovery that a disubstituted anthraquinone derivative was first able to inhibit telomerase activity *via* quadruplex induction and stabilization [24], many quadruplex-binding ligands have been proposed [5, 13, 25].

Among the molecules found capable of binding G-quadruplexes *in vitro,* only few have been evaluated in cell-based assays. Their antitumor activity has been proved by xenograft models: BRAC019 [26], RHPS4 [27, 28] and the natural product telomestatin [29] Fig. (2). In particular, telomestatin was the first compound to show both high quadruplex affinity and telomerase inhibitory potency [30].

Interestingly, another recently tested compound, quarfloxin, a fluoroquinolone-based antitumor agent with a large aromatic surface area Fig. (2), is a first-in-class G-quadruplex-interactive compound that has reached Phase II clinical trials [31]. Quarfloxin is active against neuroendocrine/carcinoid tumours and has a mixed mechanism of action as a topoisomerase II poison and a G-quadruplex interactive compound [32]. The target quadruplex is formed by purine-rich strand in the NHE III_1 of the MYC promoter and preferentially forms a parallel stranded structure (for a review see Ref. [33]).

The focus of this review is on the biophysical methodologies, both experimental and computational, available to identify new ligands as putative drugs and to investigate their interactions with G-quadruplexes. The methods reported here are not a comprehensive compilation of the numerous methodologies devoted to the study of the quadruplex ligand interaction. In particular, we focused our attention on techniques like computational methods, isothermal titration calorimetry (ITC), mass spectrometry (MS), nuclear magnetic resonance (NMR), circular dichroism (CD) and fluorescence spectroscopies.

Fig. (1). *(A) NMR structure of the anti-parallel G-quadruplex formed by the d[$AG_3(T_2AG_3)_3$] sequence in Na^+ solution (pdb code: 143D); (B) NMR structure of the G-quadruplex formed by the d[$TAG_3(T_2AG_3)_3$] sequence (hybrid-1) in K^+ solution (pdb code: 2JSM); (C) NMR structure of the G-quadruplex formed by the d[$TAG_3(T_2AG_3)_3TT$] sequence (hybrid-2) in K^+ solution (pdb code: 2JSL); (D) NMR structure of the antiparallel G-quadruplex formed by the d[$G_3(T_2AG_3)_3T$] sequence in K^+ solution (pdb code: 2KF8); (E) crystal structure of the parallel G-quadruplex formed by the d[$AG_3(T_2AG_3)_3$] sequence in the presence of K^+ (pdb code:1KF1).*

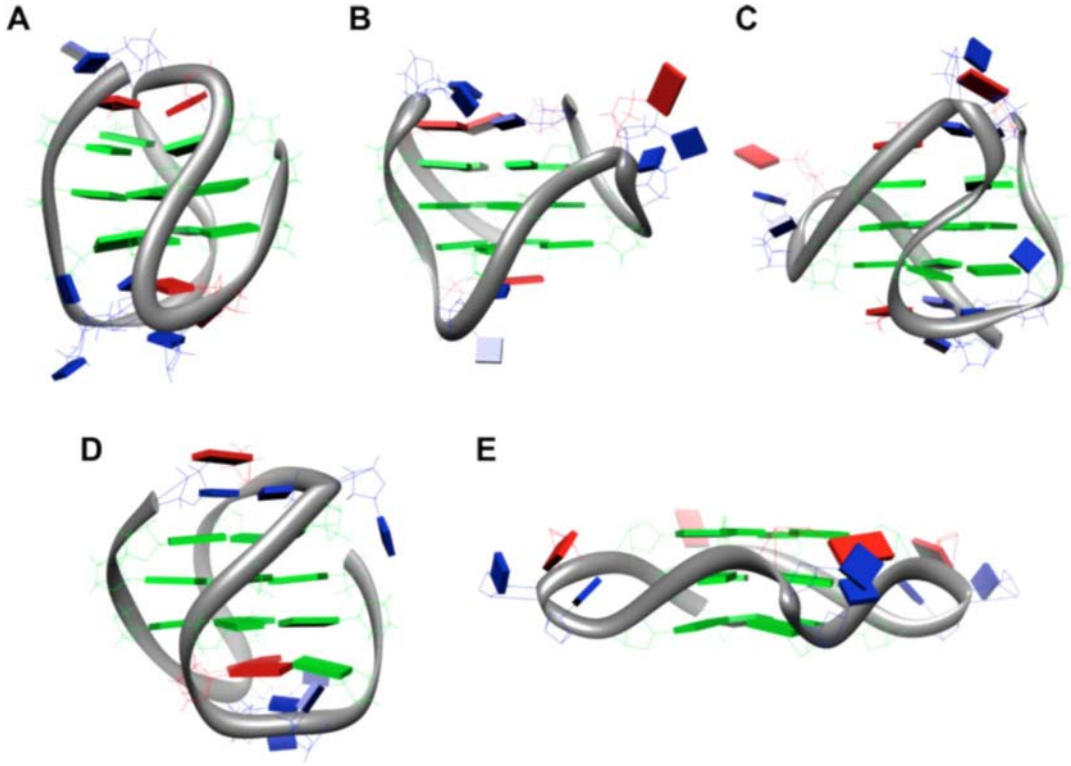
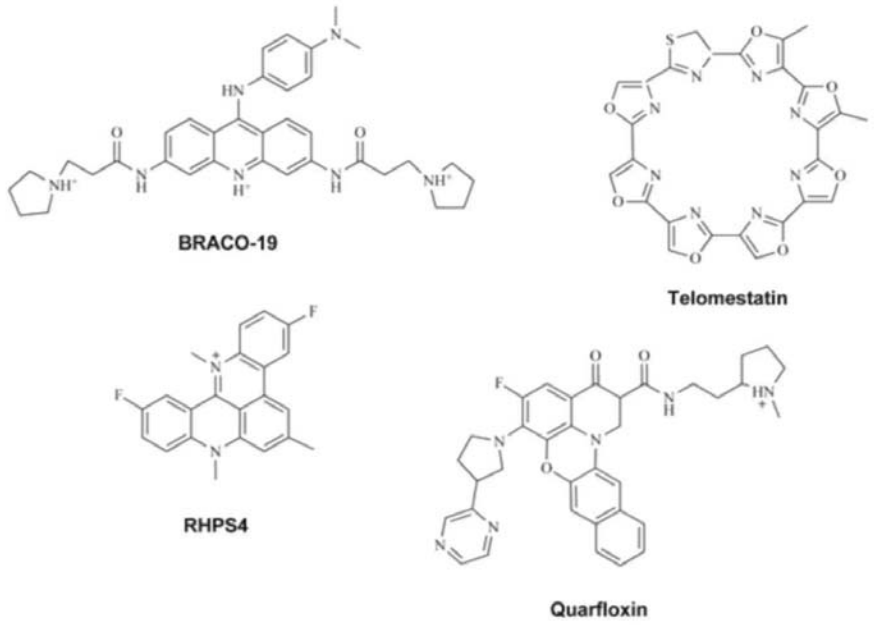


Fig. (2). *Chemical structures of quadruplex-binding ligands with proved antitumor activity.*



VIRTUAL SCREENING

In the past decades, Virtual Screening (VS) has significantly changed the way novel bio-active molecules are discovered. Such an *in silico* method allows to evaluate databases made up by hundreds of thousands of compounds with the goal of identifying the most promising compounds for further study [34]. Moreover, the rapid increases in the number of high resolution three-dimensional proteins structures [35] as well as the development of public domain repositories of compound structures and activity data [36] have given a great stride in the application of both receptor-based and ligand-based VS techniques.

Today, VS campaigns are mainly applied in the identification of new lead compounds for the inhibition of protein targets. Nevertheless, in the last decade the characterization of non-canonical nucleic acid structures as potential therapeutic targets stimulated the use of these techniques also in the identification of nucleic acid binders. Indeed, modeling non-canonical nucleic acid structures such as G-quadruplex can become a challenging task when considering its structural polymorphism, the presence of a central ion channel [37] and its intrinsic flexibility. On the other hand, accurate parameters in nucleic acid force fields have been recently developed along with new algorithms [38].

Receptor-Based VS

The early works by Evans *et al.* [39] and Holt *et al.* [40] demonstrated that molecular docking techniques can be used on nucleic acids for the design of biologically active small molecules. Since these pioneering studies, successful examples of receptor-based VS campaigns have been published [41] in which G-quadruplex three-dimensional structures (3D), solved by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, were used to perform high-throughput docking calculations of molecular databases.

A great contribution in this field has been given by Ma and coworkers. In fact, in 2008 this group published the results of a receptor-based VS campaign aimed at finding new binders of the intramolecular human telomeric G-quadruplex DNA (PDB code 1KF1) [42]. In particular, a subset of drug-like compounds (100000) of the ZINC database [43] was screened *in silico* using the ICM method (Molsoft) [44]. Briefly, this methodology allows the fully flexible ligands to be docked to a grid representation of the receptor and the calculated binding pose is associated with a score reflecting the quality of the complex (ligad/receptor fit, desolvation and hydrophobic effects and entropy loss upon binding). In these docking experiments the whole intramolecular G-quadruplex DNA molecule was considered and energy calculations were based on the ECEPP/3 force field with a distance-dependent dielectric constant. The predicted complex is then optimized through the biased probability Monte Carlo (BPMC) minimization procedure. This methodology is very fast as each ligand docking run lasts c.a. 5 min on one processor.

Through these calculations the 1H-pyrazole-3-carboxy-4-methyl-5-phenyl-(1H-indol-3-ylmethylene)hydrazide Fig. (3) was selected and evaluated for its ability to stabilize G-quadruplex DNA by using a high-throughput fluorescence resonance energy transfer (FRET) assay [45]. These experiments demonstrated that the aforementioned compound is able to selectively stabilize the quadruplex-forming human telomeric sequence (5'-FAM-d(GGG [TTAGGG]$_3$)-TAMRA-3') over the double-helix-forming oligomer (5'-FAM-d(TATAGCTATA)-HEG-d(TATAGCTATA)-TAMRA-3'). Encouraged by these results, the authors further characterized the binding between the aforementioned compound and G-quadruplex through circular dichroism (CD) experiments and additional docking experiments both indicating an end-stacking binding pose.

The above described study resulted in the discovery of a novel quadruplex end-stacker. In this regard, it should be mentioned that most of the interacting molecules discovered so far have been found to interact with the wide π-stacking surface of the G-tetrads at the 5' and/or 3' edges of the quadruplex [47]. To achieve such a binding pose quadruplex end-stacker usually feature extended planar macrocycles and cationic substituents able to interact with the anionic phosphate backbone. These structural features are present in natural products such as Telomestatin which is one of the most potent G-quadruplex binders reported so far. Thus, starting from the consideration that nature can be a vast source of chemotypes for the medicinal chemists, in 2010 Lee and co-workers identified a new natural product, Fonsecin B Fig. (3), as a potent *c-myc* G-quadruplex DNA binder [48]. In particular a database containing 20000 natural products has been used to produce VS calculations on a structural model of the nuclear hypersensitivity element III$_1$ (NHE III$_1$) which is a guanine-rich 27 base-pair sequence located upstream of the *c-myc* P1 promoter. This model was constructed starting from the X-ray crystal structure of the intramolecular human telomeric G-quadruplex DNA (PDB code 1KF1) [42]. These calculations were attained as described above with the ICM software. Five compounds were selected among the top scoring ones and tested through a polymerase stop assay to evaluate their *c-myc* G-quadruplex stabilizing properties.

Through this analysis, Fonsecin B resulted to significantly bind the *c-myc* G-quadruplex inhibiting the Taq-mediated extension (IC_{50} = ca. 20 μM), and further ICM calculations revealed that this compound is able to place its flat scaffold stacked at the ends of the G-quadruplex at the 3'-terminus.

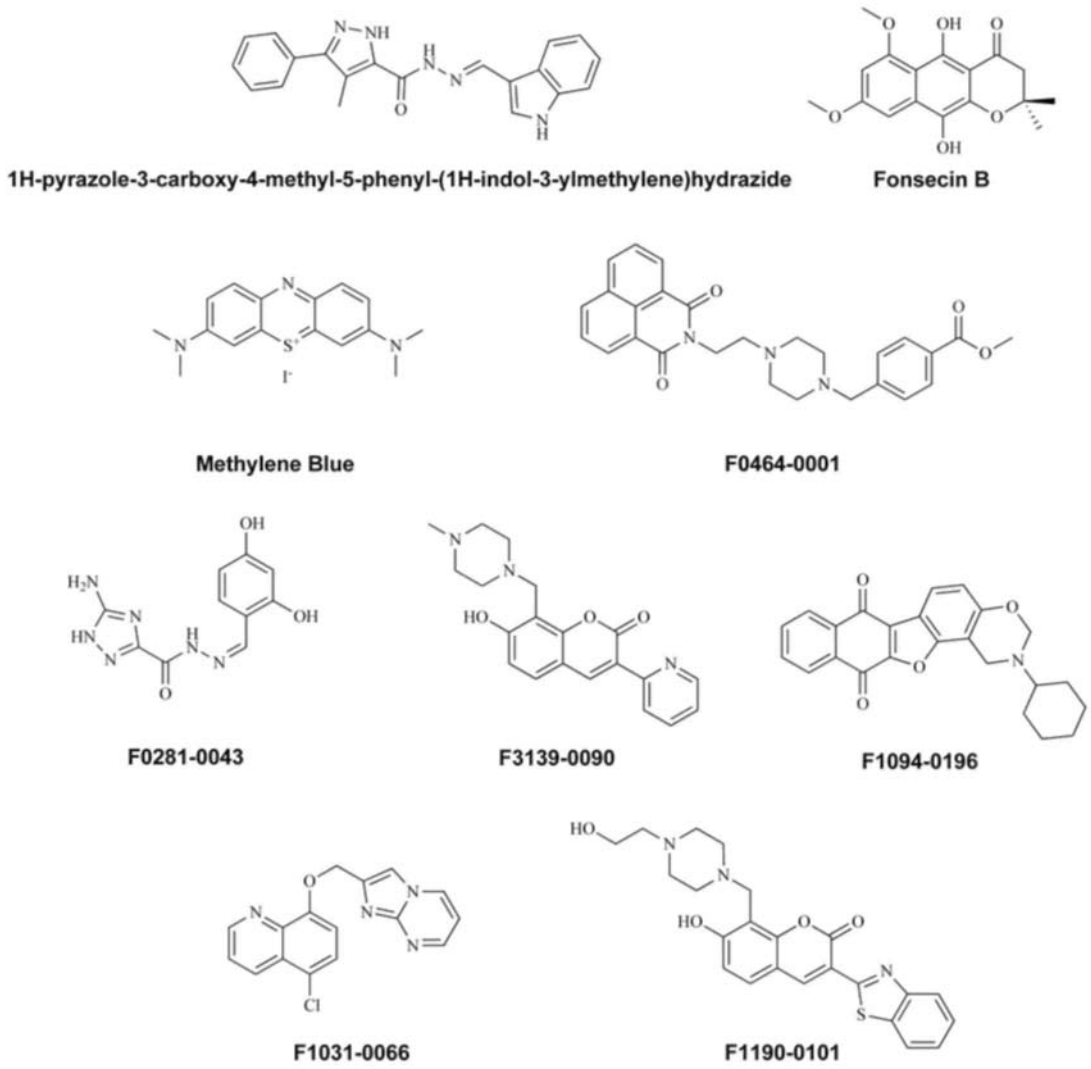
Indeed, most of the natural and synthetic compounds reported to interact with G-quadruplex structures are not drug-like [46]. Therefore, very recently, Chan *et al.* reported the results of a VS campaign in which the ICM method was used to screen a database containing over 3000 compounds of FDA-approved drugs against the above described model of the NHE III_1 [48]. Results of these docking simulations indicated the phenothiazinium salt methylene blue (MB) as a potential NHE III_1 ligand Fig. (3). This compound, featuring a plethora of biological activities (used in malaria, nitrate poisoning, methemoglobinemia, dementia and cancers treatments) was already identified as a G-quadruplex binder [49] and in 2011 Chan *et al.* [48] characterized its interaction with *c-myc* G-quadruplex. Starting from these data, the authors rationally designed a series of 50 analogues of methylene blue (MB), all featuring the phenothiazinium core structure. This database was then *in silico* screened and the best ranking ligands synthesized. In agreement with molecular modeling predictions these analogues demonstrated a good ability to bind the *c-myc* G-quadruplex in the fluorescence intercalator displacement (FID) assay. Also, the same behavior was confirmed by PCR-stop assay which also indicated that the lead-optimization step was successful as a MB analogue is able to bind *c-myc* G-quadruplex more strongly than MB.

Another contribution to the search of new quadruplex ligands was also given by our research group in 2009 [50]. Differently from the papers mentioned so far, we decided to target a very simple quadruplex, namely $[d(TGGGGT)]_4$ (PDB code 1S45) [51]. This quadruplex possesses a 4-fold symmetry with all strands parallel to each other, resulting in four grooves of identical medium width, and all nucleosides in an *anti* glycosidic conformation. The idea behind the use of this sequence resides in the assumption that the resulting structure can be experimentally (NMR spectroscopy) employed as a studying model for the inspection of the ligand interactions with human parallel quadruplexes. Moreover, this inspection was aimed at targeting only the groove area of the $[d(TGGGGT)]_4$ G-quadruplex starting from the hypothesis that grove binders can selectively recognize different DNA sequences and, in the case of quadruplex structures, can also discriminate among several quadruplex topologies, taking advantage of their different groove widths. Another difference with the other mentioned papers is the use of Autodock4 (AD4) [52], rather than ICM, as docking software. This decision was supported by a review by Trent and co-workers [53] outlining that this software optimally balances docking accuracy and ranking. Moreover, our research experience [54] increased the confidence in the use of this software in such a practical rational drug design task. The current version of AD, AD4.2, relies on a number of approximations to predict the conformation and free energy of binding during a docking simulation. The ligand is treated as flexible, but unlike traditional molecular mechanics methods, only torsional degrees of freedom are explored, holding bond angles and bond lengths constant. This allows very rapid transformations of coordinates during the search, but may cause problems if the complex requires significant distortion of the ligand upon binding. In addition, the simple tree-like structure of the data representation used for the ligand does not allow direct modeling of flexibility in rings, although several methods to reclose ring structures during a docking experiment are currently available in AD. The empirical free energy force field is based on a molecular mechanics force field, which includes typical terms for dispersion/repulsion, hydrogen bonding, electrostatics, desolvation, and torsional entropy. The force field has been calibrated against a large database of complexes with known structure and binding constant, allowing the force field to predict binding free energies. During the docking simulation, a grid-based method is used for energy evaluation, where interaction energies are pre-calculated around the target structure and then used as look-up table to allow rapid evaluation of ligand-target interaction. However, the use of this grid-based method requires that the target molecule is treated as rigid, unless specific sidechains are treated explicitly outside the grid. Several search methods are available in AD, including genetic algorithms, simulated annealing, and local search. All of these methods are stochastic, so repeated docking simulations are often used to validate the exhaustiveness of the search and the solution.

Given the stochastic nature of the software, in our inspection, 100 independent docking runs were attained for each ligand of the commercially available Life Chemicals Diversity set database (6000 compounds). This library is characterized chemotypes which are structurally unrelated thus representative of the chemical space covered by the entire Life Chemicals database. Docking results of this database were then analyzed to select all the top scoring ligands in terms of predicted binding free energy (ΔG_{bind}). This step was then followed by a more subjective selection step. Indeed, in our experience, visual inspection is one of the most critical steps in VS, as it can greatly help to increase the success rate. In fact, given that methods like AD have a typical error of ±2 kcal/mol in the prediction of free energies of binding, estimated free energy values should never be used as the sole criterion for selecting the ligands that will eventually be tested. In this case, it has been decided to discard all the individuals which were not predicted to establish tight interactions with the groove of the quadruplex structure. More precisely, compounds that were not able to form H-bonds with any of the guanine bases and/or

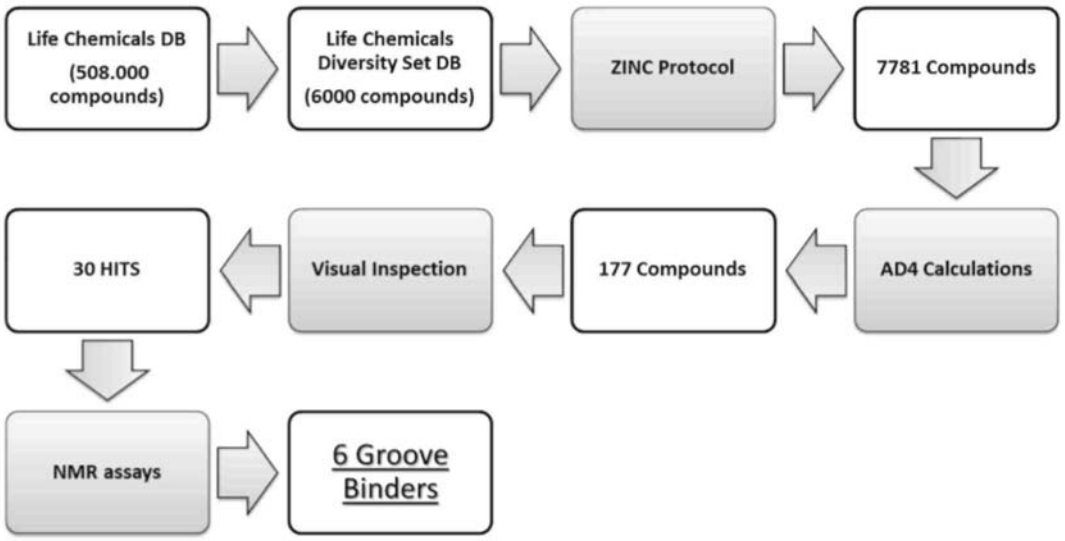
to establish an electrostatic interaction with the backbone phosphate groups were not considered. This filtering scheme (Scheme 1) allowed the selection of 30 different compounds that were subsequently purchased and tested for their ability to interact with the $[d(TGGGGT)]_4$ quadruplex through NMR spectroscopy experiments. These experiments led to the identifications of six molecules (Fig. (3): F0281-0043, F0464-0001, F3139-0090, F1094-0196, F1031-0066, F1190-0101) that are able to cause an appreciable shift of the central guanines, thus suggesting a groove binding interaction.

Fig. (3). *Structures of the G-quadruplex binders discovered by means of Receptor-based VS techniques.*



All these contributions demonstrate that receptor-based VS can now be considered a first choice technique for the discovery of new quadruplex binders as well as for their rational optimization. Surely, the existence of many compounds libraries as well as different computational techniques for evaluating ligands in these libraries greatly facilitates the challenging task of finding a "needle in the haystack". However, the limitations of the implemented scoring functions, which are mainly designed to describe binding interactions with proteins rather than nucleic acids, are such that successful outcomes are not guaranteed, and VS still requires significant proficiency and manual intervention to produce positive results.

Scheme 1. *Receptor-Based VS workflow applied in Ref. 50.*



Ligand-Based VS

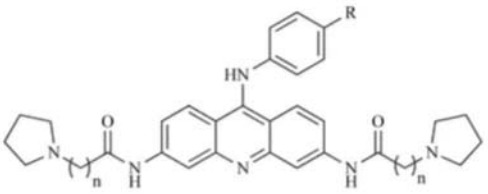
This methodology relies on the use of a given query (2D or 3D) which is used to search chemical databases to find compounds that best match such a query. In general these methods are based on the Similarity Property Principle [55], which simply states that similar molecules should have similar biological activities. Since the prior knowledge of an adequate number of molecular structures able to bind the target at the same site is a pre-requisite it is easy to understand that the lack of sufficient structural information on quadruplex binders has long hampered the application of ligand-based VS in this field. Nevertheless, two pioneering papers on the application of this approach in the discovery of new G-quadruplex binders were published.

In the study by Li *et al.* [56] the authors started from the study of series of 1,4-disubstituted anthraquinone derivatives Fig. (4) showing cytotoxicity against the telomerase positive rat glioma C6 cells indicating a possible stabilization of the G-quadruplex. These compounds were analyzed through the CATALYST software package (version 4.11, Accelrys Inc., San Diego, CA) for their conformational behavior with a Monte-Carlo-like algorithm. Thus for each ligand of this training set a maximum of 250 conformers was considered for the model generation. This model was then used to generate a "HypoGen" model, which describes the pharmacophore hypothesis. The best hypothesis (i.e. the one with the highest correlation factor) was described by the presence of one hydrogen bond donor, one hydrogen bond acceptor, one positive ionizable area and two hydrophobic areas. This 3D pharmacophore was then used to filter out all the compounds from the Chinese Herbal Medicine compounds 3D Database [57] that were not able to match it. This protocol resulted in 176 hits characterized by broad structural diversity and, of these, only the ones for which a sufficient quantity could be obtained were retained (20 compounds). Of these, the most structurally interesting ones, peimine and peiminine Fig. (4), were studied through NMR spectroscopy and CD to probe their ability to interact with the G-quadruplex. These experiments along with docking calculations unambiguously demonstrated these two compounds are able to stabilize the parallel $[d(TGGGGT)]_4$ by binding to its groove. Also, the same ligands demonstrated to preferentially bind the quadruplex over the duplex.

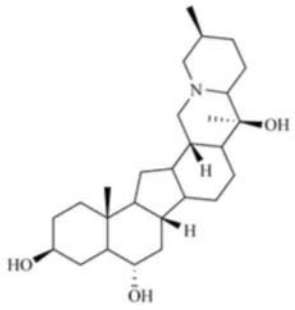
Very recently a 3D pharmacophore-based VS was also adopted by Chen *et al.* [58] and in this case a series of acridine derivates with telomerase inhibition activity (EC_{50} values) ranging from 0.067 to 6.9 μM were used to produce a pharmacophore model with the 3D-QSAR Pharmacophore Generation module/Discovery Studio (version 2.5, Accelrys Inc., San Diego, CA). HypoGen hypothesis generation process produced 10 top-scored that were then analyzed through the associated statistical parameters (null cost, fixed cost and total cost values). According to these parameters the most significant pharmacophore model featured the presence of one hydrogen bond donor, one hydrophobic and two positive ionizable sites. This model was used to an in-house compound library (5000 natural products and their derivatives). 31 chemotypes were selected through this protocol an then compounds with molecular weight over 850 were removed as well as all the molecules that could not be obtained from the compound library or re-synthesized. Of the 10 remaining compounds the six quindoline and

berberine derivatives had already been described as telomeric G-quadruplex ligands while four new triaryl-substituted imidazole derivatives were present. These compounds were subjected to experimental screening with FRET assay and one was found to significantly bind and stabilize telomeric G-quadruplex DNA. Also in this case as well a good selectivity towards telomeric G-quadruplex DNA over the duplex was proven through surface plasmon resonance (SPR) experiments.

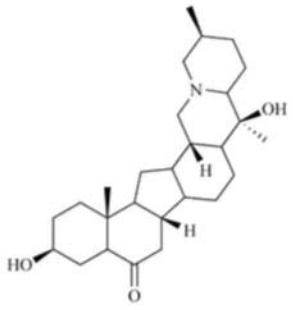
Fig. (4). *Structures of the G-quadruplex binders discovered by means of Ligand-based VS techniques.*



1,4-disubstituted anthraquinone derivatives



Peimine



Peiminine

ISOTHERMAL TITRATION CALORIMETRY (ITC)

Isothermal titration calorimetry (ITC) is a biophysical method for evaluating the thermodynamics of a molecular interaction [59]. ITC directly measures the heat of interaction (enthalpy change, $\Delta_bH°$), at a constant temperature, on titrating two compounds of known concentration forming an equilibrium complex. Moreover, it is a high-accuracy method for measuring binding affinity and stoichiometry. Of all the techniques that are currently available to measure binding, ITC is the only one capable of quantifying both enthalpic and entropic components of the interaction, revealing the overall nature of the forces that drive the molecular recognition.

ITC is a useful tool to study the energetic aspects of interaction between G-quadruplexes and other biomolecules, including small ligands [60]. A typical ITC experiment is carried out by the stepwise addition of one of the component of the complex (for example, a ligand) into the sample cell containing the other component (for example, a quadruplex molecule). At any point in the titration, the amount of free or bound ligand can be determined, establishing the equilibrium binding constant (Kb). Because the temperature (T) is kept constant throughout the entire experiment, the free energy of the binding reaction ($\Delta_bG°$) can be determined from $\Delta_bG° = -R\ T\ \ln K_b$, where R is the gas constant. Since ITC directly measures the enthalpy change, the change in entropy ($\Delta bS°$) can be calculated as $\Delta_bS° = (\Delta_bH° - \Delta_bG°)/T$. Moreover, the plot of the heat released or absorbed arising from the binding at each addition as a function of the ligand/quadruplex molar ratio, typically produces a curve with a sigmoidal shape that facilitates the estimation of the midpoint of the reaction process, and thus the stoichiometry of the binding reaction.

Thanks to the great current interest in developing molecules that can selectively bind to G-quadruplexes, the number of ITC studies on the interaction between small ligands and G-quadruplex structures is rapidly growing [5, 61-63]. Many small molecules that bind to quadruplexes have proven to be effective therapeutic agents, although the exact mode of binding and nature of thermodynamic forces that regulate DNA-ligand interactions

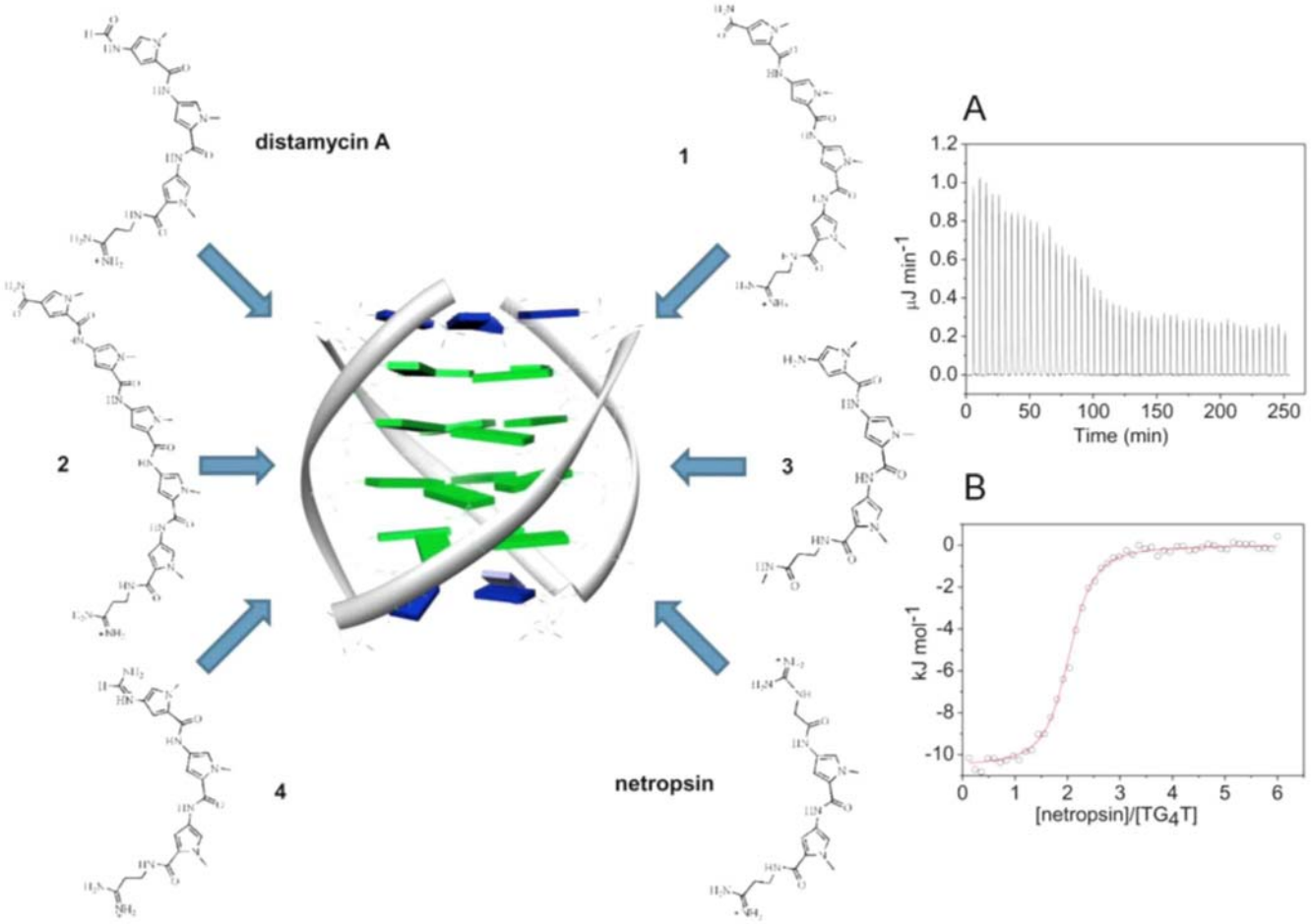
are often poorly understood. Recent advances in instrumentation, together with a broad availability of automated and high-sensitivity ITC instruments, have enhanced the opportunity to provide additional information to drug discovery and optimization processes. Indeed, drug development of lead compounds can be greatly enhanced by detailed knowledge of the thermodynamics of binding to the target [64]. Since ITC has the ability to determine the different energetic contributions to the binding affinity, it can provide a unique bridge between computational and experimental analysis. Indeed, the enthalpic and entropic contributions to the Gibbs energy of the binding process are related to the structural parameters and can be used as guide to drug design [65]. In addition, those contributions can be used to confirm the structure-based computational predictions of binding energetics, and to find structure-energy correlations.

Among the advantages of ITC is that it allows the accurate determination of thermodynamic parameters to be made with no requirement for chemical modification, labeling or immobilization of the DNA molecules and/or ligands.

ITC has been used to characterize the energetics of a variety of small molecules that have been devised to bind and stabilize G-quadruplex structures, ranging from porphyrins to acridines, anthraquinones, and other polycyclic compounds [60-63]. For example, ITC has been successfully employed to investigate the interaction of distamycin A and its derivatives with the $[d(TGGGGT)]_4$ quadruplex [66-70]. Distamycin A Fig. (5) is a small molecule which binds with high affinity to duplex DNA, however, this molecule and some its analogues have also been shown to interact with DNA quadruplex structures [71, 72]. This finding, along with the observation that derivatives of distamycin could be effective inhibitors of the human telomerase [73], has stimulated our group to carry out calorimetric investigations aimed at characterizing interactions of distamycin and its derivatives with G-quadruplexes. ITC measurements showed that the binding of distamycin to $[d(TGGGGT)]_4$ in K solution is characterized by two distinct binding events, each involving two drug molecules, to give a final 4:1 complex [67]. Furthermore, the thermodynamic parameters (entropically driven process) suggested that distamycin interacts with the grooves of the $[d(TGGGGT)]_4$ quadruplex. All these findings were confirmed by the NMR structure of the complex which shows two distamycin dimers bound to two opposite grooves of the quadruplex. These results encouraged the design and the study of new quadruplex groove binders. Then, the importance of the crescent shape extension was investigated by varying the pyrrole units number in distamycin [68]. The attention was focused on the interaction of two carbamoyl analogues of distamycin A (1 and 2, Fig. (5)), containing four and five pyrrole units, respectively. Experiments revealed that the presence of one additional pyrrole unit affect the affinity as well as the stoichiometry (2:1 ligand:quadruplex) of the binding, whereas, the addition of two pyrrole units lead to a total loss of interaction between the derivative and the $[d(TGGGGT)]_4$.

Next, to investigate the importance of the unique positive charge of distamycin A in the interaction with $[d(TGGGGT)]_4$, an uncharged analogue was synthesized [69]. The major change into the structure of the ligand was the replacement of the amidinium group by an N-methyl amide moiety (3, Fig. (5)). The binding stoichiometry was found to be 4:1 (ligand:quadruplex), as for the distamycin, but the thermodynamic parameters determined by ITC for the interaction of derivative 3 were slightly different from the ones of distamycin A to the same target. In both cases, the binding reaction was an entropically driven process. However, the binding of 3 shows a small unfavorable enthalpy change, while in the case of distamycin a small favorable enthalpy change was observed. This interesting difference is probably due to structural features of the two ligands. Indeed, the positively charged amidinium moiety of distamycin interacts with the phosphate groups of the quadruplex, providing a favorable (although small) enthalpy contribution, while in the derivative 3, the amidinium group is replaced by an uncharged moiety that cannot give this contribution.

Fig. (5). *Chemical structures of distamycin A and its derivatives targeting [d(TGGGGT)]$_4$ G-quadruplex structure. Insets show examples of raw ITC data (A) and binding isotherm (B) for titration of [d(TGGGGT)]$_4$ with netropsin.*



Finally, ITC has been employed to evaluate the effect of a second cationic group, placed at the end of the ligand molecule, on the interaction with the [d(TGGGGT)]$_4$. In particular, we characterized the interaction between the quadruplex and a dicationic derivative of distamycin A (4, Fig. (5)), where the formamide group is replaced by a charged N-formimidoil moiety [70]. We also compared the binding of compound 4 to [d(TGGGGT)]$_4$ with the binding of another dicationic analogue, netropsin Fig. (5), to the same target. ITC experiments revealed that both compound 4 and netropsin bind to the investigated quadruplex. The thermodynamic profiles of the two ligand-quadruplex interactions are qualitatively similar and in both cases the stoichiometry observed is 2:1 (ligand:quadruplex). Interestingly, the thermodynamic parameters determined by ITC indicate that the association reactions of both ligands with [d(TGGGGT)]$_4$ are entropically driven processes, even if the direct ITC measurements of the binding enthalpy change indicate that also the enthalpic contribution favours the associations. In the case of the interaction of distamycin A with [d(TGGGGT)]$_4$, similar results were obtained (entropically driven process with a small favourable enthalpic contribution), except for stoichiometry and magnitude of binding constant, suggesting a similar groove binding mode also in these cases. Interestingly, the 2:1 (ligand:quadruplex) stoichiometry suggested that 4 and netropsin are not able to bind the quadruplex in a dimeric form, most probably due to the doubly charged nature of the molecules that prevents a side-by-side arrangement into the grooves.

ITC can also be used to evaluate the selectivity of a ligand for G-quadruplex structures over duplex DNA. For example, Pilch *et al.* have characterized by ITC the binding of two synthetic oxazole-containing macrocyclic compounds (namely, HXDV and HXLV-AC) to the intramolecular G-quadruplex structural motif formed by human telomeric DNA [74]. Binding to the quadruplex was associated with a stoichiometry of two ligand molecules per DNA molecule. Moreover, thermodynamic data lend support to the proposal that HXDV and

HXLV-AC bind human telomeric G-quadruplex DNA *via* a nonintercalative "terminal capping" mode in which one ligand molecule binds to each end of the quadruplex. Interestingly, the ITC profiles for the titration of a duplex DNA into either buffer alone or buffer containing a ligand were essentially identical, indicating that the compounds bind solely to the quadruplex nucleic acid form, but not to the duplex.

Recently, some of us used ITC to compare the binding properties of the $d(TTAGGG)_8TT$ sequence, forming two adjacent quadruplex units, with the binding properties of the $[d(AGGGTT)]_4$ sequence, forming a single quadruplex structure, by using the three side-chained triazatruxene derivative azatrux and the cationic porphyrin TMPyP4 as ligands [75]. The number of binding sites per quadruplex unit available in the two-quadruplexes-forming sequence was found to be different from the one expected on the basis of the results obtained in the studies on the binding to a single quadruplex. This work suggests that the quadruplex units along a multimeric structure do not behave as completely independent. The presence of adjacent quadruplexes results in a diverse binding ability not predictable from single quadruplex binding studies. The existence of quadruplex-quadruplex interfaces in the telomeric overhang may provide an advantageous factor in drug design to enhance both affinity and selectivity for DNA telomeric quadruplexes.

It is important to bear in mind that the presence of multiple conformations in solution could invalidate the thermodynamic parameters obtained by ITC measurements. An example is just represented by the human telomeric sequences. Indeed, several papers concerning this topic do not consider the polymorphism of those sequences, causing a poor interpretation of the phenomenon. In many cases, as the previous one, the assumption that the ligand binds to a main target conformation could help to bypass the problem.

On the other hand, ITC can be used to evaluate the effect of the different quadruplex conformations on the interaction with a small molecule. In fact, an intramolecular quadruplex sequence can adopt different conformations, owing to different orientation of the loops in the structure. The differences in the loop orientation can affect their molecular recognition. To evaluate this effect, Arora and Maiti characterized the binding of cationic porphyrin TMPyP4 to three G-quadruplexes which differ in loop orientations [76]. The DNA quadruplexes used in the study were those formed by the 21-mer $d[G_3(T_2AG_3)_3]$ from human telomeric sequence, the 22-mer $d(G_4AG_3TG_4AG_3TG_4)$ and the 21-mer $d(G_3AG_3CGCTG_3AG_2AG_3)$ from the promoter region of *c-myc* and *c-kit,* respectively. The association of TMPyP4 with all the quadruplex structures exhibited negative changes in the binding enthalpies. ITC experiments showed two independent binding processes, a stronger binding (10^6 M^{-1}) of TMPyP4, probably involving end stacking, and a weaker external binding (10^6 M^{-1}). Moreover, they revealed that the TMPyP4 molecule shows preferential binding to parallel G-quadruplex over antiparallel. Indeed, the binding affinity for parallel quadruplexes (10^6 M^{-1}) was one order of magnitude higher than for the antiparallel structure (10^6 M^{-1}). They concluded highlighting that differences in the loop orientation give rise to different conformations of quadruplex, which in turn govern the binding to small molecules, playing a central role in molecular recognition.

One of the drawbacks of ITC in characterizing G-quadruplex binders is the poor solubility in water of some small molecules that sometimes hamper canonical ITC experiments, in which a solution of the quadruplex is titrated with a concentrated ligand solution in the identical aqueous buffer. An alternative approach to investigate the affinity of poor water soluble compounds for the quadruplex DNA was recently employed by some of us [77]. In particular, competition/displacement experiments were performed, by analyzing the ability of distamycin A to bind the quadruplex in the presence of another compound. Despite the solubility concerns, mixtures of quadruplex and each tested compound were successfully prepared by dissolving the molecules in DMSO and diluting them in an aqueous buffer solution containing the quadruplex. These complexes were then titrated with distamycin A, and the outcome followed by ITC. In some cases, distamycin A appeared to be no longer able to interact with the quadruplex, suggesting that the water insoluble ligand binds the DNA more tightly than distamycin A.

In summary, although the competition/displacement experiments do not give any information about the stoichiometry of the complex, they represent a possible strategy to evaluate whether a water insoluble quadruplex binder is stronger or weaker than a soluble one that could be used as reference.

ELECTROSPRAY MASS SPECTROMETRY (ESI-MS)

The major interest of mass spectrometry is to allow an assumption-free determination of the mass, and therefore of the stoichiometry of each complex present in solution. Because mass spectrometers are all based on the separation of ions in high vacuum, the complexes to be measured must be ionized and desolvated. This is the role of the ionization source. The ionization method that is most compatible with the analysis of non-covalent

complexes from native solution conditions is electrospray ionization. All types of mass analyzers that can be coupled to electrospray ionization are suitable to analyze nucleic acid complexes and G-quadruplexes. The use of mass spectrometry to study nucleic acid complexes has been reviewed elsewhere [78-82], and a recent review is devoted to mass spectrometry of G-quadruplexes [83]. Here we focus on particular aspects pertaining to the analysis of complexes between G-quadruplexes and ligands by ESI-MS, illustrate these points with example spectra, and highlight a few recent studies.

ESI-MS of G-quadruplexes

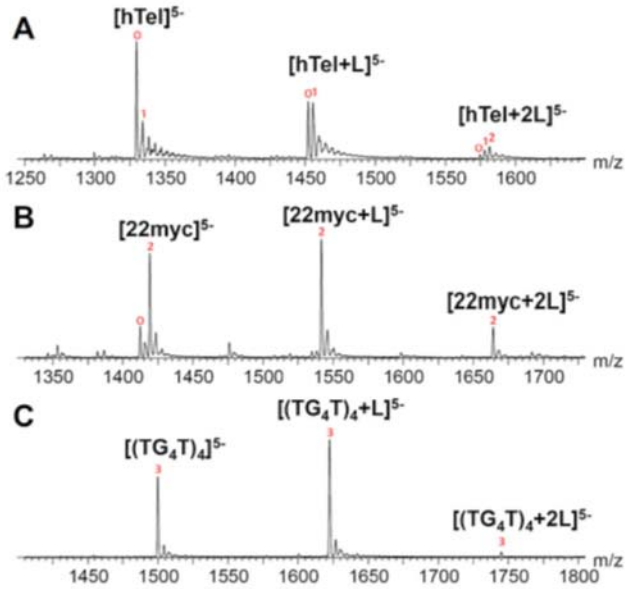
Purely aqueous solutions can be analyzed with electrospray on most instruments. Nevertheless, it remains a common habit to add 10 to 20% organic co-solvent (usually methanol or isopropanol) to increase the volatility of the solvent mixture and therefore increase the total signal-to-noise ratio. More critical than the solvent is however the other electrolytes present in solution to ensure the proper folding of the G-quadruplex structure. In native ESI mass spectrometry, for proteins as well as for nucleic acids, ammonium acetate is typically used to reach the desired ionic strength (for example, physiological ionic strength is mimicked by using 150 mM NH_4OAc). However, unlike for proteins or duplex DNA structure, the G-quadruplex structure in solution is sensitive not only to the ionic strength, but also to the nature of the cation. For example, the human telomeric sequence $dGGG(TTAGGG)_3$ folds into hybrid forms in KC1 [6], into an antiparallel form in NaCl [6], and mostly into an antiparallel form in NH_4OAc [84]. A systematic study of G-rich sequences containing four tracts of three guanines in NaCl, NH_4OAc and KC1 revealed that NH_4 has intermediate properties between K and Na in terms of type of structure formed [85].

In the electrospray source, a solution containing the complexes is infused in a thin capillary on which a high voltage is applied. As a result, the sample solution is vaporized in a mist of charged droplets. Most biomolecules are already ionic in solution; electrospray is therefore not so much here an ionization method than a method to extract pre-existing ions from the solution. Because nucleic acid complexes are polyanions in solution, they are analyzed in negative ion mode (negative voltage applied on the capillary, resulting in negatively charged droplets). The droplets are then accelerated in different regions of the source, where they collide with gas, and hence evaporate, shrink and fission into smaller charged droplets. The process repeats until each polyanionic complex becomes isolated in a shell of remaining solvent and counter-ions. The only tricky aspect of an ESI-MS experiment is to adjust the voltages, temperatures, and pressures in the mass spectrometer so as to achieve proper desolvation (removal of solvent) and declustering (removal of counter-ions) of the complexes, without further unnecessarily colliding the naked complex and cause its dissociation. A detailed protocol describing ESI source tuning was published recently [86]. The last counter-ions to disappear are the ammonium cations, which eventually give back a proton to the nucleic acid, presumably to phosphate groups, and are released as NH_3.

Unlike all other nucleic acids, for which the number distribution of ammonium ions remaining attached is statistical, G-quadruplexes show a special behavior. For most G-quadruplexes, there is indeed a range of voltages where the number of remaining ammonium ions is biased towards the number of ammonium ions present between the G-quartets. This bias is more pronounced in G-quadruplexes having a parallel structure [87]: they preserve their inner ammonium cations on a wider range of voltages than antiparallel structures. Experimental conditions that allow the preservation of these inner ammonium ions are therefore considered "soft" source conditions. Nucleic acid-ligand complexes are also analyzed in such soft source conditions, in order to preserve the ligand bound to the nucleic acid.

Fig. (6) shows three typical mass spectra, extracted from a screening by ESI-MS. Let us focus first on the distribution of number of ammonium ions preserved in the free G-quadruplexes (left peak on each spectrum), indicated in red on the figure. Despite that all three spectra are acquired in the same soft conditions, the ammonium ion distribution differs for each G-quadruplex. For the human telomeric sequences (A), which is expected to incorporate two ammonium ions between the G-quartets, these two ammonium ions are not retained and the major peak corresponds to no ammonium adduct. In contrast, for the 22myc sequence, a 22-mer oligonucleotide from the NHE III_1 of the MYC promoter (B) and for the tetramolecular G-quadruplex $[d(TGGGGT)]_4$ (C), the number of ammonium ions retained in most intense peak corresponds to the number of ammonium ions expected to be trapped between the G-quartets (2 and 3, respectively).

Fig. (6). *Example ESI-MS spectra of quadruplex-ligand mixtures. (A) 10 µM 21-mer human telomeric sequence GGG(TTAGGG)$_3$ + 10 µM PIPER. (B) 10 µM 22-mer myc promoter sequence GAGGGTGGGGAGG GTGGGGAAG + 10 µM PIPER. (C) 10 µM tetramolecular quadruplex [dTG$_4$T]$_4$ + 10 µM PIPER. All spectra were recorded on a Waters Q-TOF Ultima mass spectrometer, in negative mode and soft source conditions, from 80% 150 mM NH4OAc/20% methanol solutions.*



Stoichiometry Determination of Ligand-quadruplex Complexes

When mixing ligands with G-quadruplex nucleic acids and analyzing the mixture by ESI-MS, free and bound nucleic acid can be detected, and one can count the number of ligands bound per G-quadruplex. For example, in Fig. (6), complexes with one and two ligands are detected for all three sequences, at this particular ligand:DNA concentration ratio (10 µM each). Excess ligand can be added to test the maximum binding stoichiometry attainable. This allows to determine the stoichiometry of each complex formed, and to write down the different equilibrium constants to be considered. This assumption-free stoichiometry determination by ESI-MS is very useful for choosing a fitting model for other experimental methods that measure a signal proportional to the sum of the contributions of each component in solution, e.g. spectroscopic or calorimetric titration methods. It is also very useful to sort out complex mixtures, as illustrated by a recent study on PNA binding to G-quadruplex forming sequences: in addition to the expected PNA binding, multiple binding and then multimer formation was revealed by ESI-MS [88].

Ligand Screening

Moreover, using the relative intensities of the peaks in the mass spectra, one can also determine the ligand binding affinities directly from the mass spectra. This can be done in several ways. On a semi-quantitative level, the visual comparison of spectra recorded at the same DNA and ligand concentrations can immediately allow to spot ligand/DNA combinations that give the greatest amount of complex. Pursuing with the example of Fig. (6) and comparing the three spectra recorded at the same DNA and ligand concentration, it is straightforward to deduce from the relative intensities of free DNA and 1:1 complex that the ligand PIPER binds more to the parallel G-quadruplexes 22myc and [d(TGGGGT)]$_4$ (spectra B and C) than to the antiparallel hTel (spectrum A).

There are different ways to present the results of screening assays. One can determine the amount of complexed DNA from the relative intensities of the complexes and free DNA [89]. Alternatively, one can determine the amount of uncomplexed DNA, by monitoring the signal decrease of the free DNA compared to an internal standard (e.g., a dTn single strand to which the ligand does not bind) [90]. Finally, one can recalculate the concentration of bound ligand from the relative peak intensities (see the section below devoted to the K_d determination) and plot these into bar graphs. The latter approach was often used to have a visual comparison with equilibrium dialysis data [91-93]. To improve the speed of such assays, series of ligands or a series of nucleic acids of different masses can be used to conduct competition experiments [90,94], the only limitation

being that each complex must be distinguishable in the spectra. The most affine ligand or the preferred target can easily be deduced from the relative peak intensities.

K_d Determination

The peak areas can also be used to quantitatively determine the different equilibrium binding constants. Importantly, the equilibrium constants determined by mass spectrometry are macroscopic equilibrium constant. For example, $K_{d(1)}$ is defined as [DNA] [Ligand]/[1:1 complex], taking into account the total amount of 1:1 complex formed, independently of the ligand binding site(s). Further discussion can be found in another review [80]. The simplest approach to determine K_d's is to assume that free and bound DNA respond in the same way upon electrospray ionization, or in other words that the relative peak areas reflect the relative concentrations in solution [80,86]. This assumption of equal response can moreover be tested by adding an internal standard nucleic acid to the tested nucleic acid, for example a short poly(dT) strand to which the ligand does not bind, and recoding spectra at increasing ligand concentrations [95]. If the ratio between the intensity of the internal standard and the sum of all intensities of free and bound nucleic acid target remains the same, it means that free and bound nucleic acid have the same response. If large variations are observed, the internal standard can be used to find the relative response factors of each family of complexes, and recalculate the concentrations and binding constants more accurately [95].

For ligands binding to nucleic acid duplexes or G-quadruplexes in simple 1:1, 1:2,... (DNATigand) stoichiometries without distorting the nucleic acid structure, it is typically found that free and bound DNA respond in the same way upon electrospray ionization, and one can assume that the ratio of intensities is equal to the ratio of concentrations to determine the equilibrium binding constants. Consequently, there is usually a good agreement between equilibrium binding constants determined by ESI-MS and those determined by other methods, if the solution conditions are the same (ammonium acetate electrolyte and absence of organic co-solvents in all methods compared). Nevertheless, to have a good agreement it is necessary to calculate the peak areas by summing the contributions of all ammonium ion adduct peaks for each complex stoichiometry. Indeed, as can be seen in Fig. (6), the number of ammonium ions retained is not necessarily the same for the free and bound quadruplex. This property can be related to the ligand binding mode, as explained below.

Ligand Binding Mode

By definition, the mass of a ligand-quadruplex complex tells nothing about the ligand binding mode or binding site, and mass spectrometry is therefore not the ideal method to determine the ligand binding mode. Nevertheless, in particular cases some useful deductions can be made based only on the masses of the complexes. First, from the maximum number of ligands bound to the G-quadruplex, one can deduce the maximum number of binding sites and from the binding constants, one can deduce whether these binding sites are equivalent or not. Many G-quadruplexes are found to allow maximum two ligands bound, and this is usually interpreted as due to the two stacking sites on the extremities of the G-stack. Second, one can use the ammonium ion count in the complexes to obtain information on the ligand binding mode. Intercalation between G-quartets would necessitate the removal of one inner ammonium cation, whereas other binding modes would not. The mass spectra in Fig. (6) illustrate this point: in the complexes with one and two ligands, not only the inner ammonium ions are preserved, but in spectra A and B they seem better preserved than in the free DNA. The ligands seem to protect the G-quadruplex from losing its inner ammoniums, and this is understandable if the ligand is capping the G-quadruplex by end-stacking. Some ligands like telomestatin and other macrocyles have even showed the trapping of an additional ammonium cation upon binding, suggesting again an end-stacking of the ligand, trapping an additional cation between itself and the terminal G-quartet [96,97]. These examples illustrate that, because MS provides an assumption-free determination of the mass of each complex, the subtlety often resides in the thoughtful interpretation of unexpected peaks.

The other category of MS-based methods which have potential for determining the ligand binding mode are the so-called gas-phase probing methods, where the desolvated complexes can be subjected, inside the mass spectrometer, to a variety of probing techniques. The most affordable technique is tandem mass spectrometry (MS/MS), where an ion of given m/z is selected, collided with a gas to cause dissociation, and the fragments are measured. A few papers reported different dissociation patterns for ligands having different binding modes to G-quadruplexes [98,99]. The two caveats of MS/MS interpretation are however (1) that collision-induced dissociation is a destructive technique, and that the fragmentation pathways or collision energy at which they occur might depend on non-native conformations attained during dissociation process, and (2) that the fragmentation pathways also depend much on the charge and gas-phase basicity of the ligand [100]. To the authors' opinion, using MS/MS to determine the binding mode of unknown ligands is therefore not a suitable

approach, and non-destructive gas-phase probing techniques should be used instead. One such promising technique is ion mobility spectrometry (IMS). IMS separates ions according to their shape by electrophoretic drift of ions in a gas, and the ion mobility can be related to a shape parameter called the collision cross sections (in $Å^2$). This technique was used for several G-quadruplex ligands [101,102], and the experimental collision cross sections were compared to gas-phase structural models generated for end-stacking, intercalating, and external binding modes. End-stacking and intercalating models were compatible with the experiment while the external binding model was not, and by using the ammonium count argument explained above, external stacking of the ligands can be concluded. IMS is therefore promising, but requires extensive modeling for its interpretation.

Advantages and Drawbacks of ESI-MS

ESI-MS is a label-free technique that suits all ligands (given that the only requirement is that the ligands have a mass). For example, in one of the ESI-MS studies cited above [90] the ligands were soluble only in DMSO and photosensitive, so they could not be studied by ITC or spectroscopic techniques. The other key advantage of mass spectrometry is the assumption-free determination of the stoichiometry of each complex formed, compared to other techniques where the measurable is proportional to the sum of the contributions of all species present in solution. Its sensitivity is a key for detecting and characterizing minor species, or complex mixtures. Techniques for ligand binding mode determination by MS-based techniques are still in their infancy, but remain nevertheless the subject of fundamental research, the goal being to determine the structure of each complex stoichiometry. Finally, one must recall that the main current limitation of ESI-MS in the field of G-quadruplexes is that it can be performed only in high ammonium acetate concentrations, and not in high NaCl or KCl concentrations. ESI-MS results compare however very well with other techniques, when the latter are conducted in NH_4OAc.

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

Nuclear Magnetic Resonance (NMR) spectroscopy is a unique tool to study molecular interactions in solution, and it became an essential technique to characterize events of molecular recognition. During the last years, due to instrumental improvements in the development of higher magnetic fields and cryogenic probes, NMR became more sensitive. At the same time, the development of new experiments and pulse sequences made NMR a unique technique to obtain information about the interactions of small ligands with biologically relevant macromolecules (proteins and/or nucleic acids), key for drug discovery.

In this section, we will give an overview about most used NMR spectroscopic methods to identify and to characterize the binding activity of ligands with DNA quadruplex structures. We intentionally have decided not to report any examples of the obvious use of NOESY experiments.

The identification of small-molecule ligands for a given target can be realized by observation of changes in NMR parameters that occur upon their interactions. A commonly used strategy for fragment-based drug discovery consists in building up high-affinity ligands in a modular way, starting from small scaffolds or "fragments" and growing, linking or merging them into high-affinity ligands. Because the individual fragments generally bind with low affinity, they are difficult to identify with conventional assays. NMR spectroscopy with its intrinsic high sensitivity to detect weak interactions becomes the method of choice to pick up such low-affinity fragments by screening of a compound library.

NMR-based interaction studies can be followed by two different approaches, namely, by looking at the target spectrum and following the changes in chemical shift by ligand titration, or recording the spectra of a sample of ligand with small amounts of target.

The first approach is applicable to any class of compound, with no upper limit in affinity, in particular, compounds with dissociation constants from mM to nM and lower can be monitored. By this approach, identification of the ligand binding site is possible, and measurement of the dissociation constant (K_d) can be pursued for millimolar and micromolar ligands [103]. Chemical shift alterations due to ligand interaction can be easily followed by simple 1D ^{1}H-NMR spectra, but total or partial resonance assignment needs to be done in order to identify the residues that are involved in the interaction. In this case, NMR is limited by the size of the target.

However, for the ligand detection techniques, spectra can be easily recorded using samples with small amounts of target, which makes this second approach more attractive. In this second approach, the choice of NMR parameters is more diverse. These comprise, diffusion coefficients, intramolecular and intermolecular

magnetization transfer, including transferred NOE, saturation transfer and WaterLOGSY experiments [104-106]. However, a general drawback of the ligand-observation methods is their inability to detect high-affinity ligands. In strongly bound ligands, slow dissociation rates prevent transfer of the properties of the relative small fraction of bound ligand molecules to the bulk unbound ligand molecules, which are in effect the ones observed by the NMR experiments. Suprisingly, only a very limited number of available techniques have been applied to the DNA quadruplexes.

Chemical Shift Changes Observation

The most commonly employed method to detect interaction between a drug candidate and a target is the chemical shift perturbation method [67-70, 77, 107, 108], which, as its names suggests, analyzes the chemical shift changes observed in the target when a ligand interacts with its surface. Upon binding of a ligand to the DNA G-quadruplex, the chemical shifts of both the ligand and quadruplex proton resonance signals are affected. For the DNA, nuclei located in close proximity of the binding usually show the largest effects. Protons are the most sensitive nuclei for NMR spectroscopy. Therefore, one can, for example, follow the binding of a ligand to a DNA by observation of well separated signals like those belonging to imino and aromatic protons, and to methyls of thymines. To relate the changes observed with the definition of binding site, total or partial resonance assignment needs to be done in order to identify the residues that are involved in the interaction, and for the method to yield the maximal amount of information the three-dimensional structure of the target should be known. In the case of G-quadruplex structures, imino and aromatic protons of the Gs involved in the formation of the G-tetrad always pointing inside the quadruplex core and into the grooves, respectively. Generally, imino protons are more affected by the binding than aromatic protons, and their shift is much more intense in the case of ligand capable to stack on the edges of the quadruplex. During the NMR titration of a ligand to a target, two scenarios are possible Fig. (7). In the first case, the addition of the ligand to the target causes the appearance of another set of signals due to the presence in solution of different species (i.e. the bounded and unbounded forms) in slow exchange on the chemical shift time scale (Fig. (7)-left panel). Generally this latter case evolves with the new set of proton signals whose intensities rise up by increasing the ligand amount, along with the concomitant falling off of the original signals which completely disappeared at the saturation of the target. In the second case, the addition of the ligand causes a progressive drift of DNA signals (Fig. (7)-right panel), suggesting the formation of a shortlived complex on the NMR time scale. The two scenarios are generally associated to high and low affinity binding affinity of the ligand to the DNA, respectively. To the best of our knowledge, the only case where the first scenario is reported is the titration of the parallel quadruplex $[d(TGGGGT)]_4$ with the distamycin A [107], which NMR titration profile is almost unique. An increase of distamycin A concentration up to 2 mol equiv. caused ligand resonances to gradually grow in intensity and DNA signals to drift progressively. Nearly at 2:1 ligand:quadruplex stoichiometry, a further addition of tested ligand caused a complication of the spectrum due to the appearance of a separate set of proton resonances. The intensities of these new resonances rose by increasing the ligand amount with the concomitant falling off of the original signals which completely disappeared at a ratio of 4:1 ligand-quadruplex.

NMR Transfer Experiments

Rather than monitoring chemical shift changes, which require the use of stoichiometric amounts of target, "transfer" experiments can be used. These experiments use pulse sequences that transfer magnetization or coherence (or the lack of it) from the DNA to the ligand (or vice-versa). Typical concentrations used for this type of experiments are 1mM ligand and 50 μM DNA. For this concentration ratio, assuming that the binding constant is sufficiently high for effective saturation of the binding site, each ligand molecule spends 1/20 of its time as part of a target-ligand complex having very different relaxation and hydrodynamic properties than those of the free ligand. If the exchange between the free and bound states is sufficiently fast so as to take place many times during the mixing (or saturation) time of the experiment the property of choice will be *transferred* to the free ligand. The most successful experiment of those described for transferring magnetization from the macromolecular target to the ligand (or vice-versa) is the Saturation Transfer Difference (STD) experiment which was first described in 1999 [109] and has since found widespread use in the drug discovery industry. The experiment is carried out by subtracting the spectrum obtained when irradiation is placed on a DNA resonance from the spectrum obtained when irradiation is off-resonance. On resonance irradiation causes saturation of the DNA resonance and spread of the saturation to the rest of DNA and to any interacting ligand, whereas, off resonance irradiation should yield the 1D spectrum of the sample. In this case no coherence is transferred from the complex to the ligand; it is indeed the lack of coherence due to saturation which is very quickly and efficiently transferred in high molecular weight species. In order to prevent the appearance of false positives by direct saturation, it is important in STD spectroscopy to carefully select the irradiation frequency. One important feature of this method is the possibility of obtaining information about the binding epitope of the ligand

[110,111] through the analysis of the relative amount of saturation transferred to each of its atoms; those atoms which show a higher intensity in the transfer spectrum are those which lie at the target-ligand interface. In the case of DNA as target, novel STD-based approach has been proposed for the NMR screening of DNA binders. This method provides key information on their binding mode and allows the convenient probing of DNA recognition phenomena such as those of base-pair intercalators, minor groove binders, and external backbone binders [112]. In essence, this approach takes advantage of the difference in STD effect arising from the saturation of protons located in different DNA regions. Data from preliminary STD spectra on ligand-DNA complexes showed that saturation diffusion is far from being isotropic in long tracts of DNA. The approach is based on two parallel sets of STD experiments performed under the same experimental conditions, in which saturation is elicited by irradiating at a suitable frequency chosen from specific DNA resonances. The signal-to-noise (S/N) ratios of all the protons showing STD effects are measured with reference to an STD spectrum with an off-resonance irradiation. A comparative analysis of such effects in the two sets is then performed. For this reason, this method has been named differential-frequency STD (DF-STD) spectroscopy. Besides presenting all the typical advantages of STD spectroscopy, DF-STD spectroscopy has the distinct attribute of providing direct information on the mode of binding of the ligand onto the DNA molecule. In particular, in the case of DNA G-quadruplex a modified version of the DF-STD [112] method has been applied. This has been reported only in the study of the binding mode of distamycin A with the quadruplex $[d(TGGGGT)]_4$ [67]. In its original version, where duplex-DNA is investigated, the DF-STD method is based on the acquisition of two parallel sets of STD experiments performed under the same experimental conditions, irradiating on aromatic protons of purine/ pyrimidine bases and on deoxyribose/backbone resonances (both spectral regions where no ligand resonances were present) [113,114]. Nevertheless, in that investigation a third saturation frequency (that is in the range of the imino proton frequencies) have been used. Thus, three binding mode indexes (BMIs) have been calculated [67]:

$$\mathrm{BMI} = \frac{\sum_i \left(\frac{SN\,aromatic / SN\,rif}{SN\,aliphatic / SN\,rif} \right)}{ni} \qquad \text{(eq. 1)}$$

$$\mathrm{BMI'} = \frac{\sum_i \left(\frac{SN\,i\min o / SN\,rif}{SN\,aliphatic / SN\,rif} \right)}{ni} \qquad \text{(eq. 2)}$$

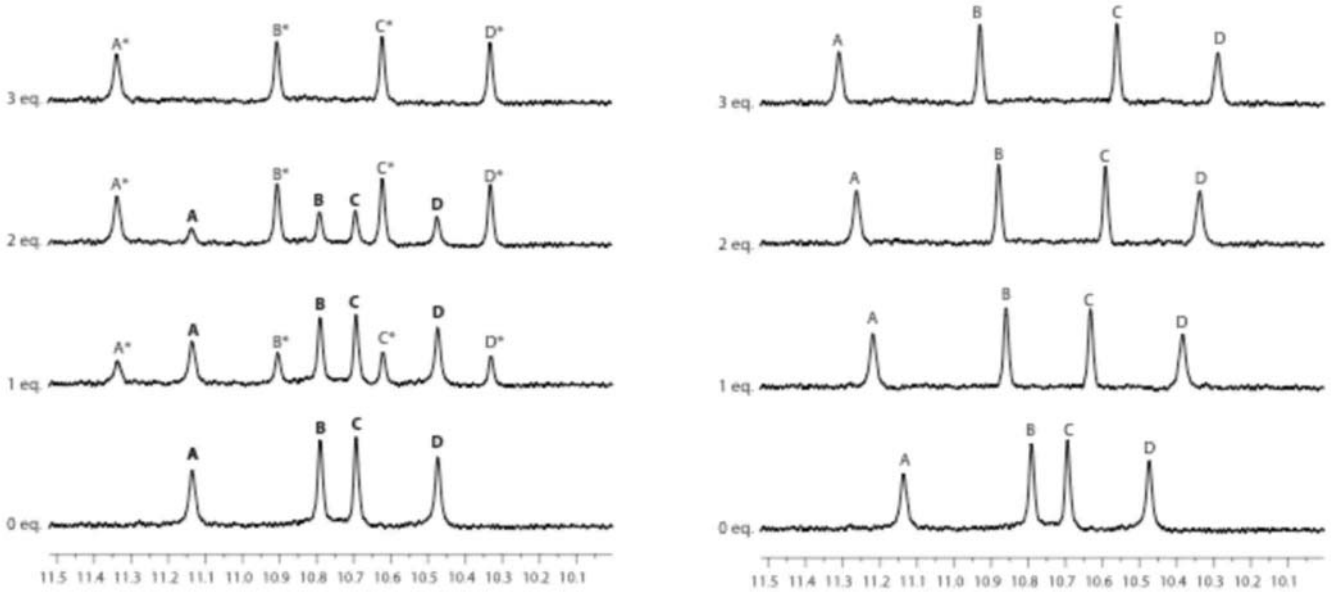
$$\mathrm{BMI''} = \frac{\sum_i \left(\frac{SN\,i\min o / SN\,rif}{SN\,aromatic / SN\,rif} \right)}{ni} \qquad \text{(eq. 3)}$$

where $SN_{aliphatic}$, $SN_{aromatic}$ and SN_{imino} are the differences between the intensities (expressed as S/N ratio) of the ligand signals when irradiated in the on-resonance STD spectrum (*i.e.* in the aliphatic, aromatic and imino regions, respectively) and that of the signals in the off-resonance NMR spectrum. SN_{rif} is instead the intensity of the same signal in the off-resonance spectrum, and n_i is the number of the signals.

There are a couple of methodologies that are similar to those just described, but they have never been used in the DNA quadruplex field. We believe useful to describe them in any case. The first is a variant of STD NMR spectroscopy that utilizes the bound water at target-ligand interfaces. It is well documented that NMR spectroscopy is well suited to study this bound water [115-117]. The observation of negative intermolecular water-ligand NOEs may be explained either by bound water squeezed in between ligand and target or by a water shell surrounding the ligand [118]. Based on these observations experiments were developed that use the bulk water to detect the binding of ligands to proteins [119]. The experimental setup utilizes the steady-state NOE experiment, where on-resonance saturation is applied to the water chemical shift. To generate the best sensitivity, the experiments are performed in H_2O containing only small amounts of D_2O. To eliminate artifacts from, for example, radiation damping, pulsed field gradients were employed for proper water suppression giving the method the name water-LOGSY (Water-Ligand Observation with Gradient Spectroscopy). The technique has successfully been applied to study the binding of a number of ligand to protein, and it appears that this approach is especially useful for complexes where either ligand or receptor are strongly hydrated, as demonstrated, for example, for RNA ligand interactions. This means that this valuable technique could in principle successfully be used also in the case of DNA G-quadruplex structures. The second methodology is based on the observation of

transferred NOEs (trNOEs). The principles of trNOEs were originally observed and described more than twenty years ago [120-124] and, since then, have found widespread use in the determination of the 3D structures of ligands bound to receptor proteins [125-129]. The observation of trNOEs relies on different tumbling times τ_c of free and bound molecules. Low- or medium-molecular-weight molecules (MW<1000-2000) have a short correlation time τ_c and, as a consequence, such molecules exhibit positive NOEs, no NOEs, or very small negative NOEs depending on their molecular weight, shape, and the field strength. Large molecules, however, exhibit strong negative NOEs. When a small molecule (ligand) is bound to a large-molecular weight protein (the protein receptor molecule) it behaves as a part of the large molecule and adopts the corresponding NOE behavior, that is, it shows strong negative NOEs, so-called transfer NOE (trNOEs). These trNOEs reflect the bound conformation of the ligand. Binding of a ligand to a receptor protein can thus easily be distinguished by looking at the sign and size of the observed NOEs. Furthermore, the discrimination between trNOEs originating from the bound state and NOEs of the ligand in solution can also be achieved by the build-up rate, that is, the time required to achieve maximum intensity, which for trNOEs is in the range of 50 to 100 ms, whereas for nonbinding molecules it is four- to ten-times as long. Therefore, the maximum enhancement for trNOEs is observed at significantly shorter mixing times than for isolated small molecules in solution. Also, several schemes have been developed that allow a quantitative interpretation of trNOEs and, thus, yield more reliable information about the conformation of bound ligands [125, 126, 129]. In general, one can observe inter- and intramolecular trNOEs. Whereas intramolecular trNOEs are the key to define bound-ligand conformations, intermo-lecular trNOEs occur between a ligand and a receptor, and therefore, in principle, allow the determination of the orientation of bound ligands in protein binding pockets [130, 131].

Fig. (7). *Simulation of two different NMR titrations. On left panel, example of binding with high affinity, where original signals of the target (bold letters) are gradually replaced by new ones (letter marked with asterisk). On right panel, example of binding with low affinity where the NMR signals of the target drift upon addition of the ligand.*



Diffusion Coefficient Analysis

Finally, a very modern NMR technique to discover new G-quadruplex ligands is based on the diffusion processes. Self-diffusion is the random translational motion of molecules driven by their internal kinetic energy. Self-diffusion coefficients and the structural properties of a molecule are connected by the dependence of the self-diffusion coefficients on molecular size and shape; therefore, it is not surprising that the determination of molecular self-diffusion coefficients has become a valuable methodology for studies of molecular interaction in solution. In the pharmaceutical industry, diffusion-based NMR techniques have been used in a wide range of applications, such as screening of chemical mixtures, determining the structures of bound ligands without physical separation, and measuring the diffusion coefficient of small metabolites in biofluids among others

[132]. In the G-quadruplex field, interestingly, Guangzhi Xu and co-workers reported a novel approach for fast screening of G-quadruplex ligands from natural plant extracts [133]. The concept behind this application of diffusion NMR techniques for screening studies is very simple and it is based on the fact that the diffusion coefficient of a small molecule is altered upon binding to a large receptor. For this type of studies, it has been sufficient to identify compounds that bind to the quadruplex $[d(TTGGGTT)]_4$, obtaining the diffusion of individual compounds from a mixture separated in different rows, resembling a chromatographic separation [134-135].

CIRCULAR DICHROISM (CD) SPECTROSCOPY AND INDUCED CD

Circular dichroism spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light by a substance [136, 137]. For its sensitivity to stereochemical variations, CD has emerged as an important technique for studying subtle conformational changes and supramolecular interactions. Nowadays, CD spectroscopy is employed mainly to investigate biological macromolecules and their perturbation by external factors [138]. In this scenario the sensitivity of CD makes this technique an useful tool to study different aspects of G-quadruplex DNA such as 3D-structures [139], ligand binding [140], kinetics of quadruplex formation [141] and thermal melting [142]. Interestingly, CD can be used to discriminate among different folding topologies of G-quadruplexes [139, 143, 144] that are characterized by distinctive marker bands: the spectra of a parallel structure (all strands with the same 5' to 3' orientation) show a positive band at ca. 260 nm, and a negative peak at ca. 240 nm [145, 146], whereas the spectra of an antiparallel quadruplex have a negative band at ca. 260 nm and positive band at ca. 290 nm [145, 147].

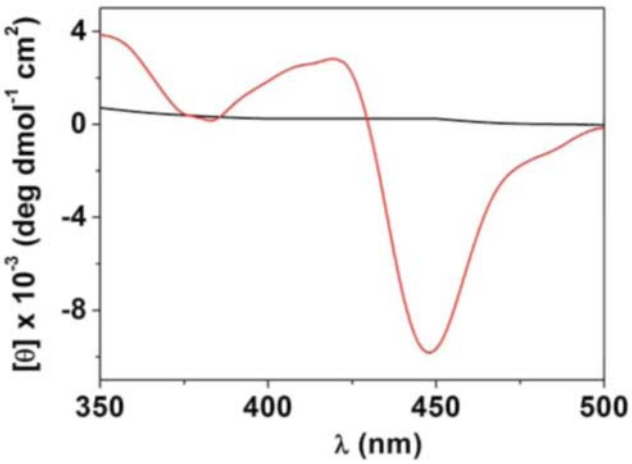
Focusing on DNA quadruplex as a target for ligand binding, CD is an elective method to explore the interaction capability of a molecule with quadruplex structures and, in favorable cases, to evaluate the selectivity of this molecule for a particular topology and/or sequence of G-quadruplex.

A typical CD titration is performed by adding the ligand into the cell containing the DNA-quadruplex sample, collecting a CD spectrum after each addition. The gradual appearance of characteristic bands in particular regions of the CD spectra provides specific information concerning the binding site and the binding mode of the ligand Fig. (8). Recently, Rodriguez *et al.* [140] reported the successful design of an anthracene derivative as first molecule able to induce the folding of the parallel human telomeric G-quadruplex from single-stranded DNA in the absence of added cations; this evidence was carried out trough CD titrations, at fixed concentration of telomeric DNA $d(TTAGGG)_4$ (telo24) and with variable concentration of the ligand. The human telomeric DNA is highly polymorphic and can exist as quadruplex structures with different strand orientations or folding topology, depending on the used experimental conditions and investigation methods [6]. The CD titration profile reported in Rodriguez's work clearly shows the formation of a parallel quadruplex because of positive and negative CD signals at 263 and 240 nm, respectively. Considering the structural variability of the telomeric DNA, these results suggest that the anthracene derivative is involved in a rather specific interaction only with a single topology of all the possible ones assumed by telo 24. Similarly, Wang *et al.* studied the binding of four new disubstituted phenanthroline-based compounds with the human telomeric G-quadruplex DNA HG-22 [148]; the appearance of a negative band at 260 nm and a positive band at 295 nm in CD spectra is consistent with the formation of an anti-parallel structure.

A more detailed investigation of the interactions between G-quadruplex and potential ligands is provided by the induced circular dichroism (ICD). ICD is emerging as useful technique to evaluate the geometry of the binding site and the binding mode of a ligand to DNA. In general the binding event of CD-inactive nonchiral molecules to a chiral host, such as DNA-quadruplex, gives induced CD signals due to the chiral enviroment of the ligand in the bound state [149]. As far as we know, ligands generally interact with G-quadruplex structures through groove and end-stacking binding modes. It was shown that the sign of the induced CD spectra of molecules bound to nucleic acids provides important information about their way of interaction: a positive induced CD band is indicative of groove binding, whereas a negative induced CD signal (or a slightly intense positive band) is indicative of end-stacking. Dash *et al.* demonstrated that bis-phenylethynyl amide derivatives interact with c-kit quadruplex as groove-binders [150]. In fact, CD titration profiles showed the presence of two marker bands attributed to the quadruplex structure, and a positive induced CD signal at 380 nm (absorbance of ligands) that strongly suggested a groove binding interaction. On the other hand, in the case of the binding of actinomycin D to human telomeric quadruplex structure $d[AGGG(TTAGGG)_3]$ [151], induced CD signals were observed at both 375 and 440 nm; however, while the 375 nm band was negative, the 440 nm band turned out to be positive. A positive band at 440 nm indicates that the complex formed by actinomycin D with quadruplex DNA is not purely intercalative but involve end-stacking on the terminal G-tetrad of the quadruplex structures.

The investigations reported here are only a few examples about the use of circualr dichrosim spectroscopy in general and induced CD data aimed to get insights into the folding topology of quadruplex structures and into the binding mode of potential DNA-quadruplex ligands.

Fig. (8). *Example of induced CD spectra of the TMPyP4 bound to the DNA quadruplex d(AG$_3$TT)$_4$ [75]. In absence of the DNA quadruplex (black line), TMPyP4 does not show CD signal.*



FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy is based on the fundamental physical phenomenon of the fluorescence, consisting in the emission of a photon upon relaxation from an electronically excited singlet state to the ground state subsequent to the absorption of a photon by the fluorescent molecule (fluorophore). The process takes place on a much slower time scale (~ ns) than absorption (~ fs) allowing a much wider range of interactions to influence the fluorescence emission spectrum. Due to its sensitivity to the fluorophore environment, fluorescence is the most efficient optical spectroscopy for following the ligand binding phenomenon. Like other optical spectroscopies, fluorescence is rapid, not destructive and relatively inexpensive. In addition, fluorescence usually has a higher intensity signal variation in comparison with the other optical spectroscopies and requires much lower DNA and ligand concentration, thereby avoiding precipitations/aggregation problems.

Commonly available fluorescence spectrometers determine the relative fluorescence ***F***, which is the emission intensity of the system measured under defined conditions. ***F*** is a function of the instrument (intensity of the excitation source, sensitivity of the detection system, etc.) and of the conditions of the measurement (fluorophore concentration, temperature and solution conditions) and often is given in arbitrary units. ***F*** is most accurately obtained from integration over the wavelengths of the emission spectrum but, for many purposes, it may be sufficient to determine the fluorescence intensity at the maximum of the fluorescence spectrum.

Several reported quadruplex ligands have intrinsic fluorescence properties and, generally, show a large change in their fluorescence intensity upon DNA binding [63, 75, 152]. In these cases, fluorescence titration experiments can be performed following the variation of the ligand fluorescence on increasing DNA quadruplex concentration. The binding curve obtained by plotting the fluorescence intensity at the maximum of the ligand emission spectrum as function of the quadruplex concentration Fig. (9) can be fitted to a specific binding model to determine the binding stoichiometry and affinity constant [75, 153]. Recently, some of us used this method to explore the binding of a triazatruxene derivative (azatrux) to the parallel quadruplex scaffold. It was found that the binding stoichiometry and affinity constant determined by fluorescence titration experiments are similar to the ones obtained by other biophysical methods such as ITC and UV-Vis spectroscopy demonstrating the reliability of the fluorescence spectroscopy in determining the binding parameters [153].

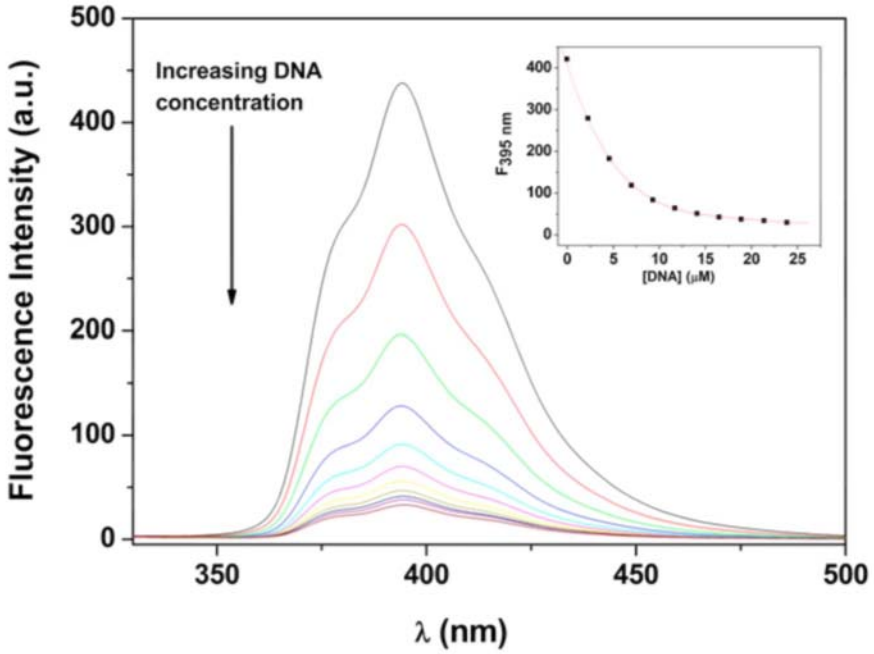
One limitation of fluorescence experiments is that the sign (increase or decrease) and the magnitude of the fluorescence change upon quadruplex binding are not correlated in a simple way to the ligand binding mode. However, useful information on the binding mode can be obtained, in some cases, taking advantage of the

fluorescence resonance energy transfer (FRET) effect between the ligand and the DNA bases or by means of fluorescence quenching experiments.

In a FRET experiment, a donor fluorophore transfers its excitation energy to an acceptor fluorophore, with an efficiency depending on their spectral properties, their distance and relative orientations. One requirement for FRET effect is that the absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor. If the requirement for a strong FRET effect is satisfied, excitation in the wavelength region corresponding to the donor fluorophore will result in fluorescence emission from the acceptor fluorophore. It was shown that FRET between DNA ligand (acceptor fluorophore) and DNA bases (donor fluorophore) occurs efficiently only if the bound ligand is in close contact with, and oriented parallel to, the DNA bases [154]. Hence, the increase of the fluorescence intensity of a ligand (due to FRET effect), when excited in the wavelength range corresponding to DNA absorbtion, can be used as strong evidence for intercalative or end-stacking binding mode to G-quadruplexes [155].

Another method to distinguish between external and intercalative binding mode is provided by fluorescence quenching experiments. In those experiments, the fluorescence intensity of the ligand in presence of the target DNA structure is monitored upon increasing the concentration of a fluorescence quencher. If the quencher is anionic, the electrostatic barrier due to the negative charges on the phosphate groups at the helix surface limits the penetration of the quencher into the helix interior. Ligands externally bound to the quadruplex scaffold are available to the quencher, while those that are intercalated are not. Hence, very little or no quenching should be observed if the binding involves intercalation or strong stacking interactions. Bhadra *et al.* employed fluorescence quenching experiments to explore the binding mode to the DNA telomeric quadruplex of various isoquinoline alkaloids having non planar and planar structures like berberine, palmatine, coralyne and sanguinarine [63]. They measured the accessibility of the quencher to the free and bound ligands and found evidence of a stronger stacking interaction of coralyne and sanguinarine compared to berberine and palmatine.

Fig. (9). *Representative fluorescence titration experiment. Fluorescence spectra on increasing DNA concentration are shown. Ligand fluorescence decreases on increasing DNA quadruplex concentration. In the inset: binding curve obtained by plotting the fluorescence intensity at the maximum of the emission spectrum as function of DNA concentration.*



Fluorescence polarization anisotropy measurement is an alternative method to get information on the interaction of G-quadruplexes with fluorescent compounds. The method is based on the idea that a fluorophore excited by polarized light will also emit polarized light, but the degree of polarization of the emitted light will depend on

the rotational motion of the fluorophore on the time scale of the fluorescence phenomenon. When the ligand is bound, its rotational motion will be restricted and consequently the fluorescence emission should be more polarized in comparison with the free ligand. The measured degree of polarization provides information on the rotational freedom of the ligand bound to the quadruplex structure and on the extent of its interactions with the DNA bases [63]. Further, time-resolved fluorescence anisotropy measurements can provide additional dynamical information about the motion of the ligand in the confined environment of the binding site [156, 157].

A major limitation of all the fluorescence experiments described above is the requirement of specific fluorescence properties of the ligand under investigation. To monitor the binding of a non fluorescent ligand, the introduction of a fluorescence probe in the system is needed. A useful probe for fluorescence sensing of G-quadruplex-ligand interactions is the thiazole orange (TO). TO has very convenient property: it is non-fluorescent when free in solution but strongly fluorescent when bound to DNA. Taking advantage of this property, Monchaud *et al.* developed a fluorescent intercalator displacement (G4-FID) assay for screening G4 ligands [158]. The method is based on the displacement of TO from quadruplex or duplex by increasing amounts of the ligand under investigation. A decrease of the fluorescence intensity on increasing a candidate ligand concentration indicates displacement of TO by the ligand. Therefore, the quadruplex-affinity of the ligand is evaluated through its ability to displace TO from quadruplex DNA. This method has been recently implemented onto 96-well plates reader and represents a powerful and rapid tool for high throughput screening of G-quadruplex ligands. Major advantages of this method are that it does not require specific ligand properties (*e.g.* fluorescence), it employs widespread materials and equipments and it easy to implement. However, some limitations have to be carefully considered: 1) compounds that are naturally fluorescent with excitation and emission maxima overlapping TO may interfere with the measurement; 2) some ligands could bind DNA without displacing TO, thus leading to underestimate the ligand affinity for the quadruplex structure.

Another method for fast screening of G-quadruplex ligands is the (FRET)-based melting assay developed by Mergny *et al.* In this method the ligand affinity for a quadruplex is evaluated by measuring the increase in the melting temperature (T_m) of the quadruplex induced by the presence of the ligand [159, 160]. The method is an indirect probe of the ligand-quadruplex binding affinity because the T_m displacement rather reflects the effect of the ligand on the displacement of the folded quadruplex <=> unfolded strand equilibrium, around the melting temperature. Fluorescence spectroscopy can be used to monitor the thermal quadruplex unfolding, providing that a fluorescent probe (*e.g.* fluorescein/FAM) and a quencher (dabcyl) or an acceptor for FRET (*e.g.* tetramethylrhodamine/TAMRA) are attached to the 5' and 3' ends of the oligonucleotide, respectively. The fluorescence intensity of the fluorescent probe depends on its distance from the quencher (or from the FRET acceptor), this distance significantly changes in the quadruplex folding/unfolding process. The large difference in the fluorescence properties of the folded and unfolded quadruplex allows one to obtain well resolved melting curves. As for G4-FID assay, this method can be easily implemented on a multi-well plate reader and requires little amounts of ligand and DNA. A disadvantage of this method is that it employs modified oligonucleotides, so the attached fluorescent probes could interfere with the ligand binding to the quadruplex structures leading to an inaccurate evaluation of the ligand affinity.

Finally, the introduction of fluorescent DNA base analogues at specific sites of quadruplex-forming oligonucleotides is a powerful tool to get detailed information on the binding mode of the ligands. A highly fluorescent adenine analogue, 2-aminopurine (2AP), has been widely used as site-specific fluorescent reporter to study local structural and dynamic properties of duplex and quadruplex structures [161, 162]. 2AP fluorescence generally increases when it is exposed to the solvent and decreases when it is hidden from the solvent due to base stacking. Moreover, it has been proved that this adenine analogue does not alter the quadruplex conformation. The fluorescence of 2AP residue changes when a quadruplex ligand binds close to it, thereby giving local information on the binding site properties. Barbieri *et al.* used oligonucleotide site-specifically substituted with 2AP to explore the molecular nature of the interaction between a macrocyclic heaoxazole (HXDV) and the $d(T_2AG_3)_4$ quadruplex [157]. They substituted the adenines 9, 15 and 21 situated in different loops of the $d(T_2AG_3)_4$ quadruplex structure and found that HXDV binding induced destacking of adenines at positions 15 and 21 but not at position 9, supporting a mode of interaction in which two HXDV molecules bind to the quadruplex, one at each end of the structure with an end-stacking binding mode.

Table 1. *Comparison of Characteristics of Experimental Techniques Reported in this Review*

	ITC	Fluorescence	CD	NMR	ESI-MS
Stoichiometry of complex	+++ (from fitting)	++ (from fitting)	+ (from fitting)	++	+++
HT screening of relative binding affinity	+++	FRET-T_m: ++ Thioazole Orange displacement: +++	+	++	+++
Binding constant determination	++ (from fitting) Possible independent determination for each binding site. (K_b values in the range 10^4-10^8 M^{-1})	++ (from fitting)	+ (from fitting)	++ Microscopic K_d, independent determination for each binding site.	+++ Macroscopic K_d, independent determination for each stoichiometry. (liMtomMÅ'd)
Binding mode assessment	+ Hints from thermodynamic signature	+ Hints from FRET or quenching experiments	++	+++	-
Major advantages	Determination of termody-namic parameters with no requirements of chemical modification, labeling or immobilization of DNA molecules and/or ligands.	Low DNA and ligand concentrations.	Very easy and fast to acquire.	Determination of binding site; characterization of 3D structure of the complex at the atomic level; determination of the conformation of the ligand.	Assumption-free determination of stoichiometry and of K_d for each complex. Suits all ligands.
Major drawbacks	Presence of multiple DNA conformations could invalidate the thermodynamic parameters; no canonical experiments with water insoluble ligands.	Requirement of specific fluorescent properties of the ligand.	Multiple DNA conformations are not easily detected.	Very fast kinetic of the binding are detrimental.	Possible only from ammonium acetate solutions (influences the conformation of the target G-quadruplex).
Sample consumption	nmol of DNA and ligand	pmol of DNA and ligand	nmol of DNA and ligand	mmol of DNA and ligand	pmol of DNA and ligand

+++: Excellent; ++: Good; +: Sufficient, -: Not applicable.

SUMMARY AND OUTLOOK

Over the past decade, the biological significance of G-quadru-plexes has been proved by numerous research investigations, that highlighted their role in the regulation of a number of gene promoter sequences as well as in the maintenance of telomeres. The pharmacological potential of quadruplex-binding ligands has also been proved. Consequently, a number of researchers has focused their attention on the discovery of new molecules able to interact with quadruplex structures and therefore able to interfere with biological processes.

Thanks to X-ray crystallography and NMR studies a great number of G-quadruplex structures have been characterized. Compared to duplex DNA, G-quadruplexes have much more compact structures containing well-defined binding sites for small molecules, thus providing an exciting opportunity for the development of selective quadruplex-binding anti-cancer agents.

Biophysical methodologies such as ITC, fluorescence, NMR, CD, virtual screening and mass spectrometry are needed to investigate the interactions between small molecules and G-quadruplex DNA and for evaluating the affinity, selectivity, as well as binding mode of such molecules, providing accurate guidelines for the optimization of ligand-target interactions. In our opinion, the techniques described here are greatly supporting

this area of research, as proved by the number of examples reported. Very interestingly, all these techniques are complementary. None of them can provide alone a complete picture of the binding activity of a ligand to a DNA quadruplex structure. Each of these techniques, in fact, can address more accurately than others to specific questions and vice-versa. We summarize the advantages and drawbacks of each reported technique in the Table 1.

We expect for the near future a more tight combination of these biophysical methods with the aim of improving our understanding of the quadruplex recognition process, to discover selective quadruplex-binding molecules with valid therapeutic applications.

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