G-Quadruplex Ligands

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Screening Potential Antitumor Agents from Natural Plant Extracts by G-Quadruplex Recognition and NMR Methods

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A currently unique G-quadruplex motif has emerged as a biologically crucial structure[1] that is implicated in several physiologically important regulatory processes, with special emphasis on carcinogenesis.[2–4] Small molecules that can facilitate the formation of or stabilize the G-quadruplex structures have the potential for the arrest of cancer-cell growth through disruption of telomere maintenance or alteration of oncogene expression levels, and may be potentially valuable as antitumor drugs.[5–7] A good case in point is the compound quarfloxin (CX-3543), which targets the parallel/mixed class of quadruplexes that are overexpressed in the ribosomal RNA (rRNA) gene during rRNA biogenesis in cancer cells and is currently in clinical trials as an antitumor agent.[8–10] There is thus a considerable interest in discovering G-quadruplex ligands that could ultimately be used against cancer cells.[11,12]

Plant-derived agents, because of their diversity in structure and bioactivity and low toxicity,[13–15] play an important role in pharmaceutical research. This is especially clear in the case of antitumor drugs, as exemplified by paclitaxel, vincristine, vinorelbine, teniposide, and various water-soluble analogues of camptothecin.[16] Several techniques applied in identifying G-quadruplex ligands, such as melting temperature fluorescence assays on oligonucleotides,[17] electrophoresis analysis of quadruplex formation,[18] electrospray ionization mass spectrometry,[19] and the telomeric repeat amplification protocol that measures telomerase activity in cell extracts,[20] mainly focus on screening molecule(s) with known structure. However, up to now, the screening of G-quadruplex ligands from natural plant extracts, a complicated mixture system with multiform unknown chemical frames, has not been reported.

Herein, we address a novel approach for fast screening of G-quadruplex ligands from natural plant extracts. This approach includes the following three steps: judging the existence of G-quadruplex ligand(s) in the test extracts by ^{1}H NMR spectroscopy; detecting the characteristic peak(s) of the G-quadruplex ligand(s) by diffusion-ordered spectroscopy (DOSY) NMR methods; and subsequently identifying the structure of the G-quadruplex ligand(s) by 2D NMR experiments, such as heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC). In this work, we verified the feasibility of this method by using the ethanol/water extracts of *Phellodendron chinense Schneid* cortexes (PE) and *Coptis chinensis Franch* rhizomes (CE). Lentinan extract was used as a background in control experiments, and berberine was employed as a standard substance to investigate the detection limit suitable for assaying the extracts whose principal constituent is berberine.

First, it should be judged whether the test extracts contain G-quadruplex ligand(s). Proton NMR spectroscopy has been widely used to study the interactions between small-molecule ligands and G-quadruplexes.[21,22] The evident differences in the imino region ($\delta = 10$–12 ppm) in the ^{1}H NMR spectra of free and bound G-quadruplexes[23] can be utilized as a spectroscopic means of indicating the existence/nonexistence of G-quadruplex ligand(s) in the test extracts. Upon the addition of PE to a solution containing d(TTGGGTT)$_4$ at room temperature, a clear upfield shift of the guanine imino proton resonance signals was observed (Figure 1). The resultant ^{1}H NMR spectra suggest that certain component(s)

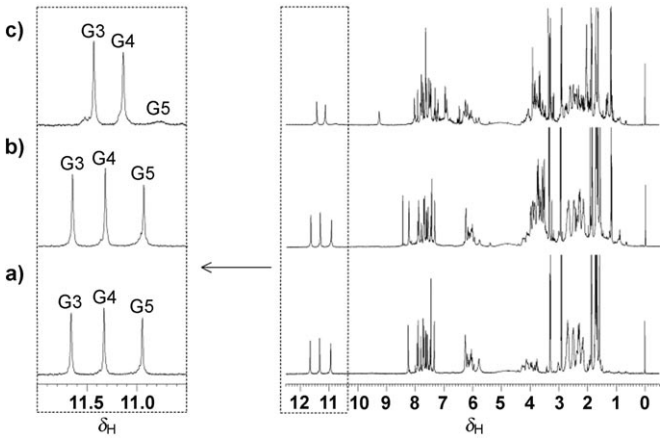

Figure 1. ^{1}H NMR spectra of a) d(TTGGGTT)$_4$, b) a mixture of d(TTGGGTT)$_4$ and lentinan extract, and c) a mixture of d(TTGGGTT)$_4$ and PE. The concentrations of d(TTGGGTT)$_4$, lentinan extract, and PE are 0.25 mM, 3.50 mg mL^{-1}, and 1.0 mg mL^{-1}, respectively. The region of $\delta = 10.5$–12.0 ppm is broadened and the imino proton resonance signals are labeled as G3–G5.

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in PE can bind to d(TTGGGTT)$_4$. Data from temperature-dependent CD (see the Supporting Information) show that PE can increase the melting temperature of the G-quadruplex, which also indicates the existence of G-quadruplex ligand(s) in PE. The next step is to determine which molecule in PE is responsible for the spectral shift.

DOSY provides a means for "virtual separation" of compounds, and is a very powerful tool for extracting information about the intermolecular interactions and local structure of solutions.[24–26] As the mobility of small molecules, in general, would decrease when bound to biomacromolecules, one may expect such molecules to be isolated by the DOSY method. The DOSY spectrum of a mixture of PE and d(TTGGGTT)$_4$ is shown in Figure 2. The component with

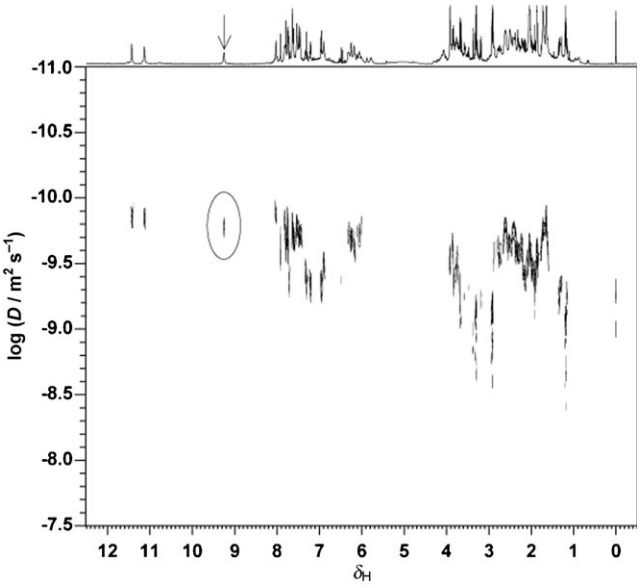


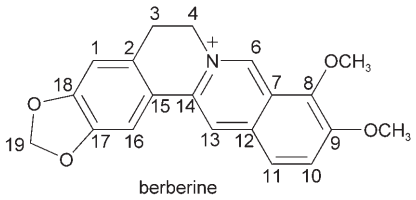
Figure 2. DOSY analysis of a mixture of PE (1.0 mg mL^{-1}) and d(TTGGGTT)$_4$ (0.25 mM). The peak of the d(TTGGGTT)$_4$ ligand(s) is designated by "↓" in the F_2 projection.

slower mobility (around 1.6×10^{-10} m^{2}s^{-1}; log$D\approx-9.8$) could be ascribed to the bound G-quadruplex because it exhibits typical imino proton peaks at the low field ($\delta=10$–12 ppm) of the NMR spectrum, while the other components with faster mobility (more than 3.2×10^{-10} m^{2}s^{-1}; log$D\geq-9.5$) could be assigned to the molecules that do not bind to d(TTGGGTT)$_4$. For the component with slower mobility, a predominant peak around $\delta=9$ ppm was observed, whereas no such peak appeared in the spectrum of the G-quadruplex alone. This result, together with a consideration of the sharp drop in the diffusion coefficient of the proton that resonates around $\delta=$ 9 ppm in the two samples (4.8×10^{-10} and 1.4×10^{-10} m^{2}s^{-1}, respectively; see Table 1), led us to reasonably conclude that this peak results from the compound in PE bound to G-quadruplex.

Further structural illumination was sought by using other NMR techniques, such as HSQC and HMBC. Based on the connectivity of the carbon atoms and the protons in the molecular frame, berberine, which stabilizes G-quadruplexes,

Table 1: Diffusion coefficients for PE and CE in the absence and presence of d(TTGGGTT)$_4$.

Sample	δ [ppm]	Peak assignment	Diffusion coefficient D [m^{2}s^{-1}]
PE	9.61	H6 of berberine	4.8×10^{-10}
	11.13	G4 proton	1.3×10^{-10}
PE + d(TTGGGTT)$_4$	9.25	H6 of berberine	1.4×10^{-10}
CE	9.37	H6 of berberine	4.3×10^{-10}
	11.12	G4 proton	1.3×10^{-10}
CE + d(TTGGGTT)$_4$	9.28	H6 of berberine	1.5×10^{-10}



berberine

could be identified from PE (see the Supporting Information). These results coincide with published results.[27,28] After these encouraging positive results, PE was replaced with CE and the experiments were repeated; similar results were obtained (see the Supporting Information).

However, the HPLC profiles of PE and CE (see the Supporting Information) indicate that these extracts contain one principal constituent, berberine, in common, whereas CE contains another principal constituent, palmatine. Data from temperature-dependent ^{1}H NMR spectroscopy reveal that both berberine and palmatine can stabilize G-quadruplexes, while palmatine is a stronger stabilizer. These results are further confirmed by CD and fluorescence analysis (see the Supporting Information). It would be interesting to look into the reason why the ligand screened from CE is still berberine.

As the amount of sample is one of the key factors that influence the sensitivity of NMR signals, quantitative analysis of PE and CE by a Waters 515 HPLC system was performed. The data for CE show that the content of berberine (24.5 %) is much higher than that of palmatine (5.9 %). These results indicate that the content of each ligand dominates the priority of ligands identified in test extracts in this study. For a multiligand system such as CE, which contains at least two ligands (berberine and palmatine) with similar G-quadruplex binding ability, the ligand with the higher content would tend to be identified by this method and the ligand with lower content may need preconcentration before it can be finally identified. Furthermore, the fractions of berberine bound to a G-quadruplex in the absence and presence of palmatine are 0.82 and 0.74, respectively, calculated according to the literature.[26] The decrease in the fraction of bound berberine might arise from competition by palmatine.

The content of ligand in the extracts is a key factor that influences the results, and therefore the detection limit of this

NMR method was further investigated. ^{1}H NMR titration of a mixture of d(TTGGGTT)$_4$ and a large amount of lentinan extract (1.58 mg) by berberine was carried out, in which lentinan extract was used as a background. As shown in Figure 3, the lentinan extract, which contains no G-quadruplex ligand, does not affect the guanine imino proton

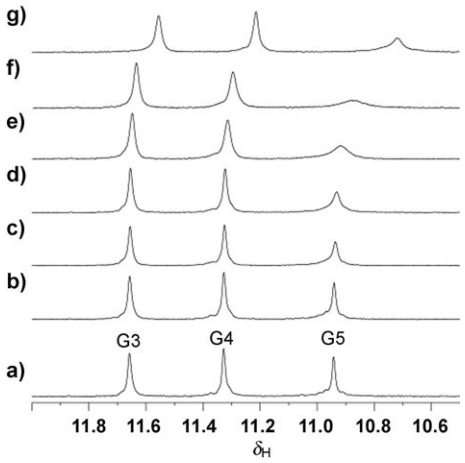


Figure 3. ^{1}H NMR spectra ($\delta = 10.5$–12.0 ppm) showing resonance signals of imino protons of G3, G4, and G5 as a function of the amount of berberine in a mixture of d(TTGGGTT)$_4$ (0.25 mM) and lentinan extract (1.58 mg). (The composition of lentinan extract was analyzed by HPLC; see the Supporting Information, Figure S7.) The mass concentration of berberine is 0 (a), 0 (b), 0.06 (c), 0.12 (d), 0.30 (e), 0.60 (f), and 3.00% (g).

resonance signals ($\delta = 10.5$–12 ppm). However, the addition of berberine to the mixture of d(TTGGGTT)$_4$ and lentinan extract leads to significant changes: the G3, G4, and G5 proton signals shift upfield, while the G5 proton resonance signal exhibits an evident broadening. Under our experimental conditions, a small quantity of berberine (0.005 mg, see Figure 3e is sufficient for a clear change of the ^{1}H NMR spectrum of d(TTGGGTT)$_4$. Thus, even if the content of G-quadruplex ligand in the extracts is very low (about 0.06%, mass concentration), it is still detected by ^{1}H NMR spectroscopy.

In summary, a G-quadruplex ligand was successfully identified in two different plant extracts by the combination of G-quadruplex recognition and NMR methods, without in vitro functional assays or binding assays. At the same time, the detection limit of this method was also investigated. Our work provides a very promising strategy for the fast screening of lead components or antitumor agents from natural plant extracts.

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