

Bis(benzimidazole)pyridine derivative as a new class of G-quadruplex inducing and stabilizing ligand†

Guorui Li,^{‡a} Jing Huang,^{‡a} Ming Zhang,^a Yangyang Zhou,^a Dan Zhang,^a Zhiguo Wu,^a Shaoru Wang,^a Xiaocheng Weng,^a Xiang Zhou^{*ab} and Guangfu Yang^b

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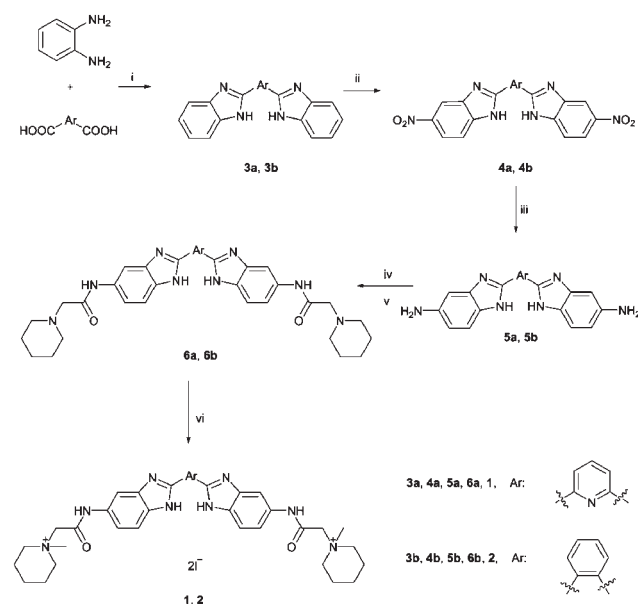
Two new bis(benzimidazole)aryl derivatives have been prepared and one of them has been shown to induce and stabilize formation of a G-quadruplex.

It has been reported that guanine-rich oligonucleotides could form G-quadruplexes *via* Hoogsteen hydrogen bonding.¹ In recent years G-quadruplexes have attracted significant attention because of their potential biological application. A large number of putative quadruplex forming sequences have been identified in many chromosomal locations, such as the telomeric region² and some gene promoters.^{3–6} The human telomeric quadruplex has been extensively studied and formation of G-quadruplexes will lead to inhibition of telomere extension.⁷ Further reports indicated that quadruplex motifs exist in the promoter regions of genes which suggests they are involved in the regulation of gene expression.^{5,8,9} So the development of G-quadruplex binding ligands has been a focus of interest and a wide range of such ligands have been found in many groups over the past few years.^{10–12}

The benzimidazole moiety is structurally related to purine bases¹³ and is found in several biologically relevant natural compounds such as vitamin B12.¹⁴ Benzimidazole derivatives have been studied and displayed a wide range of biological activity.¹⁵ In addition, bis-benzimidazoles have been extensively studied as minor groove binding agents.¹⁶ However, as far as we know, there are only few reports that applied them for G-quadruplex binding.¹⁷ These facts have prompted us to develop benzimidazole derivatives and evaluate their abilities for G-quadruplex binding. It was reported that bisquinolinium linked with pyridine could form H bonds, which leads to a molecular V planar shape that is crucial for quadruplex affinity.¹⁸ In order to evaluate the effect of a planar core on

G-quadruplex binding, we synthesized the benzimidazole derivatives **1**, **2**. The conformation optimization results, using DFT method, showed that **1** exhibits a planar central core, while **2** does not (Fig. S1, see ESI†).

The compounds **1**, **2** were synthesized *via* the route shown in Scheme 1. The bis(benzimidazole)aryls **3a**, **3b** were prepared by condensation of aromatic diamines with aromatic dicarboxylic acids.¹⁹ The dinitro compounds **4a**, **4b** were obtained by direct nitration of the unsubstituted **3a**, **3b**.²⁰ The dinitro compounds **4a**, **4b** were reduced by SnCl₂ to give the diamino compounds **5a**, **5b**. Then they were reacted with chloroacetyl chloride and then piperidine to yield the piperidineacetamide benzimidazole derivatives **6a**, **6b**.²¹ In order to increase the water solubility, they were methylated by CH₃I in CHCl₃ to yield the desired bis(benzimidazole) derivatives **1**, **2**. All the new compounds were fully characterized by NMR, HRMS (see ESI†).



Scheme 1 Synthesis of compounds **1**, **2**. *Reagents and conditions:* (i) polyphosphoric acid, 210 °C for 6 h; (ii) concentrated H₂SO₄, fuming HNO₃, 0 °C for 4 h; (iii) SnCl₂·2H₂O, glacial acetic acid, concentrated HCl, reflux for 6 h; (iv) ClCH₂COCl, DMF, pyridine, –10 °C for 0.5 h; (v) piperidine, CH₃OH, 30 °C, overnight; (vi) MeI, CHCl₃, rt, overnight.

^a College of Chemistry and Molecular Sciences, Minist Educ, Key Lab Biomed, State Key Laboratory of Virology Wuhan University, Hubei, Wuhan, 430072, P. R. China. E-mail: xzhou@whu.edu.cn; Fax: 86-27-87336380; Tel: 86-27-61056559

^b Key Laboratory of Pesticide & Chemical Biology of Ministry of Education, College of Chemistry, Central China Normal University, Hubei, Wuhan, 430079, P. R. China. E-mail: gfyang@mail.ccnu.edu.cn; Fax: 86-27-67867141; Tel: 86-27-67867706

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‡ These two authors contributed equally to this work.

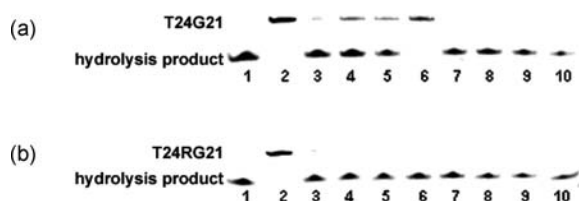


Fig. 1 Quadruplex formation by T24G21 and its resistance to hydrolysis by exonuclease I. (a) Hydrolysis of T24G21 by exonuclease I as a function of compounds concentration. Lane 1: T24G21 treated with exonuclease I; lane 2: T24G21 control; lanes 3–6: T24G21 treated with 1, 5, 10, 25 μM of compound **1** before the addition of exonuclease I; lanes 7–10: T24G21 treated with 1, 5, 10, 25 μM of compound **2** before the addition of exonuclease I; (b) Hydrolysis of T24RG21 by exonuclease I as a function of compounds concentration. Lane 1: T24RG21 treated with exonuclease I; lane 2: T24RG21 control; lanes 3–6: T24RG21 treated with 1, 5, 10, 25 μM of compound **1** before the addition of exonuclease I; lanes 7–10: T24RG21 treated with 1, 5, 10, 25 μM of compound **2** before the addition of exonuclease I.

To identify whether **1** and **2** could induce the formation of the G-quadruplex, the exonuclease I hydrolysis assay was used. This method was introduced by Tan's group for evaluating G-quadruplex stabilization by small molecules.²² It is based on the fact that oligonucleotide T24G21 ((T₂₄(G₃T₂A)₃G₃)) can be hydrolyzed by exonuclease I, while the formation of a G-quadruplex in the oligomer inhibits its hydrolysis. The results are shown in Fig. 1 and the experiment was carried out in the absence of K⁺, Na⁺ cations. With the increase of the concentration of compound **1**, the hydrolysis product decreased, while compound **2** did not resist the hydrolysis (Fig. 1(a)). A non-quadruplex-forming oligomer T24RG21 (T₂₄GTGTGAGTGGAGGTGTGAGGT) was used to discriminate the inhibitory effect from different sources. As shown in Fig. 1(b), the hydrolysis of T24RG21 was not affected by compound **1**. The compound **1** induced resistance to hydrolysis is explained by quadruplex formation of the T24G21. The hydrolysis of the T24RG21 was not inhibited because the oligomer T24RG21 does not form a quadruplex.

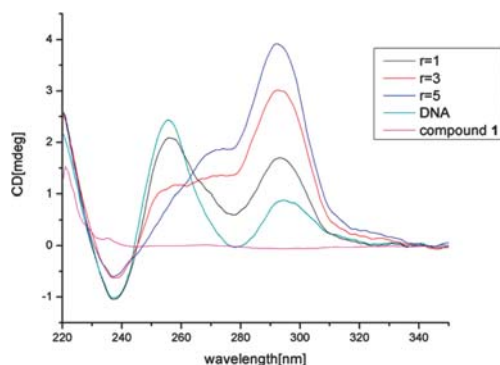


Fig. 2 CD titration of d[T₂AG₃]₄ (12.5 μM) in 10 mM Tris-HCl, 1 mM EDTA buffer at pH 7.4. (r = compound **1**/DNA strand concentration) CD spectra were recorded on a Jasco-810 spectropolarimeter (Jasco, Easton, MD), scanning speed of 100 nm min⁻¹ with a response time of 1 s, and over a wavelength range of 220–350 nm at room temperature.

To confirm the results and determine the conformation of G-quadruplex induced by the compounds, circular dichroism (CD) spectroscopy was carried out.^{12c} Without any metal cations, the CD spectra of the human telomeric d[T₂AG₃]₄ sequence exhibited a negative peak near 237 nm, a major positive peak near 256 nm, and a small positive peak around 292 nm. As shown in Fig. 2, when compound **1** was added, the peak at 256 nm was gradually suppressed, and the peak at 292 nm increased dramatically with increase of the concentration of compound **1**. At the same time, a small positive peak at about 270 nm started to appear. The results showed compound **1** induced the formation of the hybrid G-quadruplex.^{12c} Consistent with the results of the exonuclease I hydrolysis assay, compound **2** could not induce the formation of the G-quadruplex (data not shown).

These results have prompted us to evaluate their abilities of binding and stabilizing the G-quadruplex. The binding abilities of the compounds to duplex and quadruplex DNA were tested by surface plasmon resonance (SPR).²³ The chosen quadruplex-forming sequence was the human telomeric sequence. The steady-state equilibrium binding constants and kinetic constants of compound **1**, **2** with duplex and quadruplex DNA were both measured under previously described experimental conditions.^{12f,23} (Table S2 and Fig. S2, see ESI†). The results showed compound **1** had a high binding constant and showed an obvious selectivity with more than one order of magnitude in favour of quadruplex DNA. The results also revealed compound **1** stabilized the quadruplex by a factor of one order of magnitude relative to compound **2** (Table S2, see ESI†). During the experiment, compound **2** was observed to adhere to the Biacore sensor chip only at high concentrations, which indirectly proved it as a weak binding ligand. Further experimental data from the CD melting method also supported that compound **1** can stabilize the G-quadruplex more strongly than compound **2** (Table S1, see ESI†). Compared with the well-established G-quadruplex stabilizer TMPyP₄,^{12a} although compound **1** had lower affinity, the selectivity was much better.^{12b}

To get quantitative estimates of G-quadruplex stabilization by the two compounds, PCR stop assay was performed.²⁴ Since all the above experiments was performed using the human telomeric sequence, 21G (5'-GGGTTAGGGT-TAGGGTTAGGG-3') was chosen as the test oligonucleotide. The formation of the G-quadruplex in 21G will block its hybridization with a complementary strand and lead to the

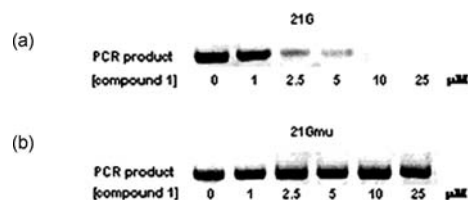


Fig. 3 Effect of compound **1** on the formation of the PCR-stop assay with G-quadruplex forming 21G oligomer (a) or with control mutated 21Gmu oligomer (b). Increasing concentrations of compound **1** (1–25 μM) were added to G-quadruplex forming 21G oligomer or mutated 21Gmu oligomers. With the mutated 21Gmu oligomer the double-stranded PCR product was formed.

double-stranded DNA PCR product being undetectable. As shown in Fig. 3(a), the PCR product was inhibited in a concentration-dependent manner by compound **1**. The IC₅₀ value, which indicates the concentration of compound **1** required to achieve 50% inhibition of the reaction, was found to be 1.8 μ M. To discriminate inhibitory effects from different sources, a parallel experiment using a mutated oligomer 21 Gmu (5'-GGGTTAGAATTAGGGTTAGGG-3') which could not form G-quadruplex was performed. In that case, no inhibition was observed even at the highest concentration of 25 μ M (see Fig. 3(b)). Compound **2** was also tested in this assay, and not surprisingly, it showed weak inhibition ability (Fig. S3, see ESI†). The IC₅₀ was about 100 μ M.

All the above results have shown compound **1** is a good G-quadruplex inducing and stabilizing ligand, while **2** is not. We suggested the tremendous difference between the two compounds was due to the structural difference of the cores. Most recently bis-indole carboxamides linked with a pyridine ring have been studied for G-quadruplex recognition.²⁵ The H-bonds between NH of the pyridine ring and benzimidazole may play an important role in the planar structure, and a planar central core may be vital for the binding of this kind of ligand with the G-quadruplex. UV-Vis titration was used to evaluate the binding mode of compound **1** with the G-quadruplex. Compound **1** demonstrated 9 nm red shifts and significant hypochromicity of 42% (Fig. S4, see ESI†). We assumed the benzimidazole group may be ideally suited for π - π stacking interactions with guanine, considering their structural similarity, and compound **1** could be bound to the ends of the G-quadruplex through π - π stacking.²⁶

In summary, two new bis(benzimidazole)aryl derivatives have been synthesized and evaluated as a new class of G-quadruplex inducing and stabilizing ligands. All the experimental results have shown that the bis(benzimidazole)pyridine derivative **1** can induce and stabilize the formation of a G-quadruplex. SPR sensorgrams showed the strong binding and obvious selectivity of compound **1** for G-quadruplex DNA vs. ds DNA. The difference of activities between **1** and **2** convinced us that, for this kind of compounds, the planar conformation of cores is vital for their binding with G-quadruplex.

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