Mix and measure fluorescence screening for selective quadruplex binders

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ABSTRACT

The human genome contains thousands of regions, including that of the telomere, that have the potential to form quadruplex structures. Many of these regions are potential targets for therapeutic intervention. There are many different folding patterns for quadruplex DNAs and the loops exhibit much more variation than do the quartets. The successful targeting of a particular quadruplex structure requires distinguishing that structure from all of the other quadruplex structures that may be present. A mix and measure fluorescent screening method has been developed, that utilizes multiple reporter molecules that bind to different features of quadruplex DNA. The reporter molecules are used in combination with DNAs that have a variety of quadruplex structures. The screening is based on observing the increase or decrease in the fluorescence of the reporter molecules. The selectivity of a set of test molecules has been determined by this approach.

There are many regions of the human genome that have the potential to form quadruplex structures (1–3) with the d(TTAGGG) repeat of the vertebrate telomere being the most familiar (4,5). Of the apparently thousands of regions in the human genome that can potentially form quadruplex structures some are in the promoters of genes important in cancer including c-myc, bel-2, e-kit, VGEF and KRAS (1–3,6–10). Quadruplex forming regions are also found in the promoters of other genes including in the insulin-linked polymorphic region (11).

Some of the quadruplex folding patterns that have been observed are depicted in Figure 1. The details of the experimental conditions, in particular the concentration of potassium, can play a significant role in determining which, if any, quadruplex structure is formed (12–18). At present it is not possible to predict which quadruplex structural type will be adopted by a particular sequence under a given set of conditions. It is known that modest changes such as the substitution of dU for dT, the addition of one or two nucleotides as a dangling end or even the addition of a terminal 5’ P can alter the quadruplex structural type (9,19,20). Thus, there are many regions of the genome that can adopt quadruplex structures and there are many types of quadruplex structure. This diversity of structures and sequences suggests that selective targeting is possible though it may be challenging to attain.

A motivation for the investigation of quadruplex DNA is the understanding of transformation and to identify targets for chemotherapy (4,5,9,21–27). The inhibition of telomerase is thought to be a potential route to cancer therapy and about 85% of human tumor cells have elevated telomerase activity (28). Molecules that bind to quadruplex DNA have been shown to inhibit telomerase and to be selectively toxic to tumor cells and at least some of the cytotoxic activity of quadruplex-binding ligands may arise from the disruption of telomere structure (4,5,9,21–27).

Proteins have been found that have high affinity for quadruplex DNAs and defects in these proteins can lead to errors in replication, transcription and recombination, as well as to increases in the rates of tumor formation and aging (29). Quadruplex DNAs have been shown to inhibit telomerase, HIV integrase and thrombin (29). There is evidence that the presence of quadruplex DNA can lead to errors in transcription, replication and recombination, while quadruplex DNA may play roles in recombination and the formation of the synaptonemal complex (30–34). There have been previous studies that have examined the binding of ligands to quadruplex DNA as described in recent reviews (5,9,21,22,35). Since there are many genomic sequences that can form quadruplex structures and there are many types of quadruplex structure there is a need for methods that allow rapid screening for selective binding molecules. Enhancing the affinity of selective binding ligands is typically easier than improving the selectivity of strong binding, nonselective ligands.

The fluorescence method presented here allows for the rapid, microplate, mix and measure screening of ligand binding to quadruplex DNAs of diverse sequence and structure. The fluorescence of the reporter molecules increases when the reporter binds to quadruplex DNA.

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and ligands can enhance or decrease the reporter molecule fluorescence. Since the fluorescence is a property of the reporter molecule, this allows the screening of almost any drug-like molecule. This methodology utilizes multiple fluorescent dyes that bind to different features of the quadruplex DNAs thus allowing monitoring of binding to different features of the quadruplex structures. The fluorescent reporter molecules used as reporters do not alter the type of quadruplex structure adopted by the DNAs. The molecules that bind to specific quadruplex structural types can be used to gain understanding about the biological roles of quadruplex DNA as well as in designing therapeutics.

**MATERIALS AND METHODS**

**DNA sample preparation**

All of the DNAs were obtained from Integrated DNA Technologies Inc. as HPLC purified samples. The DNA samples were desalted by ethanol precipitation and dissolved in 100 mM KCl, 5 mM NaCl and 2.5 mM phosphate at pH 7.0. The DNA samples were heated to 363 K, in a water bath, for 8 min and then allowed to cool to room temperature overnight. The extinction coefficients of the DNAs used to determine the concentrations are listed in Table 1.

**Dyes and test compounds**

The N-methyl mesoporphyrin (NMM), was obtained from Porphyrin Products Inc. and 1 mM stock solution was prepared in distilled water. The 3,3'-diethyloxadicarbocyanine iodide (DODC) and 3,3'-diethylthiacarbocyanine iodide (DTDC) were obtained from Sigma-Aldrich and millimolar stock solutions were prepared in DMSO. There was <1% of DMSO in the samples used for the optical experiments. The concentrations were determined using the extinction coefficients 145 000/M/cm at 379 nm for NMM; 270 000/M/cm at 582 nm for DODC and 125 000/M/cm at 557 nm for DTDC.

The sequences of the DNAs used in the screening are given along with the structural type where known (9, 20) with the structural types shown in Figure 1. The structure of ILPR2, insulin promoter region repeat, in the presence of potassium is not known. TBA is the thrombin binding aptamer; Vet 4 contains four repeats of the vertebrate telomere repeat d(GGGTTA); Vet T4 contains four repeats of the vertebrate telomere repeat d(TTAGGG); Vet T2 and Vet 2 U6 are variants of Vet 2 of known structure; Oxy 1.5 contains 1.5 repeats of the Oxytricha telomere repeat d(GGGTGGG).

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<table>
<thead>
<tr>
<th>DNA</th>
<th>Sequence</th>
<th>Fold type</th>
<th>Extinction coefficient M⁻¹ cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA</td>
<td>d(GGTTGGTGTGGTTGG)</td>
<td>A4</td>
<td>143 300</td>
</tr>
<tr>
<td>Oxy 1.5</td>
<td>d(GGGGTTTTGGGG)</td>
<td>A6</td>
<td>115 200</td>
</tr>
<tr>
<td>Vet 2 U6</td>
<td>d(TAGGGUTAGGGGT)</td>
<td>P3</td>
<td>121 500</td>
</tr>
<tr>
<td>TGGGT</td>
<td>d(TGGGTTG)</td>
<td>P1</td>
<td>57 800</td>
</tr>
<tr>
<td>22 mer</td>
<td>d(AGGGG(TTAGGG)₃)</td>
<td>P2</td>
<td>228 500</td>
</tr>
<tr>
<td>24 mer</td>
<td>d(AGGGG(TTAGGG)₃A)</td>
<td>M2/M1</td>
<td>244 300</td>
</tr>
<tr>
<td>Vet T4</td>
<td>d(TTAGGG)₄</td>
<td>M2</td>
<td>244 600</td>
</tr>
<tr>
<td>Vet 4</td>
<td>d(GGGTTA)₅</td>
<td>P3</td>
<td>246 300</td>
</tr>
<tr>
<td>Vet 2 T6</td>
<td>d(TAGGGGTAGGGGT)</td>
<td>A2/P3</td>
<td>122 800</td>
</tr>
<tr>
<td>Vet T2</td>
<td>d(TTAGGG)₄</td>
<td>P3</td>
<td>122 400</td>
</tr>
<tr>
<td>ILPR2</td>
<td>d(ACAGGGGTGTGGGG)₂</td>
<td>P3</td>
<td>283 900</td>
</tr>
</tbody>
</table>

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Circular dichroism (CD) experiments

The CD spectra were obtained using a Jasco J-810 spectropolarimeter equipped with a six cell programmable Peltier junction temperature controlled cell holder using methods previously described (19). CD experiments were carried out on DNA samples at a concentration of $1 \times 10^{-5}$ M. The CD measurements used 0.2 cm path-length cell and were carried out at 298 K. The CD spectra were averaged over three scans and data was obtained with a 1 nm slit width from 200 to 650 nm at 0.1 nm intervals.

Fluorescence experiments

The fluorescence experiments were carried out using a Spectramax M5 microplate reader. The excitation and the emission wavelengths for NMM are 399 and 610 nm, for DODC these are 579 and 610 nm and for DTDC 554 and 577 nm. The plate reader experiments were carried out at 298 K and all experiments were carried out at least in duplicate. The emission and excitation spectra for NMM, DODC and DTDC in the presence and in the absence of DNA are included in the Supplementary Data. The absorption spectra for NMM, DODC and DTDC in the presence and in the absence of DNA are also included in the Supplementary Data.

RESULTS AND DISCUSSION

The basis of the screening assay is that the reporter molecules become more fluorescent in the presence of quadruplex DNA. To be able to monitor the binding of ligands to the various structural features of quadruplex DNAs more than one reporter molecule is needed as the different reporter molecules can bind to distinct parts of the quadruplex structure. The assay uses a set of quadruplex DNAs of diverse structure and sequence to allow the screening for selectivity as to quadruplex structural type and sequence. CD experiments were carried out to show that the binding of the reporter molecules does not alter the structure of the quadruplex DNAs. Thus, the combined results from using multiple reporter molecules with a variety of DNA quadruplexes can be used to categorize ligands as to their quadruplex structural type binding specificity.

The assay is carried out at a concentration of DNA that leads to half the maximum increase in fluorescence of the reporter molecule. This allows for monitoring whether the binding of the test molecule increases or decreases the reporter molecule fluorescence. Examples of both increased and decreased fluorescence are shown below.

The reporters that are used in this version of the screening are NMM, DODC and DTDC whose structures are shown in Figure 2. All three dyes show an increase in fluorescence upon binding to quadruplex DNA. DODC and DTDC, but not NMM, also exhibit induced CD upon binding to most quadruplex DNAs.

There is no atomic resolution structural information about the complexes between any of these reporter dyes with quadruplex DNA. Since NMM has a large surface area and shows little, or no selectivity in its binding to quadruplex DNAs (36) it may well be the case that NMM binds to the dG quartets as is the case for some other large surface area binders.

Quadruplex DNA structures included in the screen

Quadruplex structural types have been recently reviewed (9,20) and the sequences and folding patterns of the DNAs used here are given in Table 1. The four strands can be parallel, anti-parallel or mixed and additional variation arises from how the strands are connected by the loops. Two of the DNAs included in the screen are the Vet T4 and Vet 4 DNAs that contain four repeats of the vertebrate telomere repeat.

Effect of quadruplex DNA on the fluorescence of NMM, DODC and DTDC

To be effective as reporter molecules the fluorescence needs to change upon binding to quadruplex DNA. The structures of the reporter molecules NMM, DODC and DTDC are shown in Figure 2. It has been known for some time that the fluorescence of NMM is enhanced by the presence of quadruplex but not duplex DNA (36) and
that NMM binds to quadruplex but not duplex DNA (37). The fluorescence of NMM was measured in the presence and in the absence of each of the quadruplex DNAs and the results are given in Table 2. In each case there is a significant increase in the fluorescence.

The fluorescence of NMM was monitored as a function of the concentration of each of the DNAs. The DNA concentration that gives rise to 50% of the maximum increase is listed in Table 2. The screening was done at the 50% mark, to allow screening for molecules that can displace the NMM can be detected, as well as those test molecules that enhance the fluorescence of NMM upon binding.

Analogous experiments were carried out for DODC and DTDC and the results are listed in Table 2. The DNA concentrations needed to obtain 50% increase are greater for both DODC and DTDC than was found with NMM. The extent of fluorescence increase for any one of the reporter molecules with a particular DNA does not correlate in any obvious way with the fluorescence increase of the other two. Similarly, the DNA concentration needed for half maximal increase for any one of the reporter molecules with a particular DNA does not correlate in any obvious way with the concentration needed for the other two reporter molecules. The effect of NMM on the fluorescence of DODC and DTDC is presented below.

### Effect of NMM, DODC and DTDC on the structures of quadruplex DNAs

If a reporter dye alters the structure of the quadruplex DNA then it will have limited usefulness in screening. Therefore, CD was used to examine the effects of the reporter dyes on the structures of the quadruplex DNAs. The CD of each of the DNAs was monitored as a function of reporter dye concentration. The results in Figure 3 show that the presence of the reporter dyes do not alter the CD spectra of the DNAs. Since the CD spectrum is a sensitive monitor of structural change these results indicate that the reporter dyes do not change the structures of the DNAs under these conditions as was previously shown for NMM under similar conditions (38).

The addition of the dyes to the DNAs before annealing can lead to changes in the structures of the DNAs (36). The results presented here indicate that under the conditions used these reporter dyes do not alter the structures of the DNAs.

### Induced CD of DODC and DTDC

The CD of the reporter dyes was also investigated to gain some information about the nature of the complexes as well as the competition between the reporter molecules. There was no observed induced CD from any of the NMM–DNA binary complexes examined. This is consistent with NMM binding to the terminal dG quartets that is likely to be an environment of low chirality.

The cyanine dyes DODC and DTDC bind to the minor groove of duplex DNA primarily as dimers, and the dimers can form higher-order structures (39–44). These dye preferentially interact with AT rich regions of duplex DNA (39–44). The interactions between the dyes in the chiral environment of the minor groove leads to the pronounced induced CD that is observed when the dyes bind to duplex DNA (39–44). The binding of DTDC to the quadruplex form was first demonstrated for (dGGTTGGTGTGGTTGG) (45) and subsequently for some other examples (46) and the biological effects of DTDC have also been examined (47). Under conditions different from those used here Shafer and co-workers (45) observed that the fluorescence of DTDC is enhanced by some quadruplex DNAs and decreased by others. We have obtained preliminary NMR data that is consistent with DODC interacting with the loops of quadruplex DNA. While the existing CD and fluorescence competition data is consistent with these cyanine dyes binding to the loops of quadruplex DNAs atomic resolution data is needed to determine the structures of the complexes of the cyanine dyes with quadruplex DNAs.

DODC and DTDC exhibited induced CD with almost every DNA examined here. The induced CD of DODC shows considerable variation from DNA to DNA as does that of DTDC. Representative spectra are shown in Figure 3. The induced CD patterns of DODC and DTDC do not follow any obvious pattern for the quadruplex DNAs examined here. There are quadruplex DNAs for which the induced CD of DODC is quite similar but the induced CD of DTDC is quite different. There are also quadruplex DNAs for which the induced CD of DTDC is quite similar and that of DODC is quite different.

The observation of the induced CD and the dependence on the quadruplex DNA is consistent with DODC and DTDC binding, at least partially, to the loops of the quadruplex DNAs since there is more variation in the loop structures of these quadruplex DNAs than there is in the dG quartets. The observation that there are differences in the induced CD spectra of both DODC and DTDC from DNA to DNA indicates that these dyes are binding to sites that reflect the differences in the loop structures of the quadruplex DNAs. Induced CD for DODC and
DTDC has been previously observed for the binding to the minor groove of duplex DNA.

**Competition between NMM, DODC and DTDC**

The effects of NMM on the fluorescence and induced CD of DODC and DTDC were examined to determine the extent to which the reporter molecules compete with one another. The induced CD of DODC and DTDC were monitored as a function of NMM concentration and selected results are shown in Figure 4.

The results on the competition experiments indicated that NMM competes with DODC and DTDC in binding to d(GGTTGGTGTGGTTGG). NMM competes with DODC in binding to Vet T4, but not DTDC. In the case of Vet 4 NMM competes with DTDC and DODC, but at different levels (data not shown). Since CD has low sensitivity and is not suitable to high-throughput screening, only a few cases were examined by this method.

Induced CD for NMM was only observed when DODC was also present and only for d(GGTTGGTGTGGTTGG) the thrombin binding aptamer. The induced CD signal occurs where NMM absorbs and has a positive band near 390 nm and a negative band near 420 nm as shown in Figure 4. This indicates that NMM and DODC can bind to the 15-mer at the same time and that the DODC binding alters the environment of NMM. This is additional evidence that these two reporter molecules have distinct interactions with DNA.

A more extensive set of comparisons was undertaken using the fluorescence of DODC and DTDC. In these experiments the fluorescence of DODC and DTDC was monitored as a function of the concentration of NMM. The results are summarized in Table 3. NMM decreases the fluorescence of DODC and DTDC in all, but one case where an increase is observed. The increase could be due to energy transfer, direct interaction between the bound ligands of other effect. These results indicate that the competition between NMM and DODC has a distinctly different pattern than the competition between NMM and DTDC. Spectral overlap precludes carrying out these experiments on the competition between DODC and DTDC. The results of the fluorescence and CD competition experiments indicate that the three reporter molecules do not bind to quadruplex DNAs in the same fashion. Thus, the combined use of the three reporter molecules will give more information about test molecule binding than using just one or two reporters.

**Screening of quadruplex-binding ligands**

As discussed above there are multiple structural types of quadruplex DNA that can be formed and quadruplex structures can also vary in the numbers of quartets and in the sequences of the loops. Thus, the screening for selectivity needs to be able to report on the binding to a variety of structural features. The results presented above indicate that the use of NMM, DODC and DTDC allow the monitoring of a variety of structural features and that...
the array of quadruplex DNAs allows monitoring the quadruplex structural type specificity.

To investigate the utility of this approach we have applied it to a select set of test molecules. A diverse set of test molecules was chosen and the structures are shown in Figure 5. N-hexanoyl-α-sphingosine is a potent apoptotic (48) and the other test molecules have all been previously investigated for their interactions with quadruplex DNA (49–53). This set of test molecules contains sugar, peptide, planar aromatic, aliphatic chains and a variety of other structural features. This set was chosen to allow preliminary examination of whether any of these structural features interfere with the screen.

The screening was carried out using a conventional microplate reader. The fluorescence of the DNA–reporter molecule samples was measured in the presence and in the absence of the test ligand. The results are presented in Figure 6 with red indicating a decrease in fluorescence and blue an increase with the darker shades indicating a larger change. The color chart mode of presentation allows visual comparison of the results obtained for the test ligands.

Both increases and decreases in fluorescence are observed. Decreases in fluorescence can be due to competition while there are a number of mechanisms that could lead to increases in fluorescence. These mechanisms include direct interactions between the test ligand and reporter molecule, as well as indirect effects such as partial charge neutralization by the test ligand which could enhance binding of the reporter molecule.

The results in Figure 6 indicate that all six of the test ligands exhibit some level of selectivity as to quadruplex
structural type and that the selectivity varies from ligand to ligand. This is not that surprising considering the diversity of quadruplex structures. Ligands that bind primarily to the quartets, rather than the loops, may show low specificity as does the reporter NMM. Berberine appears to have the highest selectivity of this group of test ligands. Apigenin has a profile that is quite distinct from that of the other test ligands examined.

The results in Figure 6 also indicate that the test ligands display a range of selectivity as to the reporter molecules. For example, daunomycin has almost no effect on the fluorescence of DODC but it does affect the observed fluorescence of DTDC and NMM. Correlations between structural features and quadruplex binding specificity and affinity will require analysis of the results on a larger set of test ligands.

**SUMMARY AND FUTURE DIRECTIONS**

The results presented here show that the combined use of multiple reporter molecules along with DNAs that exhibit a range of quadruplex structures allows selective screening not only of small molecules but proteins and other large molecules. The method presented here is more general than a recently presented fluorescence displacement approach using a single reporter molecule (54,55). This screening method can now be applied to the chemical
depositories maintained by the National Cancer Institute/Developmental Therapeutics Program and others. In addition, the structures of the quadruplex DNAs can be used in conjunction with modeling methods to identify candidate molecules to screen. Both approaches are being pursued as well as the solution state structures of the DODC and DTDC complexes. In addition, the authors would welcome candidate molecules from other research groups for screening.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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