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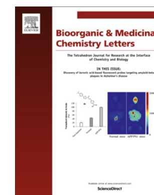

Stabilization of G-quadruplex DNA and inhibition of Bcl-2 expression by a pyridostatin analog

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ABSTRACT

The G-quadruplexes located in the P1 promoter of B-cell lymphoma-2 (Bcl-2) gene are implicated to regulate Bcl-2 expression. Here, we designed a new pyridostatin analog named PDF, which exhibited high specificity and stabilizing effect toward G-quadruplexes. The luciferase assay demonstrated that PDF could significantly suppress Bcl-2 transcriptional activation in human laryngeal squamous carcinoma cells (Hep-2) cells. Besides, PDF also induced cell apoptosis in vitro assays. These results provide an excellent G-quadruplex specific ligand as an efficient Bcl-2 inhibitor. These results also implicate that PDF may be a potential anticancer drug to head neck cancer.

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The Bcl-2 gene is known as an apoptosis inhibitor involved in the control of programmed cell death.[1,2] The overexpression of Bcl-2 gene is found in a wide range of human cancers such as head neck cancer and colorectal cancer,[3–7] reducing the rate of cell death as well as resisting chemotherapy-induced apoptosis.[8,9] Owing to the crucial role in preserving a balance between cell death and survival, Bcl-2 gene is considered to be an important target for anticancer treatment. Inhibition of Bcl-2 expression is an effective way to enhance chemotherapy and radiotherapy sensitivity.[10,11]

The transcription of the Bcl-2 gene is mainly controlled by the P1 promoter,[12–15] from which more than 90% of transcripts initiate, indicating the importance of the P1 promoter. The P1 promoter, especially the region located at −19 bp upstream of the transcription initiation site, is highly GC-rich,[16] which tends to form G-quadruplex,[15,17,18] a special secondary structure mediated by Hoogsteen hydrogen bonding. The G-quadruplex structures in some specific oncogenes like c-myc, c-kit, and k-RAS are proven to be involved in gene transcription activity.[19–27] There is also research showed that the G-quadruplexes located in Bcl-2 P1 promoter were related with Bcl-2 transcription activity,[28] in which the G-quadruplex structure disrupted by partial mutation of G → A resulted in a 2-fold increase in basal transcriptional activity of bcl-2 promoter. Further, Bcl-2 transcriptional activation was also suppressed by the highly active G-quadruplex ligands, quindoline derivatives.[28] Owing to the potential function of Bcl-2 G-quadruplexes as a transcriptional repressor element, it is an effective way to develop a highly active inhibitor of Bcl-2 gene by designing a G-quadruplex-targeting ligand with high specificity.

Here, a novel G-quadruplex ligand named PDF (Fig. 1) was designed (Figs. S1–S3) and proposed to inhibit Bcl-2 expression. PDF exhibits a much higher affinity towards Bcl-2 G-quadruplexes versus duplex DNA and also significantly improves the thermal stability of Bcl-2 G-quadruplexes. Further in vitro assays show that the transcription activity of Bcl-2 can be obviously inhibited by PDF. These results indicate that PDF as a high-specific G-quadruplex ligand will be a promising inhibitor of Bcl-2 expression.

PDF is designed by using the compound pyridostatin (PDS, Fig. 1) as a reference, which has been intensively researched as a high specific G-quadruplex ligand.[29–31] The high specificity of PDS towards G-quadruplexes is due to the factors: a flat but flexible conformation facilitated by an internal hydrogen bonding network; an optimal electronic density of the aromatic surface to enable π–π interactions with the G-tetrad; and the free nitrogen lone pairs able to lock the flat surface of the molecule and facilitate the interaction with G-tetrads.[32] PDF is similar to PDS in structure, but the only difference is that two of the amino groups of PDS were changed to nitrogen methyl groups. It is known that the main interaction mode for G-quadruplex targeting ligands is

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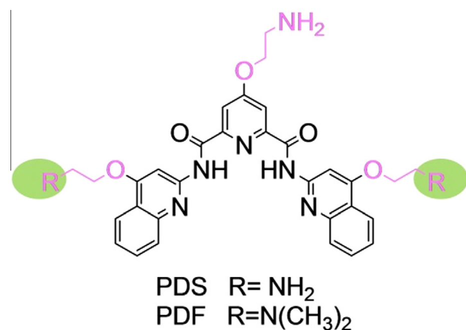

Figure 1. The structural formula of PDS and PDF.

π–π stacking with the G-tetrad[33] and for duplex ligands is intercalative binding with the grooves of duplex. The more bulky amino substituents of PDF is proposed to increase the steric-hindrance effect for PDF intercalating into duplex grooves and thus further improved the selectivity towards G-quadruplexes.

To evaluate the specificity of PDF towards Bcl-2 G-quadruplexes, the fluorescence spectra of PDF with increasing Bcl-2 G-quadruplexes (Fig. 2a) and duplex DNA (ds26 and ds20) were measured, respectively. The ds26 and ds20 sequences are selected as the competitors in according to the researches of Teulade-Fichou[34] and Neidle group.[35] The fluorescence intensity of PDF at 435 nm was sharply decreased by Bcl-2 G-quadruplexes but was not obviously changed by duplex DNA (Fig. 2b). By using the Stern–Volmer equation,[36] the binding constants (k_a) of PDF to quadruplex DNA was calculated to 4.29×10^5 L/mol (Fig. 2c), and an over 6.5-fold higher affinity to G-quadruplex over duplex DNA. As a comparison, the specificity of PDS towards Bcl-2 G-quadruplex was also measured, and PDS showed an over 4-fold higher affinity to G-quadruplex over duplex DNA (Fig. S4). The result implicates a better selectivity of PDF towards Bcl-2 G-quadruplexes than that of PDS in the presence of duplex DNA, implying the possibility of PDF targeting Bcl-2 G-quadruplexes under physiological condition.

The π–π and electrostatic interactions of PDF with the G-quadruplexes will improve the thermal stability of G-quadruplex structure. To confirm the effect of PDF on improving the thermal stability of Bcl-2 G-quadruplexes, the melting curves of Bcl-2 G-quadruplexes without and with PDF was compared based on the circular dichroism (CD) spectra. At the molar ratio (PDF/G-quadruplex) of 2, the melting temperature of the G-quadruplexes was increased by 15 °C (Fig. 3). At the molar ratio of 5, the melting temperature of the G-quadruplexes did not appear even though the measurement temperature has reached to the boiling point of the sample solution, implying a much higher stability of the G-quadruplexes. Furthermore, the stabilizing effect of PDF on duplexes was also detected. The binding of PDF caused no obvious enhancement of denaturation temperature of duplexes (Fig. S5), which further supports the high specificity of PDF towards quadruplex versus duplex DNA.

Ligands that stabilize the formation of DNA G-quadruplexes have potential as oncogene inhibition.[19] According to the excellent selectivity and thermal stabilizing effect of PDF, it could be expected that PDF may affect Bcl-2 gene transcription activity by targeting G-quadruplexes. Luciferase assay was used to test the Bcl-2 transcription activity. Hep-2 cells in 24-well plates were cotransfected with luciferase constructed or empty pGL vector as well as the internal control pRL-TK vector for 48 h. A down-regulation of Bcl-2 expression in a dose-dependent manner was observed when PDF was cotransfected with the native vector (Fig. 4), meaning PDF has efficiently inhibited Bcl-2 transcription activity.

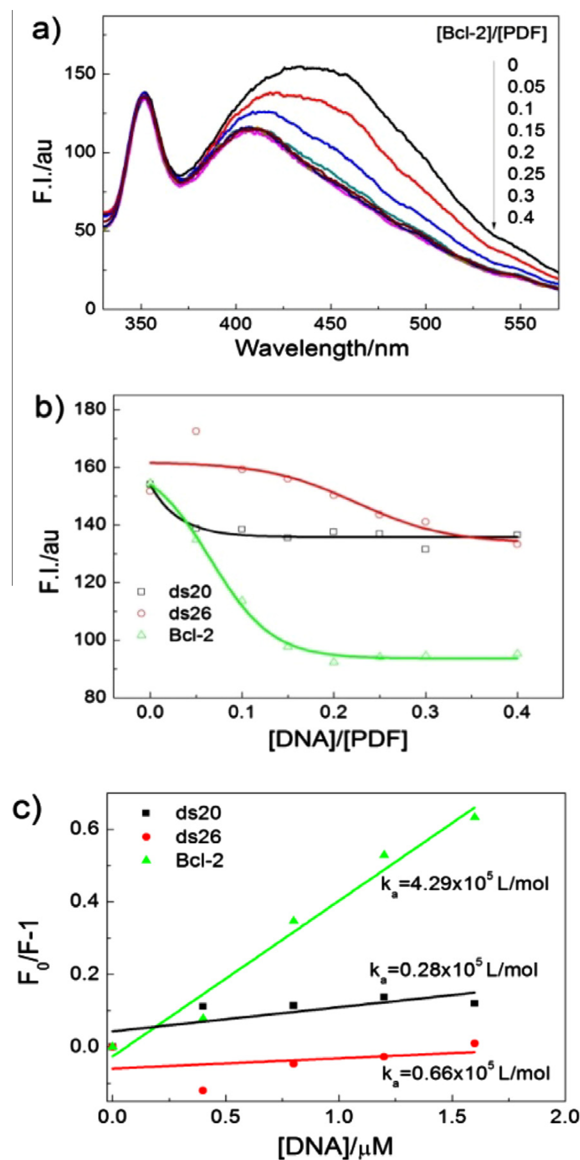

Figure 2. (a) The fluorescence spectra of 8 μM PDF with increasing amounts of Bcl-2 in 10 mM Tris–HCl buffer solution (pH 7.2) with 150 mM KCl and 12 mM NaCl when exciting at 320 nm. (b) The plots of the fluorescence intensity at 435 nm versus the ratio of [DNA]/[PDF]. (c) The plots of $F_0/F-1$ versus [DNA] to obtain the value of k_a according to the Stern–Volmer equation.

Furthermore, total RNA from Hep-2 cells was extracted and reversely transcribed to cDNA to investigate the effect of PDF on Bcl-2 gene transcription. The cDNA was then used as a template for real-time PCR amplification of Bcl-2 sequence with ACTIN as a control. After treatment by using PDF, the RNA levels of Bcl-2 significantly decreased (Fig. S6), suggesting that PDF could down-regulate the transcription of oncogene Bcl-2. Besides, proliferation assay was also carried out with Hep-2 to evaluate the effect of PDS on tumor cell apoptosis. The concentrations of 1, 2, 5, 10 μM were used in the proliferation assay. As shown in Fig. 5, PDF could effectively inhibit the proliferation of Hep-2 cells, indicating that PDF had a relatively strong inhibitory activity on proliferation.

In summary, a new compound named PDF has been designed and synthetized, which is similar to pyridostatin in structure. PDF exhibited a much higher specificity towards Bcl-2 G-quadruplexes than that towards duplex DNA and also significantly

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