**EDGE ARTICLE**

**Preferential targeting of i-motifs and G-quadruplexes by small molecules‡**

Manish Debnath, Shirsendu Ghosh, Ajay Chauhan, Rakesh Paul, Kankan Bhattacharyya and Jyotirmayee Dash

i-Motifs and G-quadruplexes are dynamic nucleic acid secondary structures, which are believed to play key roles in gene expression. We herein report two peptidomimetic ligands (PBP1 and PBP2) that selectively target i-motifs and G-quadruplexes over double-stranded DNA. These peptidomimetics, regioisomeric with respect to the position of triazole/prolinamide motifs, have been synthesized using a modular method involving Cu(1)-catalyzed azide and alkyne cycloaddition. The para-isomer, PBP1 exhibits high selectivity for i-motifs while the meta-isomer PBP2 binds selectively to G-quadruplex structures. Interestingly, these ligands have the ability to induce G-quadruplex or i-motif structures from the unstructured single-stranded DNA conformations, as observed using single molecule Förster resonance energy transfer (smFRET) studies. The quantitative real-time polymerase chain reaction (qRT-PCR), western blot, and dual-luciferase assays indicate that PBP1 upregulates and PBP2 downregulates BCL-2 gene expression in cancer cells.

**Introduction**

Cytosine (C)-rich and guanine (G)-rich sequences can adopt stable nucleic acid secondary structures such as i-motifs and G-quadruplexes, respectively. The C-rich sequences form i-motif structures at acidic pH, whereas the G-rich sequences usually form G-quadruplexes at neutral pH in the presence of metal ions (Na⁺, K⁺). These sequences are prevalent in the promoter region of oncogenes like BCL-2 and c-MYC. It has been reported that small molecules bind G-quadruplexes and modulate the gene expression. Although i-motifs are hypothesized to play important roles in gene transcription, only a few ligands are known to selectively target i-motifs in biological systems. Furthermore, i-motifs and G-quadruplexes are highly dynamic and can exist in equilibrium with unfolded DNA under physiological conditions. However, little is known about how small molecules can regulate the relative populations of these two dynamic secondary structures. In this context, we envisioned to develop small molecules that can discriminate between i-motif and G-quadruplex structures and modulate gene expression.

The single molecule Förster resonance energy transfer (smFRET) technique provides key information about the structure, relative population distribution of folded or unfolded species, and the end-to-end distance of biomolecules. The smFRET technique has been used to elucidate the conformational dynamics of G-quadruplexes in the presence of metal ions (K⁺/Na⁺), protein, and small molecules. The population equilibrium of C-rich ILPR and BCL-2 promoter sequences has been studied using laser tweezer experiments. Majima and co-workers have used smFRET to quantitatively analyse the pH-induced intra-molecular folding dynamics of i-motif DNA. However, the use of smFRET to monitor the ligand induced change in the relative population distribution of i-motif and G-quadruplex structures present in oncogenic promoters is very limited.

Hurley and Hecht have reported that the steroid ligand IMC-48 folds the BCL-2 C-rich sequence into an i-motif, while the same sequence is folded into a hairpin duplex in the presence of the related ligand IMC-76. In this study, we describe the synthesis of two flexible peptidomimetic congeners, PBP1 and PBP2, which show structure-specific recognition for G-quadruplex and i-motif structures. The interaction of these ligands with BCL-2 or c-MYC i-motifs and G-quadruplexes has been evaluated using biophysical studies like melting analysis by Förster resonance energy transfer (FRET), thiazole-orange (TO) displacement assay, fluorescence quenching assay, and circular dichroism (CD) spectroscopy. In addition, the ability of these ligands to induce the formation of i-motif and

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‡ Electronic supplementary information (ESI) available: Experimental details, synthetic procedures, characterization data of compounds, NMR spectra, FRET melting, TO displacement, smFRET, lifetime data, CD spectra, western blot, dual luciferase, caspase assay. See DOI: 10.1039/c7sc02693e

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G-quadruplex structures from the unfolded BCL-2 and c-MYC C-rich and G-rich promoter sequences has been investigated using smFRET and fluorescence lifetime studies at neutral pH. We have further demonstrated how ligand-dependent conformational changes of BCL-2 i-motif or G-quadruplex topologies can modulate the BCL-2 expression in cancer cells.

Results and discussion

Design and synthesis of peptidomimetic ligands

Peptidomimetics are designed to interact with specific biological targets as they exhibit enhanced proteolytic stability and improved cell permeability. We have anticipated that peptidomimetics containing the 2,6-pyridine dicarboxamide unit, linked to i-proline residues through triazole and arne motifs would be structurally flexible enough to adopt different conformations upon interacting with different DNA four stranded structures (i-motifs and G-quadruplexes). The proline residues play an important role in peptide conformation. The 2,6-pyridine dicarboxamide motif can adopt folded conformations due to the bifurcated H-bonding between the lone pair of pyridine nitrogen and amide -NH protons. The arene motif attached to the proline residues would provide additional flexibility to form topologically different positional isomers that could discriminate between different DNA structures such as i-motifs and G-quadruplexes. The triazole ring system could facilitate stacking interactions with the loop bases and, thus, could differentially interact with different DNA secondary structures with variations in the loop region. The triazole ring system, able to mimic the cis- or trans-conformations of amide bonds, would impart rigidity to the peptidomimetics. It has been reported that triazole containing ligands generated by “click” chemistry selectively bind G-quadruplexes.

The bis-triazole containing peptidomimetic type ligands PBPI and PBPII were assembled using a modular synthetic strategy involving a Cu(i)-catalyzed 1,3-dipolar azide–alkyne cycloaddition between azido prolinamides 1, 2 and pyridyl dialkyne 3 (Schemes 1 and S1, ESI†). The azido prolinamides 1 and 2 were obtained by amide coupling of N-Bocproline 4 with the para and meta-azido anilines 3 and 6. The dialkyne building block 3 was prepared from chelidamic acid 7. Chelidamic acid 7 was treated with oxalyl chloride to generate the corresponding acid chloride, which was subsequently coupled with propargyl amine 8, followed by alkylation of the resulting pyridyl dialkyne with 3-dimethylaminopropyl chloride 9, affording the dialkyne 3 in high overall yield. The Cu(i)-catalyzed Huisgen cycloaddition of azido prolinamide derivatives 1 and 2 with the dialkyne 3 and subsequent removal of the Boc group provided the bis-prolinamide derivatives PBPI and PBPII in high yields. The

![Scheme 1](image) The synthesis of bis-prolinamidinocyan derivatives PBPI, PBPII and the structure of PBPIII.

![Fig. 1](image) The FRET melting and TO displacement assays. The FRET stabilization potential of bis-prolinamidinocyan derivatives PBPI (1 μM), PBPII (1 μM), and PBPIII (1 μM) upon interaction with (a) 100 nM folded i-motifs (c-MYC-C, BCL-2-C, and h-TELO-C) and ds DNA in 60 mM K-cacodylate buffer, (pH 6); (b) 100 nM folded G-quadruplexes (c-MYC-G, BCL-2-G, and h-TELO-G) and ds DNA in 60 mM K-cacodylate buffer, (pH 7); thermal shift profiles for (c) PBPI (0–10 μM) and (d) PBPII (0–15 μM) upon stabilizing i-motifs and ds DNA in 60 mM K-cacodylate buffer, (pH 6). The TO displacement from 250 nM BCL-2-C, c-MYC-C, and h-TELO-C i-motifs in 60 mM K-cacodylate buffer, (pH 6); BCL-2-G, c-MYC-G, and h-TELO-G G-quadruplexes and ds DNA in 60 mM K-cacodylate buffer, (pH 7) with increasing concentrations of (e) PBPI (0–10 μM); (f) PBPII (0–15 μM).
bis-prolinamide derivative PBP3 was similarly assembled from azido prolinamide 1 and pyridine-2,6-dicarboxylic acid.

PBP1 and PBP2 exhibit differential binding between i-motifs and G-quadruplexes

The ability of these regioisomeric ligands to interact with G-quadruplexes and i-motifs was evaluated using biophysical assays. C-Rich sequences were folded into i-motifs by annealing in 60 mM K-cacodylate buffer, at pH 4.8 and then the pH was adjusted to 6 for biophysical analysis.\(^{18,22,24,45}\) Dual labeled (5’-FAM and 3’-TAMRA) C-rich and G-rich sequences present in oncogenic promoter regions (BCL-2 and c-MYC) and the telomeric region (h-TELO) were folded into i-motifs and G-quadruplexes, respectively,\(^{18,44}\) and these were used in this study along with a control double-stranded (ds) DNA (Fig. 1 and Table S1, ESI†).

Interestingly, the two positional isomers PBP1 and PBP2 exhibited a marked difference in increasing the \(T_m\) of folded G-quadruplexes and i-motifs at 1 \(\mu M\) ligand concentration (Table 1, Fig. 1a and b and S2, ESI†). Ligand PBP1, in which the prolinamide motifs are at the para position with respect to the triazole ring system, increased the \(T_m\) values of BCL-2-C and c-MYC-C i-motifs more effectively compared to ligand PBP2 at 1 \(\mu M\) ligand concentration (\(\Delta T_m = 16-29^\circ C\) for PBP1 and \(\Delta T_m = 8^\circ C\) for PBP2). In contrast, the meta regioisomer PBP2 increased the \(\Delta T_m\) value of c-MYC-G and BCL-2-G G-quadruplexes (\(\Delta T_m = 16^\circ C\) at 1 \(\mu M\) PBP2 and \(\Delta T_m = 5.2^\circ C\) at 1 \(\mu M\) PBP1) (Table 1). Ligand PBP3, which lacks the –NM2 side chain in the central pyridine ring, showed low stabilization potential (\(\Delta T_m = 3-5^\circ C\)) for both G-quadruplex and i-motif structures (Table S1, ESI†). When BCL-2-C and c-MYC-C mutant C-rich sequences were used in the melting analysis, no melting curves were observed, thereby indicating their existence in the unfolded form (Fig. S3, ESI†).

Next, FRET melting experiments were carried out for BCL-2, c-MYC, and h-TELO i-motifs and G-quadruplexes using an increasing concentration of PBP1–2. PBP1 showed high \(\Delta T_m\) values for BCL-2-C, c-MYC-G, and h-TELO-C i-motifs while PBP2 exhibited high \(\Delta T_m\) values for the corresponding G-quadruplexes in a dose-dependent manner (Fig. 1c and d, S2–S4, ESI†). PBP1 showed a \(\Delta T_m\) value of 32 ± 1°C (i.e., a \(T_m\) of 92°C) for BCL-2-C at 1.3 \(\mu M\) concentration, whereas higher concentrations of PBP1 were required to attain a \(\Delta T_m\) value of 32 ± 1°C for c-MYC-C (a \(T_m\) of 81°C at 6.5 \(\mu M\)) and h-TELO-C (a \(T_m\) of 76°C at 10 \(\mu M\)). These results indicate that PBP1 shows a preferential affinity for the BCL-2-C i-motif as it can obtain a maximum \(\Delta T_m\) value for the BCL-2-C i-motif at 5–8 fold lower concentrations than the c-MYC-G and h-TELO-C i-motifs. In contrast, BCL-2-G and c-MYC-G G-quadruplexes exhibited maximum \(\Delta T_m\) values at 3 fold lower concentrations of PBP2 than PBP1 (Fig. S2, ESI†). However, both PBP1 and PBP2 failed to alter the \(T_m\) of ds DNA even at high ligand concentrations (10–15 \(\mu M\)), indicating their selectivity for four stranded structures over double-stranded DNA.

The selectivity of PBP1 for i-motifs and PBP2 for G-quadruplexes was determined using the FRET competition assay with the competing G-quadruplex (TG\(_5\)TG\(_4\)) and double-stranded ds26 DNA (Fig. S2c and d, ESI†). The results show that no significant changes in the \(\Delta T_m\) values for PBP1 bound i-motifs and PBP2 bound G-quadruplexes were observed in the presence of 40 mol equivalent excess of the G-quadruplex and double-stranded DNA competitors. PBP1 exhibited DC\(_{50}\) values of 0.9 \(\mu M\), 2.7 \(\mu M\) and 4.0 \(\mu M\) for BCL-2-C, c-MYC-G and h-TELO-C i-motifs, respectively (Table 1).

### Table 1. The sequences used in this study and a comparison of the binding data obtained for PBP1 and PBP2 from TO displacement, fluorescence quenching, and FRET melting assay

<table>
<thead>
<tr>
<th>DNA(^{a})</th>
<th>DC(_{50}) (\mu M)</th>
<th>(K_d) (\mu M)</th>
<th>(\Delta T_m) (^\circ C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBP1</td>
<td>PBP2</td>
<td>PBP1</td>
<td>PBP2</td>
</tr>
<tr>
<td>BCL-2-C: 5’-d(CAGC(_6)GTC(_6)GC(_6)T(_6)C(_6)C(_6)G(_6)C(_6)T(_6))-3’</td>
<td>0.9</td>
<td>8.2</td>
<td>0.3</td>
</tr>
<tr>
<td>BCL-2-G: 5’-d(AAGC(_6)CG(_6)CG(_6)A(_6)G(_6)G(_6)G(_6)G(_6)CG(_6)G(_6)T(_6))-3’</td>
<td>5.7</td>
<td>2.4</td>
<td>7.2</td>
</tr>
<tr>
<td>c-MYC-C: 5’-d(TG(_6)AC(_6)T(_6)AC(_6)G(_6)G(_6)T(_6)G(_6))-3’</td>
<td>2.7</td>
<td>6.8</td>
<td>2.4</td>
</tr>
<tr>
<td>c-MYC-G: 5’-d(TG(_6)AG(_6)A(_6)G(_6)A(_6)G(_6)A(_6)G(_6))-3’</td>
<td>8.5</td>
<td>1.3</td>
<td>12.5</td>
</tr>
<tr>
<td>h-TELO-C: 5’-d(TA(_6)TG(_6)TG(_6)TG(_6)TG(_6)TG(_6))-3’</td>
<td>4</td>
<td>&gt;15</td>
<td>n.d.</td>
</tr>
<tr>
<td>h-TELO-G: 5’-d(G(_6)TTAG(_6)TTAG(_6)TTAG(_6))-3’</td>
<td>9.8</td>
<td>4.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>ds DNA: 5’-d(TATAGCTATA-HEG-TATAGCTATA)-3’</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt;25</td>
</tr>
</tbody>
</table>

\(\Delta T_m\) values for the corresponding i-motifs \(\Delta T_m \geq 3\) and 5°C are denoted as ‘n.d.’. **Table S2** in the ESI lists concentrations of the ligands required to displace TO by 50% (DC\(_{50}\)) from the investigated DNA structures.

### (a) Melting analysis using FRET.

The FRET based melting assay was carried out to evaluate the stabilization potential of PBP1–3 for G-quadruplexes and i-motifs.\(^{18,22,24,45}\) When G-quadruplexes and i-motifs at 1 \(\mu M\) were added to the melting analysis, no melting curves were observed, thereby indicating their existence in the unfolded form (Fig. S3, ESI†).

### (b) TO displacement assay.

The affinity of PBP1–3 for the folded G-quadruplexes and i-motifs was further investigated by measuring the ability of the ligands to displace the bound thiazole-orange (TO) from pre-folded G-quadruplexes\(^{49}\) or i-motifs\(^{49}\) (Fig. 1e and f). Table S2† lists the concentrations of the ligands required to displace TO by 50% (DC\(_{50}\)) from the investigated DNA structures.

PBP1 exhibited DC\(_{50}\) values of 0.9 \(\mu M\), 2.7 \(\mu M\) and 4.0 \(\mu M\) for BCL-2-C, c-MYC-G and h-TELO-C i-motifs, respectively (Table 1).
In comparison, the meta-isomer PBP2 displayed significantly lower affinity for BCL-2-C (DC_{50} = 8.2 \mu M), c-MYC-C (DC_{50} = 6.8 \mu M), and h-TELO-C (DC_{50} > 15 \mu M) i-motifs. On the other hand, PBP1 showed higher DC_{50} values for BCL-2-G, c-MYC-G, and h-TELO-G G-quadruplexes compared to PBP2 (Table 1). These results are in agreement with the FRET melting data suggesting the higher affinity of PBP1 for the BCL-2-C i-motif as compared to that of PBP2 and the preferential binding of PBP2 for c-MYC-G and BCL-2-G G-quadruplexes as compared to PBP1. However, ligand PBP3 exhibited high DC_{50} values for G-quadruplexes (DC_{50} = 8.4–10.2 \mu M) and i-motifs (DC_{50} = 7.9–10 \mu M), which indicates the weak affinity of PBP3 for both four stranded structures (Fig. S5, ESI†).

(c) Fluorescence binding titrations. Next, fluorescence spectroscopy was employed to determine the dissociation constants (K_{d}) of PBP1 and PBP2 with BCL-2 and c-MYC i-motifs and G-quadruplexes (Tables S3 and S4, ESI†). Here, i-motifs and G-quadruplexes are labeled at either the 5’-end or at 3’-end with TAMRA dye. Binding of the ligand in the vicinity of the labeled site facilitates proximity induced quenching of the dye through non radiative methods (Scheme S2, ESI†). For a comparison, ds DNA was used as a control. We observed a dose-dependent decrease in the fluorescence emission of TAMRA labeled DNA structures upon titration with PBP1 and PBP2 (Fig. 2 and S6, ESI†). From the level of quenching, K_{d} values of the ligands for the i-motif and G-quadruplex structures were determined. PBP1 showed a 20 fold higher affinity for the 5’-TAMRA-BCL-2-C i-motif with a K_{d} value of 0.3 \mu M over PBP2 (K_{d} = 5.8 \mu M) (Table 1). Similarly, PBP1 exhibited a lower K_{d} value (2.4 \mu M) for the 5’-TAMRA-c-MYC-C i-motif compared to PBP2 (K_{d} = 9.5 \mu M).

When 5’-TAMRA labeled BCL-2-G and c-MYC-G G-quadruplexes were titrated with PBP1 and PBP2, a marked difference in their affinity was observed. PBP2 exhibited a 7 fold preference for the 5’-labeled c-MYC-G (K_{d} = 1.3 \mu M) G-quadruplex over the i-motif counterpart. Similarly, a 3 fold higher affinity of PBP2 was observed for the BCL-2-G G-quadruplex (K_{d} = 1.9 \mu M) over the BCL-2-C i-motif. It is intriguing to note that PBP1 showed a 24 fold higher selectivity for the BCL-2-C i-motif over BCL-2-G (K_{d} = 7.2 \mu M) and a 40 fold higher selectivity for the BCL-2-C i-motif over c-MYC-G (K_{d} = 12.3 \mu M) G-quadruplexes. To the best of our knowledge, this is one of the highest levels of selectivity reported by a small molecule ligand for i-motif over G-quadruplex structures.

Similar binding titrations with 3’-TAMRA labeled BCL-2-C and c-MYC i-motifs and G-quadruplexes revealed that both PBP1 and PBP2 displayed a higher affinity (lower K_{d} value) for 5’-labeled G-quadruplexes and i-motifs over 3’-labeled structures (Fig. S6, Scheme S2, Tables S3 and S4, ESI†). Therefore, the 5’-end of G-quadruplex and i-motif structures is the preferred binding site for PBP1 and PBP2. In comparison, PBP3 induced a considerably lower level of fluorescence quenching (>40%) in TAMRA labeled G-quadruplexes and i-motifs (Fig. S7, ESI†), suggesting the weak affinity of PBP3 for these DNA structures. The weak affinity of PBP3 may be attributed to its poor solubility in aqueous buffer and the lack of a cationic side chain, and hence PBP3 was not selected for further studies. Importantly, PBP1 and PBP2 preferentially bind to the four stranded DNA structures over ds DNA, as control experiments with TAMRA labeled ds DNA showed no significant quenching upon addition of the ligands (Fig. 2 and S6, ESI†).

**PPB1 and PBP2 induce the formation of i-motifs and G-quadruplexes, respectively**

(a) SmFRET analysis. SmFRET was used to study the conformational changes of folded and free i-motif and G-quadruplex forming sequences in the presence and absence of ligands via monitoring the FRET between donor and acceptor fluorophores. Dual labeled sequences of highest purity (Table S1, ESI†) were used to exclude the signals from the donor only sample and, further, the donor shot noise contributions were found to be negligible (Table S5, ESI†). We observed that the donor–acceptor fluorescence intensities of the dual labeled BCL-2-C and c-MYC-G i-motif sequences produced anti-correlated fluctuations (Fig. 3 and S8, ESI†). The FRET histograms obtained from the time traces were fitted with bi- and single Gaussian distributions. The FRET histogram of the BCL-2-C i-motif at pH 4.8 showed a narrow distribution with a mean r_{FRET} ~ 0.95 (Fig. 3). Using eqn (S5),† the distance (R_{DA}) between the donor and acceptor dyes of the BCL-2-C i-motif was determined to be ~33.7 Å (Table S6, ESI†), thereby indicating the existence of a compact structure. The single narrow distribution of the BCL-2-C i-motif was preserved even after the addition of PBP1 and PBP2 (1 equiv.) (Fig. S9, ESI†). The pre-folded BCL-2-C i-motif at pH 6 also exhibited a high r_{FRET} value (~0.88) with a correspondingly low R_{DA} ~ 39 Å, suggesting the presence of folded i-motif structures (Fig. S10, ESI†). The FRET histogram of the free BCL-2-C i-motif sequence at pH 7 showed two population distributions, a wide distribution with FRET efficiency (r_{FRET}) centered at ~0.64 (91%) and a narrow distribution centered at r_{FRET} ~ 0.45 (Fig. 3). The distribution with r_{FRET} ~ 0.45 was ignored due to the contribution of shot noise (Table S5, ESI†). The lower r_{FRET} ~ 0.64 value with a large R_{DA} (~50 Å) suggests that the free BCL-2-C sequence remains in the unstructured form at pH 7. Upon addition of PBP1 (1 equiv.), the histogram of the free BCL-2-C sequence (pH 7) was shifted to
a higher value ($\epsilon_{\text{FRET}} \sim 0.9$) with a $R_{\text{DA}}$ of $\sim 36.6$ Å, which suggests that PBP1 induces folding of free C-rich sequences into i-motif structures at pH 7. However, PBP2 (1 equiv.) induces only a partial shift in the population distributions of the free i-motif sequence at pH 7, exhibiting two major populations with $\epsilon_{\text{FRET}}$ values of $\sim 0.67$ ($R_{\text{DA}} \sim 49$ Å) and $\sim 0.95$ ($R_{\text{DA}} \sim 33.7$ Å). The mutant BCL-2-C C-rich sequence exists in an unstructured form, showing low FRET efficiencies ($\sim 57\%$) in both Milli-Q water (pH 7) and in 10 mM Na-cacodylate buffer (pH 4.8) ($R_{\text{DA}} \sim 53$ Å) (Fig. 3).

The FRET histogram of the free c-MYC-C i-motif sequence at pH 7 showed two major population distributions having mean $\epsilon_{\text{FRET}}$ values of $\sim 0.55$ and $\sim 0.8$ with $R_{\text{DA}} \sim 53.2$ Å and $\sim 43.7$ Å, respectively (Fig. S8, ESI†). Upon addition of PBP1 (1 equiv.), the $\epsilon_{\text{FRET}}$ distribution was shifted towards higher value ($\sim 0.93$) ($R_{\text{DA}} \sim 35.7$ Å), indicating the formation of a compact i-motif structure; whereas PBP2 (1 equiv.) did not significantly alter the FRET distribution pattern of the free c-MYC-C i-motif sequence at pH 7. Similar to the BCL-2-C i-motif, the folded c-MYC-C i-motif at pH 4.8 showed a single population with a mean $\epsilon_{\text{FRET}}$ value $\sim 0.95$, indicating the formation of a more compact structure with lower end-to-end distances ($R_{\text{DA}} \sim 33.7$ Å).

Similar to the folded i-motifs, the folded G-quadruplexes are known to exhibit lower $R_{\text{DA}}$ values compared to unstructured G-rich sequences. The free BCL-2-G G-quadruplex sequence showed a wide distribution centered at $\epsilon_{\text{FRET}} \sim 0.6$ with
a corresponding $R_{DA}$ of $\sim$51.4 Å (Fig. S11a and b, ESIF). PBP2 (1 equiv.) could significantly shift the populations of free BCL-2-G sequence towards higher values ($\tau_{FRET} \sim 0.95$) with a low $R_{DA}$ value of $\sim$33.7 Å, indicating PBP2 could induce a compact G-quadruplex structure similar to the K'-folded G-quadruplex. However, the free BCL-2-G sequence showed two major populations with $\tau_{FRET}$ values of $\sim$0.6 ($R_{DA} \sim 51.4$ Å) and $\sim$0.85 ($R_{DA} \sim 41.2$ Å) in the presence of PBP1 (1 equiv.). The addition of PBP2 decreased the $R_{DA}$ value of free c-MYC from $\sim$57 Å to $\sim$41 Å in the absence of K' ions (Fig. S11c, d and Table S6, ESIF). However, the non-G-quadruplex forming mutated BCL-2-G sequence did not exhibit any notable change in $\tau_{FRET}$ values upon the addition of ligands PBP1 and PBP2 ($\tau_{FRET} \sim 0.6$), suggesting that the mutated sequences are unstructured even in the presence of the ligands (Fig. S12, ESIF).

Collectively, the smFRET results suggest that ligand PBP1 can completely shift the dynamic equilibrium of C-rich BCL-2-C and c-MYC-C sequences towards the folded i-motifs from the unstructured form under physiologically relevant neutral pH conditions. However, PBP2 induces only a partial shift in the population distributions of free i-motif sequences at neutral pH but it has the ability to trigger the formation of G-quadruplexes from the unstructured G-rich sequences in the absence of K' ions. (b) Fluorescence lifetime analysis. The differential folding behaviour of free c-MYC-C and BCL-2-C i-motif sequences upon binding to PBP1 and PBP2 was further investigated by measuring the donor decay of dual labeled sequences (Fig. S13–S16, ESIF). The folding states of the dual labeled sequences were assigned on the basis of the $R_{DA}$ determined from the average lifetime ($\tau_{avg}$) of donor (D) labeled c-MYC-C and BCL-2-C (R) and donor–acceptor (DA) dual labeled c-MYC-C and BCL-2-C (RDA) i-motif sequences using eqn (S10) (Tables 2 and S7, ESIF). The free BCL-2-C i-motif sequence at pH 7 exhibited a $R_{DA}$ value of $\sim$52 Å, which decreased to $\sim$43 Å for the folded BCL-2-C (pH 4.8). A similar decrease in $R_{DA}$ value was observed for the free c-MYC-C i-motif sequence upon decreasing the pH from 7 ($R_{DA} \sim 54.3$ Å) to 4.8 ($R_{DA} \sim 44$ Å). As observed from smFRET, the $R_{DA}$ values of the free BCL-2-C and c-MYC-C i-motif sequences decreased to $\sim$40 Å and $\sim$47 Å, respectively, upon binding to PBP1, at pH 7 (Table 2). However, no sharp decrease in $R_{DA}$ values was observed after the addition of PBP2 to BCL-2-C and c-MYC-C i-motif sequences at pH 7.

Conversely, the addition of PBP2 decreased the $R_{DA}$ value of free BCL-2-G and c-MYC-G from $\sim$55 Å to $\sim$41 Å in the absence of K' ions (Table S8, ESIF), which indicates that PBP2 folds single stranded BCL-2-G and c-MYC-G G-rich sequences into G-quadruplex structures. However, no significant changes in the $R_{DA}$ values of mutant BCL-2-C-rich and G-rich sequences were noted upon addition of PBP1 and PBP2 (Tables 2, S8 and Fig. S15, ESIF). These results suggest that the observed changes in $R_{DA}$ values of the investigated sequences are due to the formation of folded G-quadruplex or i-motif structures in the presence of ligands.

In agreement with the smFRET and lifetime analyses, the CD spectroscopy also supports the idea that the ligand PBP1 triggers the formation of BCL-2-C and c-MYC-C i-motif structures and PBP2 induces the formation of G-quadruplex structures (Fig. S17–S24, ESIF†). Moreover, the change in CD intensity with

<table>
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<th>System</th>
<th>$\tau_{avg}$</th>
<th>$\epsilon_{FRET}$</th>
<th>$R_{DA}$ (Å)</th>
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<td>BCL-2-C (pH 7)</td>
<td>D</td>
<td>3.78</td>
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<td></td>
<td>DA</td>
<td>1.56</td>
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<td>BCL-2-C-mut (pH 7)</td>
<td>D</td>
<td>3.62</td>
<td>0.53</td>
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<tr>
<td></td>
<td>DA</td>
<td>1.7</td>
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<td>BCL-2-C (pH 4.8)</td>
<td>D</td>
<td>4.32</td>
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<td>DA</td>
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<td>BCL-2-C + PBP1 (pH 7)</td>
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<td>DA</td>
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<td>BCL-2-C-mut + PBP1 (pH 7)</td>
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<td></td>
<td>DA</td>
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<td>BCL-2-C + PBP2 (pH 7)</td>
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<td>DA</td>
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<tr>
<td>c-MYC-C (pH 7)</td>
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<td>3.32</td>
<td>0.52</td>
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<tr>
<td></td>
<td>DA</td>
<td>1.6</td>
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<tr>
<td>c-MYC-C (pH 4.8)</td>
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<td>c-MYC-C + PBP2 (pH 7)</td>
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<td>0.62</td>
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<td>DA</td>
<td>1.02</td>
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* $\pm$ 10%, b BCL-2-G-mut: 5'-FAM-d(AG2TGCG2TCGC G2AAG2A2G2 TG2 C GTAA GCG2TGCTG)-TAMRA-3'.

(a) qRT-PCR analysis. To investigate the ability of PBP1 and PBP2 to regulate the expression of the BCL-2 gene in biological...
systems, we measured the level of BCL-2 expression at transcriptional and translational levels. After a 24 h treatment with IC_{50} dose (24 h) of PBP1 and PBP2, the total RNA was isolated from MCF-7 and HCT116 cells. The level of transcription of BCL-2 was quantified using qRT-PCR. Gene expression was normalized against the expression of the constitutively expressed house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Treatment with the PBP2 reduced BCL-2 mRNA level to 0.3-fold (by 70%) and 0.24-fold (by 76%) in MCF-7 and HCT116 cells, respectively, compared to the control (Fig. 4a and Table S14, ESIF). In contrast, when cells were treated with PBP1, the BCL-2 mRNA expression was upregulated by 1.45-fold (45%) and 1.35-fold (35%) in MCF-7 and HCT116 cells, respectively, compared to the control (Fig. 4a and Table S12, ESIF). In addition to GAPDH, gene expression was also normalized using 18S rRNA as a control gene (Fig. S29, ESIF). Treatment with an IC_{50} dose (24 h) of PBP1 upregulated the BCL-2 mRNA level by 1.5-fold (50%), whereas treatment with IC_{50} dose (24 h) of PBP2 reduced BCL-2 mRNA level to 0.13-fold (87%) with respect to the 18S rRNA control in HCT116 cells (Tables S11 and S13, ESIF). However, GAPDH mRNA and 18S rRNA were equally expressed in the untreated control and ligand treated MCF-7 and HCT116 cells, indicating the gene specific behaviour of the bis-prolinamide derivatives.

(b) Western blot analysis. Having assessed the expression of BCL-2 at the transcriptional level, we employed western blot analysis to observe the effect of these ligands at the translational level (Fig. 4b, c and S30, ESIF). Protein levels of BCL-2 and GAPDH were measured in MCF-7 and HCT116 cells treated with PBP1 and PBP2 for 24 h at their respective IC_{50} doses (24 h). The western blots exhibited the differential effect of PBP1 and PBP2 on the expression of BCL-2 compared to the control cells, which is in good agreement with the qRT-PCR analysis data. The protein expressions calculated from densitometric analysis of western blots were normalized for ligand treated cells against untreated control cells. In PBP2 treated MCF-7 and HCT116 cells, the BCL-2 protein expression was downregulated by 70% and 85%, respectively (Fig. 4c). In contrast, the BCL-2 protein was upregulated by 40% and 50% in PBP1 treated MCF-7 and HCT116 cells, respectively. On the other hand, negligible reduction in GAPDH expression was observed in both treated and control cells. These results suggest that the meta-prolinamide PBP2 can downregulate BCL-2 expression, whereas the treatment with para-prolinamide PBP1 results in upregulation of the BCL-2 expression at both the mRNA and protein levels in cancer cells.

(c) Dual-luciferase assay. In order to investigate the influence of the ligands (PBP1 and PBP2) on the BCL-2 gene expression, we employed a dual-luciferase reporter assay (Scheme 2 and Fig. 4d). Reporter vectors containing wild-type BCL-2 promoter sequences (i-motif and G-quadruplex forming sequences) in the upstream region of the firefly luciferase coding gene (LB322) were co-transfected with the Renilla luciferase vector containing a non G- or C-rich promoter sequence (pRL-TK) into HCT116 cells. After cellular uptake of the reporter luciferase vectors, 5 μM of PBP1 or PBP2 was added to the cells. As expected, the Renilla luciferase expression was unaffected by the ligands due to the absence of C-rich or G-rich sequences. Hence, the expression of BCL-2 firefly luciferase was normalized relative to the Renilla luciferase expression.

Upon treatment with PBP2, the BCL-2 promoter-linked luciferase expression was decreased by 58% relative to the untreated control. In contrast, treatment with PBP1 exhibited a 42% increase in BCL-2 promoter-linked luciferase expression compared to the control. To further validate our results, we also investigated the effect of PBP1 and PBP2 on a firefly luciferase vector (pBV-Luc) containing non i-motif or G-quadruplex sequence (Fig. S31, ESIF). Interestingly, we did not observe any notable change in firefly luciferase expression in pBV-Luc.
treated HCT116 cells upon treatment of PBP1 and PBP2 compared to untreated control. In addition, ligand PBP1 did not show any significant change in the expression of reporter vector containing other promoter i-motif or G-quadruplex forming sequences such as c-MYC (Del 4 plasmid, Fig. S32, ESI†). These results indicate that PBP1 and PBP2 may regulate BCL-2 expression by targeting BCL-2 promoter i-motifs or quadruplexes in cancer cells.

Detection of apoptosis using Annexin V and caspases 3/7

To further investigate the influence of bis-prolinamides on cell survival, flow cytometry was employed using Annexin V and PI dual staining assays (Fig. 4e and S33, ESI†). Since PBP1 did not influence the healthy cancer cells in an apoptosis assay (unpublished data), we prepared model apoptotic cells via 48 h serum starvation in order to investigate the anti-apoptotic properties.33 MCF-7 and HCT116 cells were treated with 5 μM PBP1 and PBP2 after serum starvation. Control MCF-7 cells show a significant percentage of apoptotic cells (~22%) due to serum starvation. Interestingly, treatment with PBP1 reduced the percentage of apoptotic MCF-7 cells to ~14% whereas PBP2 efficiently increased the percentage of apoptotic MCF-7 cells to ~60%. Similar results were obtained for PBP1 and PBP2 treated HCT116 cells (Fig. S33, ESI†).

Since activation of caspases is an important process during apoptosis, the quantitative detection of executioner caspases 3 and 7 in HCT116 cells upon treatment with peptidomimetic ligands PBP1 and PBP2 was investigated using FLICA reagent based flow cytometry assay (Fig. S34 and Table S15, ESI†).24 The serum starved control cells exhibited moderate levels of active caspases 3 and 7 (~18.4%). At 24 h post treatment with ligand PBP1 (5 μM), the level of active caspases 3/7 was significantly decreased (~5.1%). However, cells incubated with ligand PBP2 (5 μM) for 24 h exhibited a higher level of active caspases 3/7 (~44.7%). These results suggest that PBP1 decreases the level of active caspases 3 and 7 in cancer cells whereas PBP2 induced apoptosis is associated with the activation of caspases 3 and 7. However, the exact molecular mechanism of this behaviour is under investigation.

Conclusions

We have demonstrated that two flexible peptidomimetic congeners, PBP1 and PBP2, synthesized using ‘click chemistry’, can exhibit distinguishable recognition between i-motifs and G-quadruplexes. FRET melting and fluorescence spectroscopic studies reveal that both ligands show high selectivity for i-motifs and G-quadruplexes over duplex DNA. These studies also indicate that PBP1 preferentially binds to the BCL-2-C i-motif over G-quadruplexes and PBP2 selectively binds to G-quadruplexes over i-motifs. In addition, smFRET studies indicate that PBP1 folds the unstructured BCL-2 and c-MYC C-rich DNA sequences into i-motif structures at neutral pH; whereas PBP2 promotes G-quadruplex formation from single stranded BCL-2 and c-MYC G-rich sequences in the absence of metal ions. Cellular studies revealed that PBP1 upregulates BCL-2 gene expression while PBP2 inhibits BCL-2 gene expression. Furthermore, PBP2 triggers apoptosis via activation of caspases 3 and 7; whereas PBP1 reduces the level of active caspases 3/7 and decreases the percentage of apoptotic cancer cells. These results indicate that a small change in the ligand structure can have a dramatic effect on the molecular recognition properties, providing a new platform to achieve differential recognition of G-quadruplexes and i-motifs. These observations further suggest that ligand induced folding of i-motifs or G-quadruplexes may provide an attractive way to control gene expression and to develop therapies for cancer and neurodegenerative diseases.

Conflicts of interest

The authors declare no conflict of interest.

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References