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Use of competition dialysis in the discovery of G-quadruplex selective ligands

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Abstract

G-quadruplex DNA can exist in a rich variety of structural forms, ranging from unimolecular folded structures containing diverse types of loops and strand orientations, to bimolecular dimeric structures, and finally to tetramolecular parallel-stranded structures. These diverse structures present numerous potential small molecule binding sites with distinctive properties. There is mounting evidence for important functional roles for G-quadruplex structures in biology. G-quadruplexes may participate in the maintenance of telomeres, in transcriptional regulation and, in mRNA, may act to modulate translation. G-quadruplexes thus represent an attractive target for new small-molecule therapeutic agents. Competition dialysis provides a useful tool for the discovery of small molecules that selectively recognize the unique structural features of G-quadruplexes. The principles and practice of the competition dialysis experiment are described here.

Keywords

G-quadruplexDNA; binding; dialysis; drug discovery; structural-selectivity

1. Introduction

The G-quadruplex structure was first described in 1962 in studies of guanosine gels completed in the Davies laboratory(1). While quadruplex DNA continued to be studied over the subsequent decades (2), the structure was regarded as a biophysical oddity, with little functional relevance. That situation changed when G-rich sequences capable of forming G-quadruplexes were found to be highly conserved in telomeres (3,4). The finding that G-quadruplexes prevented replication of telomeric DNA by telomerase (5) suggested a new strategy for chemotherapy (6–10). Small molecules that would stabilize the quadruplex structure would lock the telomere into an unfavourable conformation for replication, and would interfere with normal telomere biology. The strategy was validated using a G-quadruplex interactive compound (11). G-quadruplex structures were subsequently found to be important structural elements in the promoters of many genes (12–15), and more recently a quadruplex structure was found in mRNA that appeared to modulate translation (16). The G-quadruplex represents an important new potential drug target.

DNA quadruplexes present a diversity of targetable structures (17,18). Scheme I shows, in highly schematic form, some possible quadruplex structures. Individual strands can fold

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unimolecularly into a variety of structures, with diverse strand segment orientations and loop geometries. Two strands can combine to form bimolecular structures, again with diverse strand and loop orientations. Four strands can combine in a tetramolecular reaction, almost inevitably and exclusively featuring parallel strand orientations. All of these structures are characterized by unique groove geometries, loop structures, and base stacking geometries. Collectively these features present a variety of nooks, crannies and surfaces that might be selectively recognize by complementary structural features in small molecules. The challenge is to discovery such G-quadruplex selective molecules that recognize these structural features that might serve as lead compounds in a drug discovery effort. A few such small molecules exist, but a systematic effort to discover additional compounds is urgently needed.

Competition dialysis provides a useful quantitative tool for the discovery of structural-selective compounds that recognize particular nucleic acid structures (19–23). In the competition dialysis experiment, an array of nucleic acid structures is dialyzed against a common test ligand solution. When equilibrium is reached, the array of structures is in contact with the identical free ligand concentration, and measurement of the amount of ligand bound to each structure provides a direct measure of affinity for that structure. Structural preferences are immediately and simply visualized. The competition dialysis method has been widely used in a number of laboratories world wide. The principles and practice of the method as applied to quadruplex DNA will be described here.

2.1 Principle of method

The physical process of dialysis involves the diffusive flow of one or more solutes through a semi-permeable membrane. Molecules smaller in size than the membrane pores can freely diffuse across the membrane while larger molecules are retained inside the membrane. Dialysis is used routinely in basic science to remove impurities or to change solution conditions, for example, during the purification of nucleic acids and proteins (24). Competition dialysis is an extension of the dialysis method to study the equilibrium specificity of a test ligand for different nucleic acid forms (25,26).

In a simple equilibrium dialysis assay, two chambers are separated by a semi-permeable membrane. A known concentration of receptor is placed in one of the chambers and a known ligand concentration in the other. An appropriate membrane is selected such that the ligand is free to pass from one chamber to the other, but the receptor is retained in one chamber. As the ligand diffuses, some of it will bind to the receptor and some will remain free in the solution. If the ligand affinity for the receptor is nonzero, an appreciable concentration of the ligand bind to the receptor. When equilibrium is reached, the concentration of free (unbound) ligand will be identical in all chambers, but the total concentration of ligand will be higher in the receptor chamber by the amount bound. How much is bound depends on the ligand affinity. In the competition dialysis assay, a number of receptors are placed in individual closed membrane chambers in a beaker containing a solution of the test ligand (the dialysate). Once equilibrium has been attained, the ligand concentration in each chamber is measured spectrophotometrically.

Muller and Crothers (26) made the first report of a competition dialysis method that was designed to study the preference of a ligand for GC or AT base pairs. Natural DNA samples of differing base composition were placed in two chambers separated by a central chamber in which the ligand was placed. After allowing the ligand to equilibrate between the three chambers, more ligand accumulated in the receptor chamber containing the DNA with the higher percentage of the preferred base or base pair. Analysis using simple probability concepts allowed surprisingly detailed inferences about the exact nature of the preferred ligand binding site (25).

The competition dialysis assay (22) is a simple and straightforward extension of Crothers' original method. Instead of a three-chambered dialysis apparatus, disposable dialysis units are used to contain a wide variety of nucleic acid structures at identical concentrations. These units are simply placed into a beaker containing ligand solution. At equilibrium, the free ligand concentration is identical throughout the system, and preferential binding by a particular structure leads to a greater accumulation of total ligand within that dialysis unit. Structural selectivity can be easily measured by measuring total ligand concentration within each dialysis unit. The method is further extended as described here by using a custom-made prototype provided by Linden Biosciences (Woburn, MA) based on the Rapid Equilibrium Dialysis Device, shown in Figure 1. In this new apparatus, 96 dialysis membrane chambers (fitted to holders containing two chambers each) are inserted in a 48 well rack and placed into a Teflon® Base Plate filled with a solution containing the test drug. All membrane chambers are in contact with the common dialysate solution. The sample volume in each well is 150 – 200 μ L, and the volume of the dialysate reservoir is 150 mL. During the experiment, the dialysate is continuously circulated in a closed loop using a peristaltic pump in order to enhance the rate of equilibration. The apparatus conforms to standard 96-well plate geometry, permitting the use of standard multitype pipettors and other 96-well plate technology. In principle, the unit could be used with robotics to automate the procedure.

2.2 Overview of the Competition Dialysis Experiment

An overview of the competition dialysis experiment is shown in schematic form in Figure 2. Once stock solutions of the nucleic acid array are prepared, experiments may be conducted rapidly and efficiently. Experimental set up takes approximately two to three hours, and involves primarily preparing the membrane wells and loading the desired array into the dialysis apparatus. Once set up, the experiment runs unattended. After dialysis equilibrium is reached, samples are removed from the dialysis unit, SDS added to dissociate bound drug, and concentrations determined by absorbance or fluorescence. Use of 96-well technology greatly facilitates these liquid handling steps, and use of a 96-well plate reader dramatically accelerates the acquisition of data.

2.3 Array of structures used in the competition dialysis assay

The first challenge to be met in the competition dialysis assay is to find a suitable buffer in which the structures of interest are all stable. The method is amenable to a wide range of buffer conditions including sodium cacodylate, sodium phosphate, potassium phosphate, tris-based buffers, MOPS, MES, HEPES, as well as different concentrations of NaCl or KCl. In the first generation assay (22), 13 structures were found to be stable in a simple phosphate buffer containing 200 mM NaCl. The assay was expanded to include 19 structures in the second-generation assay (27), using the same buffer conditions. Table 1 lists the samples used in the current version of the assay.

The samples listed in Table 1 provide an array that includes a wide variety of nucleic acid structures. Single-stranded DNA and RNA are represented. Duplex DNA is represented by both natural DNA samples and synthetic polydeoxynucleotides of defined sequence. These samples cover a range of base composition and simple dinucleotide repeat sequences. Duplex RNA is represented, as are DNA:RNA hybrid structures. Left-handed Z-DNA represents an extreme secondary structural variant. Multistranded triplex, quadruplex and i-motif structures are represented by several samples, including several G-rich promoter sequences of current interest.

It should be emphasized that the array of structures listed in Table 1 is but a point of departure. The competition dialysis method is completely general, and arrays of structures of particular interest can be designed as desired. The only limitations are that the structures be large enough

to be retained by the dialysis tubing chosen for use, and that they are verified to be stable under the ionic conditions of the experiment. Essential quality control experiments to characterize nucleic acid samples were described in detail (19,23), and include UV absorbance and circular dichroism spectroscopy, and thermal denaturation studies.

2.4 Materials and Sample Preparation

2.4.1 Nucleic acids—The most time-consuming step is the preparation of nucleic acid stock solutions. Synthetic polynucleotides (Sigma-Aldrich, St. Louis, MO or GE Healthcare Bio-Sciences Corp., Piscataway, NJ) or oligonucleotides (Integrated DNA Technologies Inc., Coralville, IA) were dissolved in the buffer of choice at 4°C overnight to achieve complete hydration. Single- and double-stranded polynucleotides and oligonucleotides were then used after filtration with 0.22 µm filters (cat# 141128, Whatman Inc., Florham Park, NJ). Triplex solutions were prepared by direct mixing of the corresponding duplex and single strand. Triplexes and quadruplexes were annealed by heating in a water bath to 90°C for 10 minutes followed by slow, overnight cooling to room temperature. Samples were equilibrated at 4°C for 48 hours, extensively dialyzed to remove any short fragments, and finally filtered before use. Commercially available natural DNA may contain significant amounts of contaminants, such as proteins and RNA, requiring careful purification (28). First, the DNA was dissolved by continuous stirring at 4°C (this can take a number of days if the GC content is very high). Samples were then sonicated for a total of 30 minutes to produce DNA fragments of ~ 200 base pairs. Five minute periods of sonication were followed by 5 minutes of rest, with a continuous purge of nitrogen. Following sonication, the solution was extracted several times with phenol and chloroform to remove protein impurities followed by the addition of 10% (v/v) of 3M sodium acetate pH 5.5 and precipitation with ethanol. After drying in a stream of air or in a desiccator overnight, the DNA was redissolved in the appropriate buffer, dialyzed extensively and filtered prior to use. Left handed Z-DNA was prepared by bromination of poly (dGdC)-(dGdC) as previously described (29). All nucleic acid samples were characterized by UV absorbance and circular dichroism spectroscopy, and thermal denaturation studies to confirm purity and structure. Circular dichroism spectra were recorded using a JASCO J-810 spectropolarimeter (Jasco, Tokyo, Japan) and thermal denaturation studies were performed using a Hewlett Packard HP 8452A spectrophotometer equipped with a Peltier temperature controller unit (Agilent, Palo Alto, CA).

Nucleic acid samples used in the competition dialysis array were prepared at identical concentrations of 75 µM in terms of the monomeric unit: this means nucleotides for single-stranded forms, base pairs for duplex forms, triplets for triplex forms and quartets for quadruplex forms. The intent of using the monomeric unit was to normalize the concentrations of potential binding sites, although differences in the concentration of ends might be problematic for end-binding ligands. Nucleic acid solutions prepared at a concentration of 75 µM in BPES can be stored for up to 6 months at 4°C. Concentrations of nucleic acid samples are determined by UV absorbance spectroscopy using the extinction coefficients and absorbance maxima listed in Table 1. The addition of a new nucleic acid structure to the assay requires a thorough characterization of the stability of the structure in the chosen buffer as well as the determination of an experimental extinction coefficient, typically by the colorimetric phosphate assay (30).

2.4.2 Dialysis units—A number of possibilities exist for a choice of dialysis unit. Our first generation competition dialysis assay utilized 0.5 or 1.0 ml DispoDialyzer units (Spectrum Medical Industries Inc., Houston, TX) providing 6.28 mm²/ml of membrane area for dialysis. We then found a more suitable alternative in the Pierce Slide –A – Lyzer MINI dialyzer units (Pierce, Rockford, IL). Re-using these units is possible by removing the membrane after each experiment and replacing it with SnakeSkin® membrane (MWCO: 3,500; cat# 68035; Pierce).

Each unit is evaluated before experiments by dialysis against water for 24 hours. The volume in each unit is measured and units with volumes exceeding the original 200 μl are discarded; these units provide 1.4 mm^2/ml of area for contact with the dialysate. Our third generation of competition dialysis employed initially a Spectra/Por 96-well Microdialyzer (cat.# 132326, CA, USA) using membrane frames designed for the Spectra/Por microdialyzer (MWCO: 3,500; Spectra/Por® CE, cat# 132974, CA, USA), these membranes exposed a surface for dialysis exchange of 0.6 mm^2/ml . The low surface-to-volume ratio presented some problems due to slow equilibration times. After equilibrating for more than 24 hours; air dissolve in the circulating dialysate leading to bubble formation.. These difficulties were improved by replacing the set up with a custom made device provided by Linden Biosciences (Woburn, MA) based on the Rapid Equilibrium Dialysis Device pictured in figure 1. Each chamber unit is composed of a Spectra/Por RC membrane, (MWCO 3,5000; cat 133104, Rancho Dominguez, CA) and offers 7.1 mm^2/ml for dialysate exchange. Membranes are treated prior to use in order to remove any traces of urea, sodium azide or glycerol stabilizers. Reuse of the membranes is not recommended because of the possibilities of leaks or ligand binding to the membrane.

2.4.3 Ligands—The competition dialysis assay is amenable to the study of any ligand that is both soluble and stable in the chosen buffer. Preparation of micromolar concentrations can be achieved with many ligands, but where solubility is limiting DMSO can be added up to 1% (v/v). A necessary requirement for the competition dialysis assay is that the ligand possesses a convenient spectroscopic monitor to determine the ligand concentrations after dialysis. UV/Visible absorbance or fluorescence spectroscopy has been routinely used in our application of the assay. For absorbance spectroscopy, ligand extinction coefficients must be determined or taken from the literature. It is important that the extinction coefficient refer to the buffer of choice, many extinction coefficients quoted in the literature have been determined in organic solvents such as DMSO, chloroform, methanol, ethanol and acetonitrile. When using fluorescence spectroscopy to determine ligand concentrations, a standard curve is constructed from which to determine the concentrations corresponding to a given fluorescence intensity. Any adjustment of collection parameters, such as the slit width or gain, requires the construction of a new standard curve. It is important to note that some ligands bind to glass or plastic materials and precautions must be taken when working with these ligands. Careful mass-balance measurements of total ligand would be recommended for initial experiments.

3. Experimental Procedures

3.1 Nucleic acid and dialysate solutions

Our first and second generation assays typically employed 200 mL of a 1 μM ligand solution into which were placed dialysis units containing 0.5 mL of 75 μM monomeric unit of each nucleic acid sample. Extension of the assay to the 96-well microdialyzer utilizes a 200 μl volume of 75 μM of each nucleic acid sample with the dialysate reservoir charged with 150 mL of ligand solution. Initial experiments in the 96-well format explored a range of ligand concentrations. The upper limit was determined to be 5 μM and the lower concentration limited by the ability to observe a reliable ligand signal; the optimal concentration was found to be 1–2 μM . Typically the dialysis chamber is filled with 250 ml of a 2 μM solution of the test ligand using a Reliable Scientific peristaltic pump with the solution passing through a 0.2 μm filter to remove fine particles and air. After loading each well of the microdialyzer with nucleic acid solution using standard multtip pipettors, the sample chamber is sealed with a Titer-Tops™ adhesive film (Diversified Biotech, Boston, MA) to prevent any evaporation from the wells.

3.2 Incubation time

The nucleic acid array was allowed to equilibrate with dialysate with mixing by using a continuous flow of 12.25 ml/min by a Mini-Peristaltic Pump (Harvard Apparatus, Holliston, MA), for 24 h at room temperature (20–22 °C).

3.3 Determination of bound ligand

At the end of the equilibration period, 180 µl of each nucleic acid sample was carefully removed from each well and transferred to a 96-well microtiter plate (Costar® cat# 3915; Corning Inc., Corning, NY). To each sample, 20 µl of a 10% (w/v) sodium dodecyl sulfate (SDS) stock solution was added to give a final concentration of 1% (w/v) SDS, sufficient to dissociate the ligand from the DNA structures and ensure that there are no complexities arising from differences in the optical properties of free and bound ligands. When working with potassium-based buffers, the addition of SDS results in a precipitate and it is necessary to use other detergents, such as triton X100 or tween 80. The total ligand concentration (C_t) within each dialysis well was determined spectrophotometrically using an appropriate absorbance wavelength and extinction coefficient or by construction of a standard fluorescence curve. Appropriate corrections were made for the small dilution resulting from the addition of the SDS stock solution. The free ligand concentration (C_f) was determined from an aliquot of the dialysate solution, which typically did not vary appreciable from the initial 2 µM. Absorbance and fluorescence measurements were made using a Safire² microplate reader (Tecan US, Durham, NC). The bound ligand concentration (C_b) was then determined by difference as:

$$C_b = C_t - C_f \quad (\text{Eq 1})$$

4. Results and Interpretation

In the first generation of the competition dialysis assay 13 DNA samples were tested (22), this was expanded to 19 samples for the second generation (27), and currently, the third generation has 46 nucleic acids samples covering a wide range of nucleic acid structures encompassing single-stranded and duplex DNA and RNA, DNA-RNA hybrids, left-handed Z-DNA, triplex and quadruplex DNA. The new array includes a considerable expansion of quadruplex DNA sequences and structures. Several promoter regions play an important role in the transcription of proteins and many of them in the over-expression of undesired proteins in cancer cells; many of them as quadruplex forms. The array includes the following promoter regions: promoter C-Myc (31), promoter BCL-2 (32), promoter VEGF (33), promoter HER-2/*neu* (34), Retinoblastoma promoter (35) and Ki-Ras proto-oncogene (36).

4.1 Sample data

It is useful to review some results obtained from the earlier, 19-structure array that contained three quadruplex forms. These are the Na⁺ form of the human telomere quadruplex, a 20 – quartet- parallel stranded tetramolecular form (T₂G₂₀T₂)₄ and a “G-wire” that was inadvertently formed using the sequence 5’G₁₀T₄G₁₀. The structures present in the 19-structure array are indicated by an asterisk (*) in Table 1. Results obtained using six ligands are shown in figure 3A; compound structures are shown in figure 3B.

The six ligands chosen for study are ethidium, methylene blue, berberine, DODC, NMM, and PIPER. Ethidium bromide binds to DNA via an intercalation mode, usually to double stranded DNA, with preference for alternating purine-pyrimidine sequences (37), but can also bind single-stranded DNA, triplex, quadruplex and DNA-RNA hybrid structures; Methylene blue is another intercalator with unknown structural preferences (38,39). Berberine (40) is an alkaloid derived from traditional Chinese herbal remedies; it proved to have binding activity towards triplex DNA and in a minor extent to quadruplex DNA. Cyanine derivatives such as

DODC (41), can form stacked complexes in the minor groove of duplex DNA, but has been shown to bind more selectively to triplex DNA over quadruplexes (27). *N*-methylmesoporphyrin IX (NMM) (42), behaves as a stable transition-state analogue for porphyrin chelataes and can discriminate between different structures of DNA, prevailing selectivity for quadruplexes. Amongst the G-quadruplex binders family; PIPER, a perylene derivative has proved to bind to quadruplex DNA structures with relative selectivity (13,43, 44).

Competition dialysis results for ethidium bromide, methylene blue, berberine, DODC, NMM and PIPER are shown in Figure 3A as bar graphs representing the amount of compound bound to each nucleic acid sample tested for the second generation of DNA structures.

Panel A shows the preference of ethidium bromide for duplex polydApolydT and polyrArU, as well as sequence selectivity for the parallel quadruplex (5'G₁₀T₄G₁₀)₄. Panel B shows that NMM has a remarkable selectivity for only G-quadruplex DNA structures with particular specificity for the “G-wire” formed by 5'G₁₀T₄G₁₀. Panel C shows 3,3'-diethyloxadicarbocyanine iodide (DODC) to have specificity for triplex DNA (poly dAdT)₂-poly dT, but with some binding to G-quadruplexes with preference for the “G-wire” for (5'G₁₀T₄G₁₀)₄ over the antiparallel human telomere sequence and the parallel G-quadruplex (T₂G₂₀T₂)₄. Panel D indicates for PIPER selectivity and affinity of this compound for the x human telomereG-quadruple over the other quadruplexes, and over triplex, duplex and single strand DNA. A preference of methylene blue for the “G-wire” (5'G₁₀T₄G₁₀)₄ is indicated in the panel E. The groove binder berberine shows a clear preference for the triplex DNA polydAdTpolydT and to a lesser preference for the “G-wire”.

4.2 Examples of 96-well format data

Extension of this assay into this new 96 well-plate format, provides a more detailed comparison of binding to G-quadruplexes as shown in figure 4 for the compound NMM. In this format, this porphyrin derivative showed remarkably a preference of binding for quadruplex DNA structures for over all other structures in the assay. There are modest (approximately 2-fold) preferences among the various quadruplex forms. NMM most prefers the antiparallel quadruplex (5'G₄T₄)₃, but has similar affinities for the “G-wire formed by (5'G₁₀T₄G₁₀) and the i-motif formed by 5'TC₄T and 5'C₄T₄C₄. By using the data of Figure 4 to calculate apparent binding constants (as described below), the binding of NMM to quadruplex forms over all other forms seems to be more favourable by at least 2 kcal mol⁻¹.

4.3 Quantitative Analysis of Competition Dialysis Data

Competition dialysis data may be used to derive a number of quantitative parameters that characterize binding affinity and selectivity. These have been discussed in detail in earlier publications (19–21,23). For example, the apparent binding constant, K_{app} , can be simply obtained from the equation

$$K_{app} = C_b / \{C_f \times ([NA]_{total} - C_b)\} \quad (\text{Eq 2})$$

Where C_b and C_f are the bound and free respectively (in our experiment the free ligand is 2 μM) ligand concentrations, $[NA]_{total}$ is the total nucleic acid concentration (in our experiment 75 μM). This treatment assumes that potential binding sites are in excess. The unit of this apparent binding constant refers to the monomeric unit of each polymer. Binding free energies can then be calculated by using the standard equation

$$\Delta G_{app} = -RT \ln K_{app} \quad (\text{Eq 3})$$

Binding constants and free energy parameters obtained in this way are only an indication as they are derived from a single set of reactant concentrations and would correspond only to a single point on a more complete binding isotherm. Nonetheless, binding constants obtained in the way correlate well with more complete binding constants determined by spectrophotometric analysis (20,21).

Additional metrics, such as the specificity sum and Tukey box plots (20,21) are useful measures of binding selectivity and binding mode, but are limited in use for comparison of the properties of ligands studies using the identical array of structures. Since the library of ligands studied using the 46 structure array is limited, the reader is referred to discussions of the use of these metrics as applied to earlier generations of the assay (20,21).

4.4 Difference plots

Competition dialysis data obtained on libraries of compounds can be analysed by *difference plots* to select compounds with particular affinity and selectivity for a given target. The procedure to construct these graphs is very simple and straight forward and was described in an earlier publication (20). Differences in the amounts of compound bound to the structure of interest and other structures are simply calculated by subtraction. Positive values indicate those compounds with higher affinity for the structure of interest, while negative values indicate those compounds with lower than affinity for the structure. Similarly, differences relative to the average amount bound to the structures of interest reveals compounds with higher or lower affinity relative to the mean.

Difference plots can be constructed to obtain an overview of binding of a test compounds for a particular DNA structure and as a tool to select compounds with the most marked preference for a particular target. A collection of difference plots is shown in Figure 5 to reveal binding preferences for the human telomere G-quadruplex DNA over other quadruplex structures and duplex poly (dGdC). Though this figure seems at first glance to be hopelessly complex, it provides an valuable insight into compound selectivity with only a little effort. Panel A shows the difference in binding of 67 compounds for the human telomere G-quadruplex over the tetramolecular parallel quadruplex ($5'T_2G_{20}T_2$)₄. Panel B shows the difference in binding to the human telomere quadruplex over binding to the “G-wire” formed by $5'G_{10}T_4G_{10}$. Panel C shows binding to the quadruplex over duplex polydG-dC. Panel D shows differences in affinity for the human telomere quadruplex over the average value for all 67 compounds.

The value of these plots may be illustrated by looking at the differences seen for PIPER, indicated by the arrow nearest the x-axis. All of the differences across the five plots are positive. This indicates that PIPER prefers to bind to the human telomere quadruplex over the parallel quadruplex and the “G-wire”, over duplex poly (dGdC), and that it binds with slightly higher affinity relative to the mean.

Data for ditercalinium (figure 6), a dimer of 7H-pyridocarbazole, is indicated by the arrow near the top of the graphs in Figure 5. The difference plots indicate that ditercalinium, like PIPER, prefers to the human quadruplex structure over other quadruplexes and duplex DNA. Its relative affinity for the human telomere quadruplex is slightly greater than that of PIPER. Binding of ditercalinium to quadruplex DNA was reported previously (45).

Another utility of difference plots is in comparison of closely related compounds. Examination of difference plots may allow rudimentary structural-activity relationships to be established. For example, several ethidium bromide derivatives (provided to us by Jean-Louis Mergny) were studied. Their structures are shown in figure 7 and their difference plots are shown in figure 5 by the brackets.

Etidium (asterisk [*] in figure 5) shows little selectivity toward the human telomere quadruplex, and less than average affinity. But addition of the substituents in derivative 9944 (double asterisk [**] in figure 5) yields a compound selective for the human telomere quadruplex over other quadruplex forms and duplex DNA. Its affinity for the human quadruplex is greatly enhanced. Substituents changes as in compounds 8361 and 8362, however, lead to considerable lesser selectivity and affinity. These examples indicate the utility of difference plots in making obvious the effects of substituent changes.

5. Conclusions

Competition dialysis has proved to be a powerful and versatile tool for the study of the molecular recognition of new targets. The method is based on firm thermodynamic principles, is simple to implement and allows the rapid identification of structure-selective binding interactions. The current implementation of the competition dialysis experiment has been adapted to a 96-well plate format, which has allowed the study of a greatly expanded array of nucleic acid forms. The data presented here showed the interaction of six well-known DNA ligands, etidium bromide, berenil, PIPER, DODC, methylene blue and NMM, with an initial array of 19 different structures and sequences, obtained in only 24 hours and a subsequent array of 46 different DNA samples for the G-quadruplex binder NMM. The competition assay is versatile, and can be focused to study proteins or other targets of functional importance. This technique not only allows the identification of structure-selectivity interactions, but provides quantitative parameters that can be used to characterise binding interactions.

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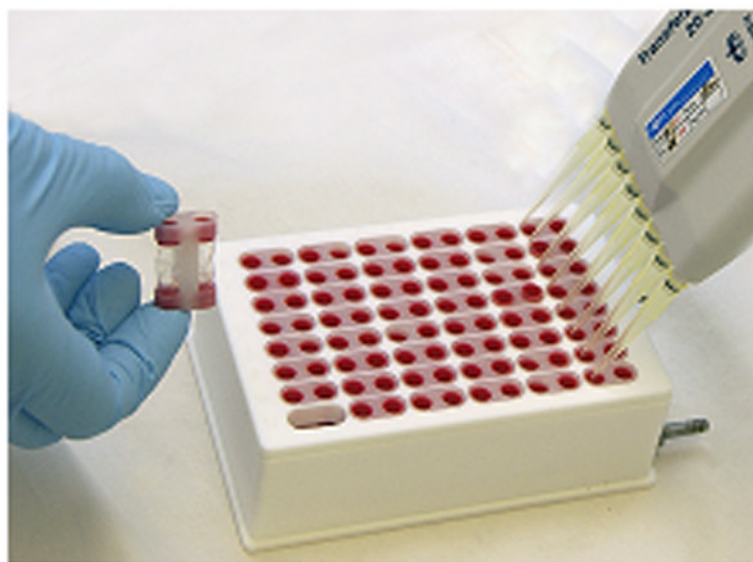


Figure 1.
Microdialyzer for 96 samples

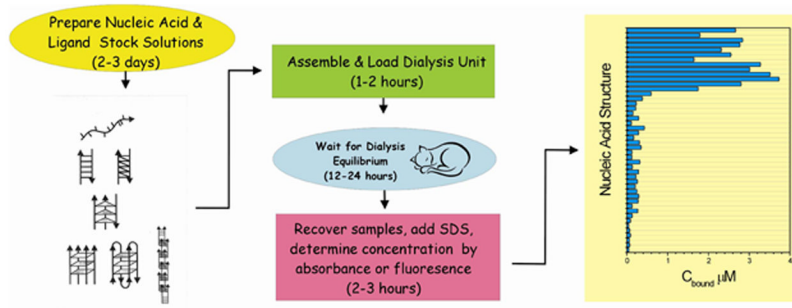


Figure 2.
Overview of the competition dialysis experiment

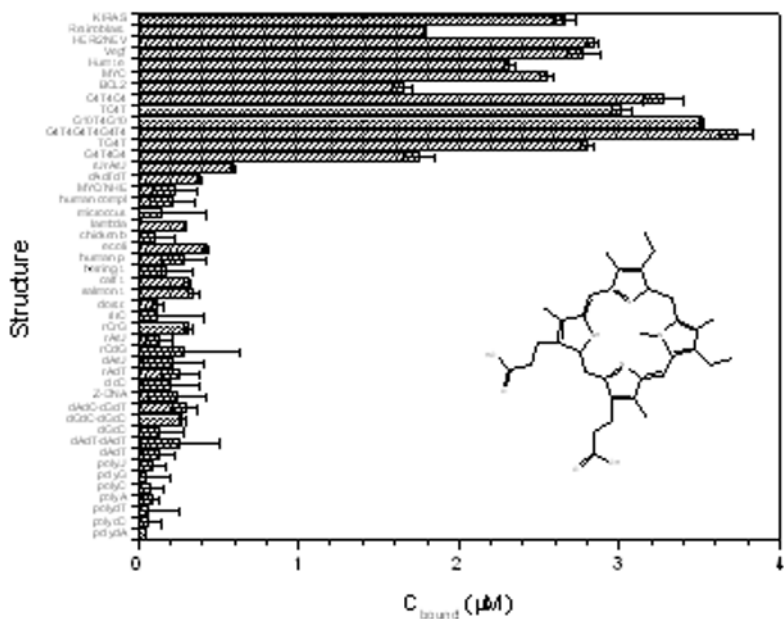


Figure 4. Third generation of competition dialysis for NMM. This porphyrin derivative prefers the G-quadruplex (5'G₄T₄)₃, but displays similar affinities for the quadruplex (5'G₁₀T₄G₁₀) and i-motif quadruplex (5'C₄T₄C₄). Amongst the quadruplex forming oncogene sequences, it preferred the oncogene sequence Her/2Neu but with a relatively similar binding to KiRas and Vegf oncogene sequence.

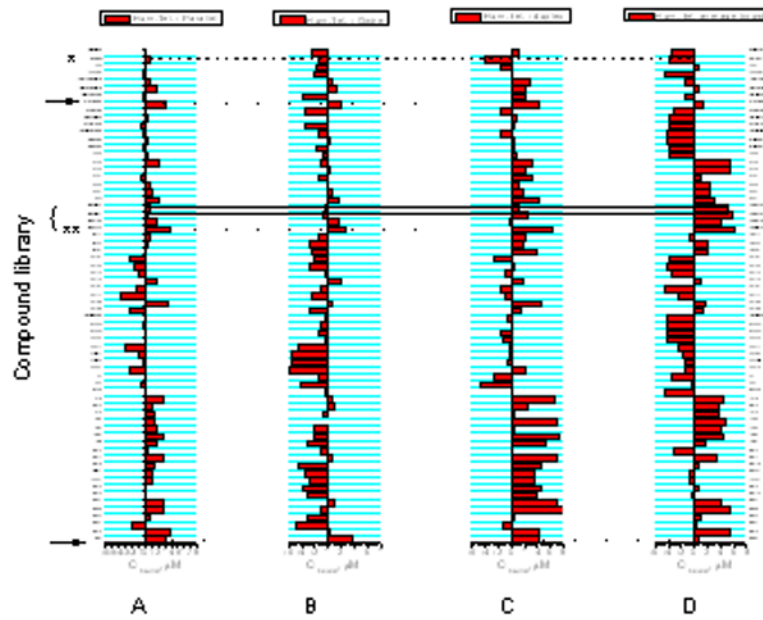


Figure 5. Difference plots for binding to different quadruplex structures. Panel A represents the difference plot for $(T_2G_{20}T_2)_4$. Panel B represents the difference plot for $(G_{10}T_4G_{10})_4$. Panel C represents the difference plot for polydGdC. Panel D represents the difference plot for the human telomere sequence.

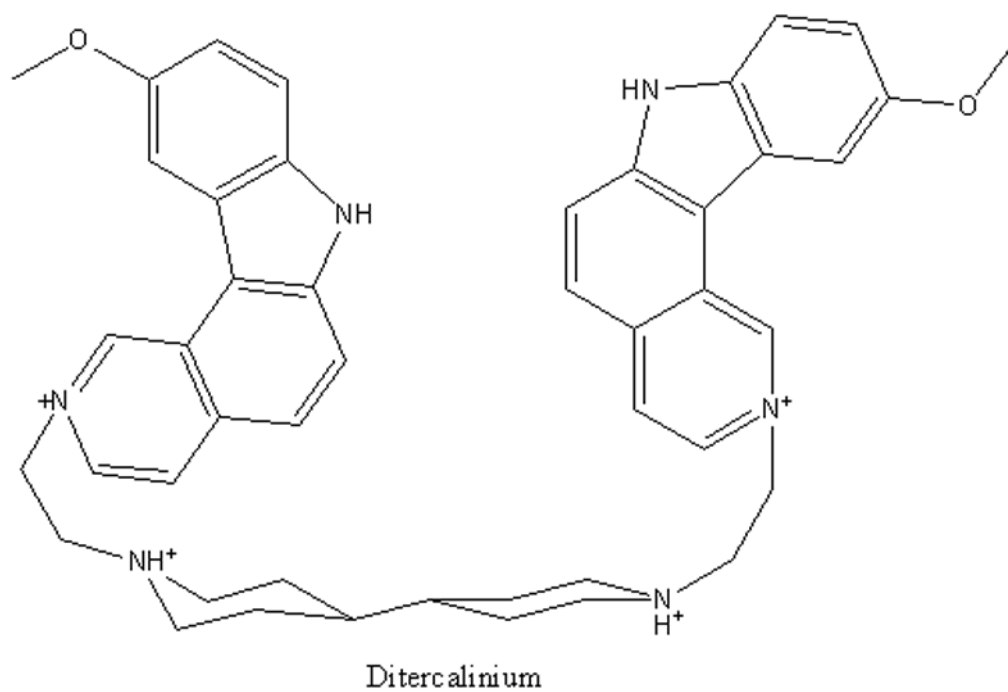


Figure 6.
Schematic representation of ditercalinium.

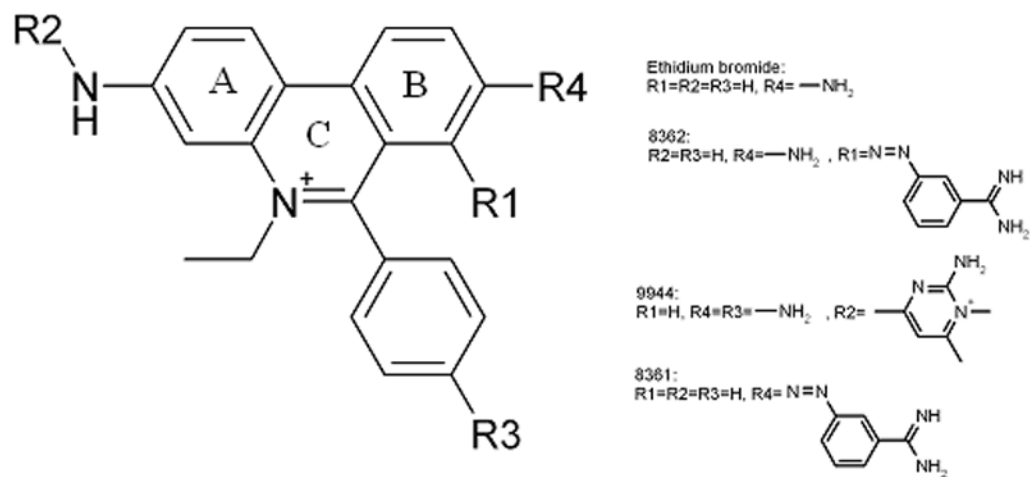


Figure 7.
 Schematic representation of ethidium bromide and its derivatives.

Table 1

Nucleic acid samples used in the current competition dialysis assay

Conformation	Index	Nucleic acid	λ (nm)	ϵ ($M^{-1}cm^{-1}$)	Monomeric unit
Single-stranded DNA	1	poly (dT) [*]	264	8520 ^b	Nucleotide
	2	poly (dA) [*]	257	8600 ^b	Nucleotide
Single-stranded RNA	3	poly (rG) [*]	253	6200 ^c	Nucleotide
	4	poly (rC) [*]	269	6200 ^c	Nucleotide
	5	poly (rA) [*]	258	9800 ^c	Nucleotide
	6	poly (rU) [*]	260	9350 ^b	Nucleotide
	7	poly (dAdT) [*]	260	12000 ^b	base pair
Double-stranded DNA	8	poly (dAdT)-(dAdT) [*]	262	13200 ^b	base pair
	9	poly (dGdC)	254	14800 ^b	base pair
	10	poly (dGdC)-(dGdC) [*]	254	16800 ^b	base pair
	11	poly (dAdC)-(dGdT)	258	13000 ^b	base pair
	12	Z-DNA [*]	254	16060 ^b	base pair
	13	<i>Clostridium Perfringens</i> (31% GC) [*]	260	12476 ^b	base pair
	14	Human placenta (41% GC)	260	12777 ^a	base pair
	15	Salmon testes (41.2% GC)	260	13200 ^b	base pair
	16	Herring testes (42% GC)	260	12858 ^b	base pair
	17	Calf thymus (42% GC) [*]	260	12824 ^b	base pair
	18	Chicken blood (42% GC)	260	12743 ^c	base pair
	19	<i>Escherichia Coli</i> (50% GC)	260	13085 ^c	base pair
	20	Lambda DNA (66% GC)	260	13441 ^a	base pair
	21	<i>Micrococcus lysodeikikus</i> (72% GC) [*]	260	13846 ^b	base pair
	22	Promoter Myc/NHE (TG ₄ AG ₃ TG ₄ A ₂ G ₂ /(AC ₄ TC ₃) ₂ AC ₄ T ₂ C ₂)	260	12355 ^a	base pair
	23	Human telomere/complementary (AG ₃ T ₂) ₃ AG ₃ (TC ₃ A ₂) ₃ TC ₃	260	11403 ^a	base pair
	24	poly (dIdC)	251	13800 ^c	base pair
	25	poly (rCdG)	269	12400 ^c	base pair
	26	poly (rAdT) [*]	260	12460 ^c	base pair
	27	poly (dArU)	257	13000 ^c	base pair
28	poly (rArU) [*]	260	14280 ^c	base pair	
DNA-RNA hybrid	29	poly (r/rC)	245	10600 ^c	base pair
	30	poly (rCrG)	260	13600 ^c	base pair
Triplex DNA or RNA	31	poly (dAdT)-(dT) [*]	260	17200 ^b	Triplet
	32	poly (rArU)-(rU) [*]	260	17840 ^b	Triplet
Quadruplex DNA	33	C ₄ T ₄ C ₄	260	27481 ^a	Quartet
	34	G ₄ T ₄ G ₄	260	16426 ^a	Quartet
	35	TG ₄ T	260	12672 ^a	Quartet
	36	G ₄ T ₃ C ₄ T ₄ G ₄ T ₄	260	46988 ^a	Quartet
	37	G ₁₀ T ₄ G ₁₀	260	42009 ^a	Quartet
	38	TC ₄ T	260	7566 ^a	Quartet
	39	Promoter BCL2 AG ₄ CG ₃ CGG ₃ AG ₂ A ₂ G ₅ CG ₃ AGCG ₄ C	260	41591 ^a	Quartet
	40	Human telomere (AG ₃ T ₂) ₃ AG ₃ [*]	260	73000 ^b	Quartet
	41	Promoter CMYC TG ₄ AG ₃ TG ₄ AG ₃ TG ₄ A ₂ G ₂	260	45309 ^a	Quartet
	42	Promoter VEGF G ₃ CG ₃ C ₂ CG ₄ TC ₃ G ₂ CG ₄ CG ₃ AG	260	35778 ^a	Quartet
43	Promoter HER-2/ <i>neu</i> AG ₂ AG ₂ (AG ₂) ₂ TG ₃ (AG ₂) ₃ GC	260	42985 ^a	Quartet	
44	Promoter RETINBLASTOMA CG ₆ T ₄ G ₃ CG ₂ C	260	9407 ^a	Quartet	
45	Promoter KIRAS G ₃ A ₂ GAG ₃ A ₂ GAG ₃ AG ₂	260	24646 ^a	Quartet	
46	i-motif poly (dC)	274	7400 ^b	Nucleotide	

^a determined experimentally²⁴

^b from reference 27

^c GE Healthcare Bio-sciences, technical information

* nucleic acids structures used in the 19 format of the assay