# Bisbenzimidazole to benzobisimidazole: from binding B-form duplex DNA to recognizing different modes of telomere G-quadruplex†

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*Received (in Cambridge, UK) 5th November 2008, Accepted 5th January 2009*
*First published as an Advance Article on the web 22nd January 2009*
**DOI: 10.1039/b819789j**

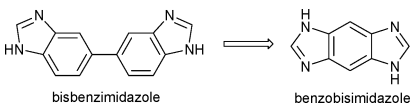


**A bisbenzimidazole was discovered to bind helix DNA, while related benzobisimidazole derivatives were found to bind and induce different G-quadruplex isomers.**

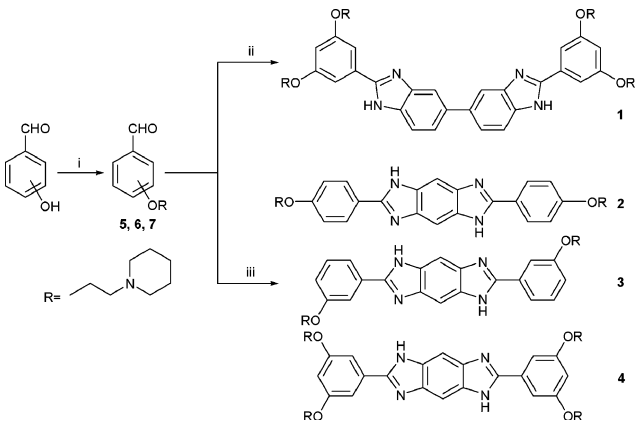
G-Rich strands can fold into G-quadruplexes using Hoogsteen hydrogen bonds and exist in telomeres and gene promoters.[1] Many groups have reported their results targeting G-quadruplex nucleic acids with small molecules as antitumor agents, since they might block telomerase activity by stabilizing G-quadruplex nucleic acids.[2–4] Therefore, most of the G-quadruplex binding ligands have been reported as potential anticancer drugs.[5]

As a strategy for G-quadruplex ligand design, high selectivity between dsDNA and G-quadruplex DNA is the crucial step for drug design.[6,7] Additionally, the polymorphism of G-quadruplex structures present in the genomes of organisms may be associated with different biological function; thus the challenge of discriminating between different G-quadruplexes with small molecules is also significant.[3,8] Benzimidazole derivatives attracted our attention not only because of their pharmacological effects in many drugs,[9] but because of their functional role in DNA ligands.[10] A previous report has indicated that bisbenzimidazole ligands preferentially bind in the minor groove of A–T-rich sequences of B-form duplex DNA.[10a] In 2003, Chowdhury *et al.* reported that Hoechst 33258 could bind c-myc G-quadruplex with modest affinity but low selectivity.[10b] Recent data suggested that bisbenzimidazole, linked with pyridine, could form a planar central core *via* H bonds for G-quadruplex-selective binding.[11] Considering the importance of a planar central core in the G-quadruplex–ligand interaction, we modified the bisbenzimidazole core to a benzobisimidazole (Scheme 1). In this study, benzobisimidazole derivatives were found to induce different types of G-quadruplexes, and bisbenzimidazole selectively bound to the B-form duplex.

Compounds **1–4** can be conveniently synthesized *via* two steps, as shown in Scheme 2.[12] First, the appropriate hydroxybenzaldehyde was treated with chloroethylpiperidine hydrochloride to afford the substituted aldehydes **5–7**. The molecules were then allowed to react with 3,3′-diaminobenzidine or 1,2,4,5-benzenetetraamine tetrahydrochloride, using 1,4-benzoquinone as the aromatizing agent, to give the desired compounds **1–4** in moderate yield. All of the new compounds were fully characterized by NMR, HRMS and HPLC (see ESI Fig. S1 and S2†).




**Scheme 1** Structures of bisbenzimidazole and benzobisimidazole.




**Scheme 2** Synthesis of compounds **1–4**. Reagents and conditions: (i) 1-(2-chloroethyl)piperidine hydrochloride, $K_2CO_3$, NaI, acetone, reflux for 18 h; (ii) 3,3′-diaminobenzidine, 1,4-benzoquinone, ethanol, reflux for 24 h; (iii) 1,2,4,5-benzenetetraamine tetrahydrochloride, NaOH, 1,4-benzoquinone, ethanol, reflux for 18 h.

The G-quadruplexes formed from DNA strands in the presence of compounds **1–4** was monitored by circular dichroism (CD). CD spectroscopy has been very useful for distinguishing DNA secondary structures. As is known, the human telomeric quadruplex is polymorphic. Parallel, antiparallel, and mixed-type parallel–antiparallel G-quadruplex structures, depending on strand orientation, exhibit distinct CD spectra.[13] Surprisingly, we found that the benzobisimidazoles with substituents at different positions appear to induce the formation of all three types of G-quadruplexes (Fig. 1A and Fig. S3, see ESI†). In this study, after addition of compound **2**, with a *para* orientation, into human telomeric sequence $d[T_2AG_3]_4$, a positive peak at around 295 nm was observed in the CD spectrum, which is characteristic of a hybrid G-quadruplex. Compound **3**, which is

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† Electronic supplementary information (ESI) available: Synthesis of compounds **1–4**, ESI HRMS spectra, $^1$H NMR and HPLC spectra of compounds **1–4**, CD titration, melting experiments, exonuclease I hydrolysis assay, and TRAP-LIG assay. See DOI: 10.1039/b819789j

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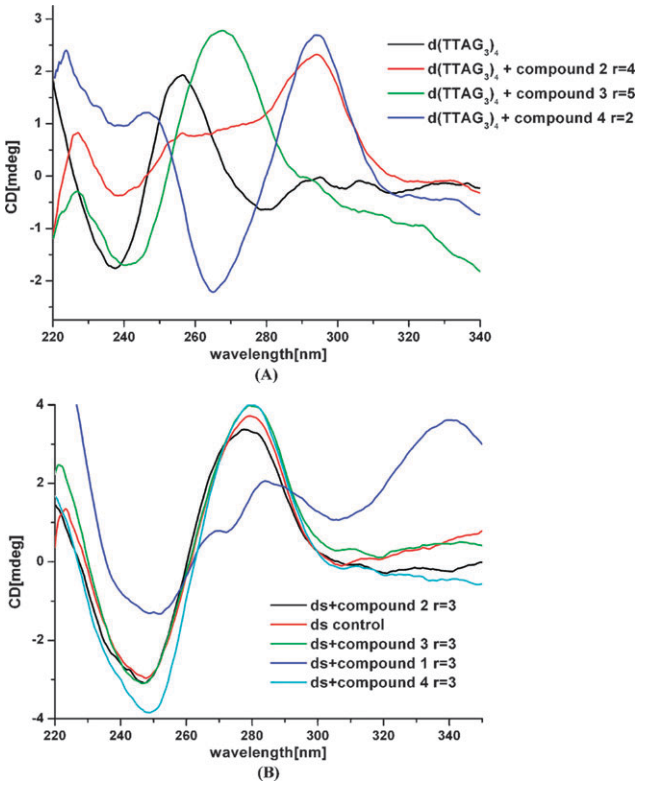


**Fig. 1** CD spectra of d[$T_2AG_3$]$_4$ (12.5 μM) (A) and dsDNA (5 μM) (B) in 10 mM Tris-HCl, 1 mM EDTA buffer at pH 7.4, in the presence of different compounds ($r$ = compound/DNA strand concentration). The steady state of the telomere G-quadruplex induction required 2 equivalents of compound **4**, while compounds **2** and **3** were less effective.

*meso* substituted, induced a parallel type G-quadruplex in the absence of stabilizing salts; the peak at 256 nm shifted to 265 nm and were accompanied by uplifting. When the titration experiments were carried out with compound **4**, it was noteworthy that the ligand increased the CD signal at 295 nm and suppressed the peak at 265 nm in the CD spectroscopic analysis, supportive of the selective induction of an antiparallel G-quadruplex structure. However, there are no significant peaks of the G-quadruplex in the presence of compound **1**. This suggests that the bis-benzimidazole **1** does not show any preference towards inducing a particular quadruplex structure (Fig. S3, see ESI†).

CD was also used to characterize the B-form of DNA. The duplex DNA (5′-GCATTGGTAACTGTCAGACC-3′) exhibits a positive Cotton effect at 280 nm and a negative Cotton effect at 250 nm, which is identical to the canonical CD spectrum of B-DNA reported in a previous paper.[14] The CD spectra of dsDNA with compound **1** at various molar ratios are shown in Fig. S4 (see ESI†). We considered that compound **1** would dramatically change the spectral profile of dsDNA. According to a previous report, these kinds of bis-benzimidazole compounds could selectively bind in the minor groove of B-form duplex DNA.[10a] One reasonable explanation is that the selective binding alters the right-handed B-form conformation. In contrast, the benzobisimidazole derivatives, which were apt to induce folding of the G-quadruplex structures, have no obvious effects on the duplex DNA (Fig. 1B). These non-homogeneous possibilities of induced CD for benzobisimidazole and bisbenzimidazole were attributed to the structures of the compounds. DFT geometry optimization of compound **1** confirmed that the twist of the dihedral angle between the two benzimidazole planes is 36.8° (Fig. S5, see ESI†), which favors helix DNA binding.[15] Benzobisimidazole derivative **4** is proposed to have an aromatic conjugate surface, binding a G-quadruplex *via* π–π stacking.

To validate the CD results, an exonuclease I (Exo I) hydrolysis assay was carried out. It is known that Exo I degrades single-stranded DNA in the 3′ to 5′ direction, releasing deoxyribonucleoside 5′-monophosphates in a stepwise manner and leaving 5′-terminal dinucleotides intact. In the presence of the ligands, the induction of the G-quadruplex structure at the 3′ terminal DNA T24G21 ($T_{24}(G_3T_2A)_3G_3$) blocks the hydrolysis reaction.[11,16] In this case, the exonuclease I hydrolysis assay could further testify to the induction of the G-quadruplex structure by the compounds (Fig. S6, see ESI†). A parallel experiment was also performed using the oligomer T24RG21 ($T_{24}$GTGTGAGTGGAGGTGTGAGGT) that contains mutations (Fig. S6, see ESI†). Consistent with the CD results, compounds **2–4** selectively stopped the degradation of T24G21 by inducing G-quadruplex structures. Unlike the other three compounds, compound **1** also inhibited the hydrolysis of the mutated oligomer T24RG21, implying that quadruplex-irrelevant inhibition was present.

We also evaluated the thermodynamic parameters to predict the stability of G-quadruplex and duplex DNA in the presence of these compounds. Based on the CD melting behavior patterns, melting temperatures were calculated as described previously.[17] According to the data shown in Table S1 (see ESI†), the stabilization potential of compound **1** for the duplex ($\Delta T_m$ = 17.1 °C) is much higher than that of the benzobisimidazole derivatives. There is also a large variation between the benzobisimidazole derivatives ($\Delta T_m$ = 13.6 °C for compound **4**) and bisbenzimidazole **1** ($\Delta T_m$ = 4.1 °C) for telomere G-quadruplex in $K^+$ buffer. The results indicated that benzobisimidazole compounds **2–4** were prominent G-quadruplex ligands, while compound **1** preferred to bind B-form duplex DNA.

Finally, all of the above encouraging results prompted us to investigate the telomerase inhibiting ability of these compounds. The TRAP-LIG assay provided qualitative and quantitative estimates of telomerase inhibition by the small molecules.[18] Considering that the presence of the compounds in the extended products would possibly interfere with the PCR step, the ligands were removed prior to the amplification step. Compounds **2–4** have significant telomerase inhibition activity, with $IC_{50}$ values of 10.7, 10.2 and 4.7 μM, respectively (Fig. 2 and Fig. S7, see ESI†); compound **1** has an $IC_{50} > 50$ μM, correlating with the results from the G-quadruplex binding studies.

In conclusion, the results obtained using compound **1** supported the fact that bisbenzimidazole is bound to the duplex, and that the other three benzobisimidazole derivatives induce three different modes of G-quadruplexes. As reported previously, the G-tetrad arrangement and the orientation of the loops are important factors for the interaction,[19] and the

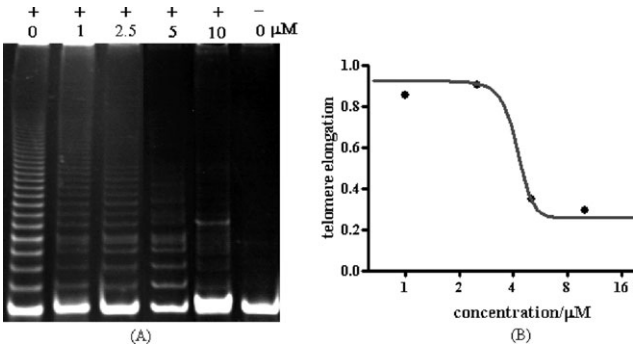


**Fig. 2** (A) 12% non-denaturing gels showing elongated telomeric DNA products of telomerase extension produced from the TRAP-LIG assay with compound **4** at concentrations of 1–10 μM. + and − labels indicate the presence and the absence of telomerase extracts. (B) Quantification of the elongated telomeric DNA products from (A).

ligand side chains play a significant role in selecting DNA topology.[20] We identified that the conformation of the aromatic core is determinate of binding selectivity; the strong and selective binding of compounds **2–4** with intramolecular G-quadruplexes may be attributed to their large ring systems, which overlap completely with the G-tetrad plane. Conversely, the twist of the dihedral angle between the two benzimidazole rings probably makes compound **1** suitable to interact with helix double-stranded DNA. The formation of G-quadruplex isomers, depending on the orientation of the loops, is closely correlated with the side chain of the ligands, especially the substituent positions. Such interesting results provide us with another new entry into G-quadruplex ligand design and conformation studies.

This work was partially supported by the National Science of Foundation of China (No. 20672084, 20621502, 90813031), the National Science Fund for Distinguished Young Scholars (No. 20425206), the Cultivation Fund of the Key Scientific and Technical Innovation Project, the Ministry of Education of China (No. 706040).

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