A new method for the study of G-quadruplex ligands†‡

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A new method for the study of G-quadruplex ligands was developed, in which the interaction of G-quadruplexes with ligands can be judged by the naked eye, eliminating the need for any expensive machines.

Telomerase has been regarded as a key target for anti-cancer therapy.1 A novel approach to the inhibition of telomerase involves the stabilization of the G-quadruplex form of telomeric DNA by G-quadruplex ligands. The 3-terminal region of the G-rich strand of human telomeres is single-stranded and may adopt a G-quadruplex conformation.2 The formation of the G-quadruplex structure may effectively hinder telomerase from adding further repeats.3 A key element in the development of potent G-quadruplex ligands, as in the development of any class of drug, is the screening of large chemical libraries of potential candidates.4 Several screening methods based on circular dichroism (CD),5 UV-spectroscopy,6 or fluorescence7 have been developed. In these methods, CD and UV absorbance require relatively high DNA and G-quadruplex ligand concentrations, and may therefore suffer from precipitation/aggregation problems and absorbance interference of some G-quadruplex ligands.4 A novel fluorogenic probe, G-quadruplex molecular beacon,7 has been used in fluorescence assays. This method may screen G-quadruplex ligands with high throughput, but it needs expensive real-time PCR machines. Therefore, the development of a simple, inexpensive, and high throughput screening method is required.

In the present work, such a novel screening method is developed based on the enhanced peroxidase activity of a G-quadruplex, hemin complex, which can catalyze the oxidation of 2,2′-azinobis(3-ethylbenzo thiozoline)-6-sulfonic acid (ABTS) by H2O2.8 The reaction mixture produces a characteristic green color. When a G-quadruplex ligand is added, the ligand competes with hemin to bind to the G-quadruplex and the color intensity of the reaction mixture decreases. Consequently, the competitive ability of the given ligands can be easily monitored according to the decrease of the color intensity of the reaction mixture by the naked eye.

In order to clearly distinguish the change in the color of the reaction mixtures in the absence or presence of G-quadruplex ligands by the naked eye two basic requirements must be fulfilled. First, the reaction mixture without G-quadruplex ligands must have a deep enough color. And second, the color of the reaction mixture in the presence of G-quadruplex ligands must fade to some degree. To establish these baseline parameters several important features, such as the concentrations of some reactive components and the reaction time, were investigated (see ESI, Fig. S1-S3).‡ The concentrations of hemin, the G-quadruplex (AG4: 5′-TG3TAG3CG3TTG3AAA) and ABTS are selected as 0.5 μM, 0.9 μM and 1.28 mM, respectively. The reaction mixtures could reach a stable green color within 5 min, and the color stays stable for at least 30 min.

Under the experimental conditions we selected, the feasibility of the screening method was tested. In this experiment, eight candidate drugs were used (see ESI, Fig. S4).‡ Four of them gave positive results (Fig. 1A–1D). Of these positive drugs, TMPyP4 (5,10,15,20-tetra-(N-methyl-4-ppyridyl) porphine) has been demonstrated to be a good G-quadruplex ligand9 and it also showed good competitive ability against hemin. With increasing TMPyP4 concentration the color of the reaction mixture began to fade gradually and became very weak at 6 μM. At higher concentrations the mixtures turned yellow, which is the color of free TMPyP4. Ethidium bromide (EB) is known to be a weak G-quadruplex ligand.10 It can also displace hemin from the AG4-hemin complex and make the color of the reaction mixture become weaker with increasing concentration. But its competitive ability is obviously weaker than that of TMPyP4; only when the concentration of EB reached 25 μM did the color of the reaction mixture become nearly unnoticeable. Methyl green is a triphenylmethane dye. The interaction of malachite green, another triphenylmethane dye, with G-quadruplex has been reported.11 Compared to both TMPyP4 and EB, methyl green showed a very weak competitive ability. The color of the reaction mixture showed a pronounced change only when very high concentrations of methyl green were added. Isoconazole nitrate is similar in structure to the triphenylmethane dyes but it has a slightly longer distance between the three aromatic groups, which may favor a stronger stacking with the n-system of the guanine quartet. The screening assay showed that isoconazole nitrate may be a good G-quadruplex ligand. Its competitive ability was much stronger than those of EB and methyl green, but a little weaker than that of TMPyP4. As negative controls, four other candidate drugs, Fe(dmbpy)2(BF4)2 (dmbpy = 4,4′-dimethyl-2,2′-pyridine), sodium tetrathylboron, 4,4′-dimethyl-2,2′-bipyridine and dimethyl-2,2′-(oxalyldiimino)bisphenylyloxylate, gave negative results (Fig. 1E–1H). With increasing drug concentrations,

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‡ Electronic supplementary information (ESI) available: Experimental details, the chemical structures of candidate drugs, the selection of concentrations of hemin, AG4 and ABTS, and the representative results of fluorescence tests. See DOI: 10.1039/b804627a

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Table 1 The results of screening and G-quadruplex stabilization experiments

<table>
<thead>
<tr>
<th>Candidate drugs</th>
<th>Screening results</th>
<th>IC₅₀/µM</th>
<th>Densitometric assay</th>
<th>UV-vis assay</th>
<th>∆Tₜₐₜ/C°</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMPyP4</td>
<td>positive</td>
<td>2.90</td>
<td>—</td>
<td>—</td>
<td>3.27(2 µM)</td>
</tr>
<tr>
<td>EB</td>
<td>positive</td>
<td>6.44</td>
<td>7.58</td>
<td>3.20</td>
<td></td>
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<tr>
<td>Methyl green</td>
<td>positive</td>
<td>21.45</td>
<td>29.04</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Isoconazole nitrate</td>
<td>positive</td>
<td>5.68</td>
<td>5.98</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Fe(dmbpy)₂(BF₄)₂</td>
<td>negative</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Sodium tetraphenylboron</td>
<td>negative</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>4,4'-dimethyl-2,2'-pyridine</td>
<td>negative</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Dimethyl-2,2'-(oxalylidimino)bis (phenylglyoxylate)</td>
<td>negative</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

*ΔTₜₐₜ = Tₜₐₜ(F22D + drug) - Tₜₐₜ(F22D). The concentration of the candidate drugs is 25 µM except TMPyP4. *ΔTₜₐₜ = Tₜₐₜ(F22D + drug) - Tₜₐₜ(F22D +5% (v/v) DMSO). The drug concentration-dependent color evolution of the reaction mixtures can also be detected by following the absorption intensities at λ = 414 nm (Fig. 1J). According to the absorption-concentration plots, relatively precise IC₅₀ values can be obtained, which were 5.98, 7.58 and 29.04 µM for isoconazole nitrate, EB and methyl green, respectively (Table 1). These values are comparable to those obtained by densitometric analysis. The IC₅₀ value of TMPyP4 was not determined by absorption spectroscopy because of the absorbance interference of free TMPyP4 at the detection wavelength. These results demonstrated the availability of densitometric analysis, which needs no experimental apparatus but a computer. Another advantage of densitometric analysis is that it can be used to evaluate the candidate drugs that exhibit absorbance interference in the absorption spectroscopy assay.

In order to investigate whether the decrease of the color intensities observed upon the addition of the positive drugs was due to their displacement behaviors or just to their bleaching abilities, another experiment was designed. In this experiment, two reaction tubes containing the AG₄-hemin complex were prepared. When H₂O₂ and ABTS were added, both of the reaction tubes displayed the characteristic green color. Then, the proper amount of the studied drugs was added into one reaction tube immediately, and the colors of the two reaction tubes were compared. The results show that the addition of the studied drugs did not cause any color difference between the two reaction tubes, indicating that the decrease of the color intensities really can reflect the competition between the drugs and hemin.

In order to give a semiquantitative evaluation of the abilities of the candidate drugs to bind to G-quadruplex, densitometric quantitation of the images in Fig. 1A–1D was performed using Glyko BandScan software (Version 4.30) (Fig. 1I). According to the plots of normalized optical density versus drug concentration, the IC₅₀ values, which represent the drug concentration required for 50% decrease of the color intensity, can be calculated. The obtained IC₅₀ values were 2.90, 5.68, 6.44 and 21.45 µM for TMPyP4, isoconazole nitrate, EB and methyl green, respectively. (Table 1)
In this report, an artificial G-quadruplex (AG4) is used. AG4 is a 20-nucleotide DNA oligomer and the enhanced peroxidase activity of AG4-hemin has been demonstrated. Under the same conditions, the complex formed by hemin and a human telomeric sequence (Hum24: 5'-(TTAGGG)₃) displays only very weak peroxidase activity, and the color intensity of the reaction mixture is too weak to be used for the screening of G-quadruplex binders (data not shown). In order to investigate whether the positive drugs in the screening assay can be used as stabilizers of human telomeric G-quadruplex, the stabilizing abilities of the studied drugs to a human telomeric G-quadruplex were demonstrated using the fluorescence method, in which a G-quadruplex molecular beacon (F22D: F AM-AGGG(TTAGGG)₃-DABCYL) that consists of human telomeric G-quadruplex was used. G-quadruplex molecular beacons are single-stranded oligonucleotide probes with guanine-rich sequences. A fluorophore (6-carboxyfluorescein, FAM) is attached to one end of the oligonucleotides and a quencher (4-(4′-dimethylaminophenylazo) benzoic acid, DABCYL) is attached to the other end. When the oligonucleotides are folded into G-quadruplex structures, the fluorophore is kept in close proximity to the quencher, causing the fluorescence of the fluorophore to be quenched. However, at higher temperatures the G-quadruplex structures give way to a random-coil configuration, separating the fluorophore from the quencher, restoring fluorescence. By recording the temperature–fluorescence profile of the probe (see ESI, Fig. S5A), the melting temperature (Tₘ) of the G-quadruplex structure can be determined by taking the maximum of the first derivative (see ESI, Fig. S5B). The two methods gave nearly comparable results. All of the drugs that were negative in the screening assay did not exhibit any stabilizing abilities to the human telomeric G-quadruplex in the fluorescence test (Table 1). Of the four drugs that were positive in our initial screen, TMPyP4, EB and methyl green showed different abilities to stabilize the G-quadruplex molecular beacon. The observed melting temperature changes (ΔTₘ) of the beacon were 3.95 °C for 3 μM TMPyP4, 3.20 °C for 25 μM EB, and 0.67 °C for 25 μM methyl green, which is consistent with their observed abilities to displace hemin from the AG4-hemin complex. The one exception to this correlation is isoconazole nitrate, which displays strong competitive ability against hemin but gives a ΔTₘ of 0.30 °C under the same conditions. This value is too small to be significant and may therefore be regarded as negative. Further studies are required to elucidate the reasons for this discrepancy. One possibility is that the solution of isoconazole nitrate is prepared by dissolving the drug in dimethyl sulfoxide (DMSO), and the fluorescence test indicates that the presence of DMSO will increase the Tₘ of F22D. In the presence of isoconazole nitrate, the ΔTₘ is obtained by subtracting the Tₘ value in the presence of a certain amount of DMSO from the Tₘ value in the presence of the drug. That is to say, the stabilizing ability of DMSO to the G-quadruplex molecular beacon may impair the apparent stabilizing ability of isoconazole nitrate. Under the studied conditions, the use of DMSO did not show any influence on our screening experiments. The results mentioned above indicate that the screening method described in this paper may produce some false-positive results, but no false-negative results are observed. Considering that the false-positive results can be picked out in subsequent experiments, and that false-negative results may make us miss a good candidate drug, our method may be used as a primary screening technique for G-quadruplex ligands.

In order to demonstrate the availability of the new method, several candidate drugs, including a strong G-quadruplex ligand (TMPyP4) and a weak G-quadruplex ligand (methyl green), were used. The screening results show that the method can work well on both of these drugs.

In conclusion, a simple screening method for G-quadruplex ligands was developed. The results of the screening can be judged by the naked eye alone, eliminating the need for any expensive machines. Another advantage of the method is that it is inexpensive, as no expensive reagents (dual-labeled fluorescent probes for example) are needed and this method allows for low reagent concentrations and small reaction volumes. In this paper, the experiments were performed in 100 μL reaction volume, but 25 μL (or even smaller) can also work well. This method may provide a useful tool for the primary screening of G-quadruplex ligands with high throughput. It is especially suitable for laboratories in which expensive analytical machines are not available.

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Notes and references

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1. Experimental Section

1.1 Materials

The DNA oligonucleotides AG4: 5′-TG₃TAG₃CG₃TTG₃AAA-3′ and Hum24: 5′-(TTAGGG)₄-3′ were obtained from TianGen Biotech Co. Ltd (Beijing China), The G-quadruplex molecular beacon F22D: FAM-AGGG(TTAGGG)₃-DABCYL was synthesized and purified by Invitrogen Ltd. (Shanghai, China). All other chemicals were purchased commercially and used without further purification unless otherwise noted.

1.2 Screening studies of G-quadruplex binders

All experiments were performed in 10 mM Tris-HCl buffer solution, pH=8.0, that included 1.6 mM KCl, 0.8 mM MgCl₂, 0.0017% (v/v) Triton X-100, 0.90 μM AG4, 0.50 μM hemin, and different concentrations of candidate drugs. The reaction mixtures were held overnight in ambient temperature, then 1.28 mM of ABTS and 1.28 mM of H₂O₂ were added. The color of the reaction mixture was recorded by a digital camera. Densitometric quantitation of the photographed images was performed by Glyko BandScan software, Version 4.30.

1.3 G-quadruplex stabilizing abilities assays
Thermal melting studies of G-quadruplex in the presence or absence of the candidate drugs were carried out on Rotor-Gene 3000 (Corbett Research) with 100 μL of reaction mixture (10 mM Tris-HCl buffer (pH 8.0), 100 mM NaCl, 0.5 μM F22D and an appropriate concentration of the candidate drug). The temperature was increased in steps of 1 °C, from 30 °C to 95 °C, with the first step lasting 30 s and the remaining steps each lasting 5 s. Fluorescence was measured at each step.

2. Selection of hemin concentration

![Figure S1](image1.png)

**Figure S1.** Oxidation of ABTS by H₂O₂ in presence of AG4 and different concentration of hemin. 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0 μM hemin was added in tube 1-13, respectively.

3. Selection of AG4 concentration

![Figure S2](image2.png)

**Figure S2.** Oxidation of ABTS by H₂O₂ in presence of hemin and different concentration of AG4. 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5, 1.7, 2.1 μM AG4
was added in tube 1-13, respectively.

4. Selection of ABTS concentration

**Figure S3.** Oxidation of different concentration of ABTS by H$_2$O$_2$ in presence of AG4-hemin complex. 0, 0.32, 0.64, 1.28, 1.92 mM ABTS was added in tube 1-5, respectively.

5. Candidate drugs used for screening experiments.

**Figure S4.** Candidate drugs used for screening experiments.
5. Respective results of fluorescence tests.

Figure S5. Stabilization of F22D in the presence of TMPyP4 or Fe(dmbpy)$_3$($\text{BF}_4$)$_2$.

(A) The thermal denaturation profile of F22D recorded alone (black) or in the presence of TMPyP4 (blue, 2 μM; red, 3 μM) or Fe(dmbpy)$_3$($\text{BF}_4$)$_2$ (green, 25 μM).

(B) First derivative of the melting curves.