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Down-regulation of the human VEGF gene expression by perylene monoimide derivatives

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ABSTRACT

The proximal promoter region of the human vascular endothelial growth factor (VEGF) gene contains a guanine-rich strand that can act as a transcriptional silencer by forming an intramolecular G-quadruplex. In this study, we compared two perylene monoimide derivatives, PM1 and PM2, with the well-studied perylene diimide derivative, PIPER, and the well-studied porphyrin derivative, $TmPyP_4$, with regard to G-quadruplex formation, G-quadruplex binding selectivity, and human VEGF gene silencing in A549 lung cancer cells. The results show that these perylene derivatives can preferentially induce intramolecular G-quadruplex formation from a duplex containing the VEGF G-quadruplex motif in vitro. Incubating A549 lung cancer cells with these perylene derivatives, especially PM2, led to the reduction of both VEGF mRNA and VEGF protein. This study might provide the foundation for the rational design and development of new perylene derivatives as effective anti-angiogenesis agents for cancer therapy.

Angiogenesis is essential for tumor growth and metastasis. When a solid tumor grows to a certain size, it becomes starved due to the limited supply of nutrients and oxygen.[1] One major factor that responds to oxygen deprivation is the hypoxia-inducible factor (HIF), a transcription factor that activates the expression of many angiogenic factors and other proteins important for cellular adaptation to hypoxia.[2] Among these angiogenic factors, vascular endothelial growth factor (VEGF) is one of the most potent regulators of angiogenesis. VEGF binds to VEGF receptors on the surface of endothelial cells, inducing a variety of signal transductions that lead to the formation of new blood vessels. These new blood vessels not only provide supply of nutrients and oxygen, but also provide an escape route for tumor cells to spread to other locations distant from the primary tumor site.[3] VEGF plays a limited role in normal adult human physiology; therefore, VEGF is an attractive therapeutic target for cancer therapy.[4]

The production of VEGF is regulated primarily at the transcriptional level.[5a,b] The proximal promoter region of the VEGF gene between −85 and −50 has been shown to be the binding site for Sp1, and is necessary for both platelet-derived growth factor (PDGF) induction and basal expression.[6] This region contains a guanine-rich strand that has the capacity to form a stable G-quadruplex structure both in vitro and in vivo.[7] Treating cancer cells with $TmPyP_4$ or Se_2SAP, the G-quadruplex interacting agents, suppresses VEGF transcription, presumably by transcriptional inhibition through G-quadruplex formation.[8]

Perylene diimide derivatives, exemplified by PIPER, have been well characterized with regard to their G-quadruplex formation,[9a–d] G-quadruplex binding selectivity,[9e,f] and telomerase inhibition through G-quadruplex formation.[9a,c,d] However, the broad planar aromatic system of perylene, while important for the G-quadruplex binding, also causes the molecules to aggregate in aqueous solution.[9b] Attempts to render perylene diimides more water-soluble include adding polyethylene glycol (PEG) as side chains,[10a] or adding one or two more cationic side chains at the bay area of the perylene diimide.[10b] In the present study, we investigated two perylene monoimide derivatives: PM1, the N-substituted perylene monoimide that was originally designed as a self-organized anisotropic (direction-dependent) fluorescent material,[11a] and PM2, the N,9-disubstituted perylene monoimide.[11b] We compared these derivatives with PIPER, and $TmPyP_4$ (a porphyrin derivative) with regard to their G-quadruplex formation and selectivity on the VEGF promoter sequence. The structures of these compounds and the DNA sequence of oligonucleotides used in this study are shown in Figure 1 and Table 1, respectively. Finally, we investigated whether these compounds could down-regulate VEGF gene expression in living A549 lung cancer cells.

We first tested our compounds for their ability to form G-quadruplex on the G-rich strand of the VEGF promoter using the well established DNA polymerase stop assay.[8,12] This assay is based on the principle that DNA polymerase cannot traverse

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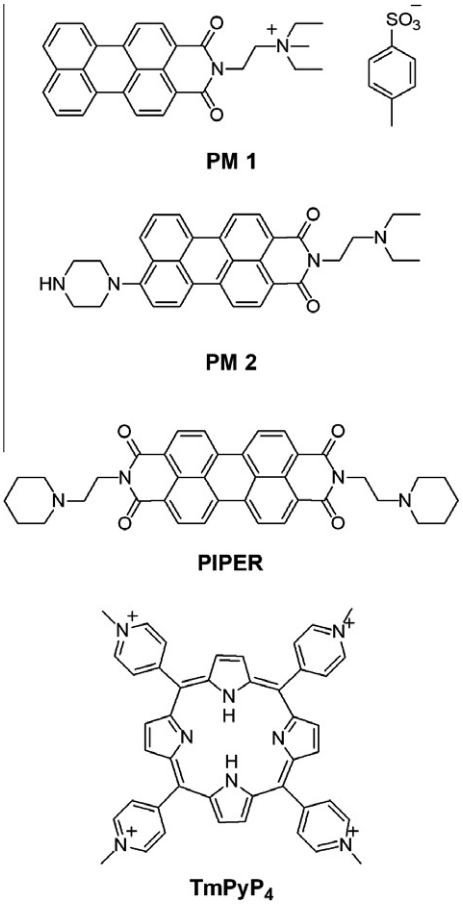

Figure 1. Structures of perylene derivatives and $TmPyP_4$.

Table 1
DNA sequence of oligonucleotides used in this study

Name	Sequence
VT-66	5′-GCCTGTCCCCGCCCCCCGGGGCGGGCCGGGGGCGGGGTCCCTTCCCGAGCCATGCGCCACCTCCTT-3′
P16	5′-AAGGAGGTGGCGCATG-3′
V32G	5′-AGTATAGGGGCGGGCCGGGGGCGGGGTTAGTA-3′
V32C	5′-TACTAACCCCGCCCCCGGCCCGCCCCTATACT-3′
VEGF(F)	5′-TGCATTGGAGCCTTGCCTTG-3′
VEGF(R)	5′-CGGCTCACCGCCTCGGCTTG-3′
GAPDH(F)	5′-CCACAGTCCATGCCATCAC-3′
GAPDH(R)	5′-CCACCACCCTGTTGCTGTA-3′

The bold and underlined sequences are the G-quadruplex motif found on the VEGF promoter.

through an intramolecular G-quadruplex structure; therefore, primer extension stops when the DNA polymerase encounters a stable intramolecualr G-quadruplex on the template. Figure 2A shows that $TmPyP_4$ and our perylene derivatives can arrest primer extension prior to the G-quadruplex motif of the VEGF gene promoter sequence (Band S) in a concentration-dependent manner, suggesting that these compounds can induce G-quadruplex formation. The gel images in Figure 2A were quantified, and the graphs between ratio of the stopped product to the total extended products $[\Delta S/(S+F)]$ and compound concentration were plotted (Fig. 2B). In comparison, $TmPyP_4$ induces G-quadruplex formation at a lower concentration than the perylene derivatives. However, at higher concentration (>1 μM), $TmPyP_4$ starts to inhibit DNA polymerase, and no extended products are observed at higher

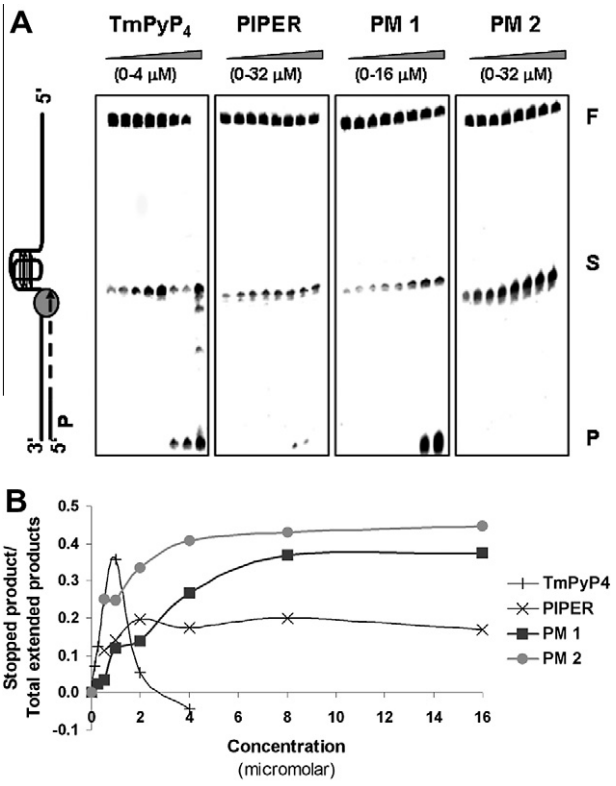

Figure 2. G-quadruplex formation determined by DNA polymerase stop assay. (A) Gel images of the DNA polymerase stop assay. The 10 μl G-quadruplex-forming mixture consisting of the test compound (twofold dilution), a DNA template (VT-66, 100 nM), and a FAM-labeled primer (P16, 100 nM) in 10 mM Tris-HCl buffer (pH 7.4) was denatured at 95 °C for 5 min before slow cooling to 37 °C. The primer extension reaction was initiated by adding a 10 μl reaction mixture consisting of dNTPs (100 mM), $MgCl_2$ (3 mM), and DNA polymerase I Klenow fragment (1 unit), and allowed to proceed at 37 °C for 30 min. The samples were then separated by electrophoresis in 12% denaturing polyacrylamide gels and visualized with a Typhoon™ imaging system. DNA bands are indicated as full-length product (F), stopped product (S), and primer (P). (B) Graph between the ratio of the stopped product to the total extended products and the compound concentration.

concentrations. Among the three perylene derivatives, the VEGF G-quadruplex binding preference follows the order: PM2 > PM1 > PIPER.

An ideal G-quadruplex ligand should bind exclusively to the G-quadruplex DNA, but not to the double-stranded DNA. To assess the binding preference between double-stranded DNA and G-quadruplex DNA from the VEGF promoter sequence, we employed a duplex/G-quadruplex competition assay. Previously we showed that $TmPyP_4$ bound preferentially to double-stranded DNA, while PIPER bound preferentially to G-quadruplex DNA.[9e] As illustrated in Figure 3, without the test compound, these oligonucleotides prefer to form duplex. However, when they were incubated with perylene derivatives, the G-rich strand forms an intramolecular G-quadruplex in a concentration-dependent manner. In contrast, incubating these oligonucleotides with $TmPyP_4$ led to a shifted duplex band, suggesting that $TmPyP_4$ preferentially binds to double-stranded DNA. The results here also show that PM2 induces G-quadruplex formation at a much lower concentration than other perylene derivatives, confirming the previous result that PM2 has the greatest binding affinity to this particular G-quadruplex DNA.

Furthermore, we used spectroscopic methods to examine the self-association and binding characteristics of these perylene derivatives in the absence and presence of the preformed VEGF quadruplex as well as the salmon sperm DNA duplex

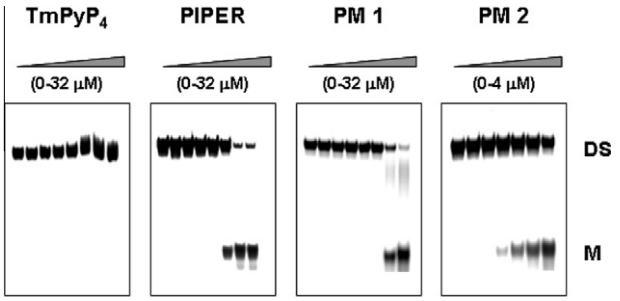

Figure 3. Duplex/G-Quadruplex competition assay with VEGF promoter sequence. The 20-μl reaction mixture consisting of FAM-labeled G-rich strand (V32G, 2 μM), the complementary C-rich strand (V32G, 2 μM), and the test compound (twofold dilution) in Tris–HCl buffer (pH 7.4) containing 100 mM KCl, was first denatured at 95 °C for 5 min before slow cooling to 55 °C and incubation there for 10 h. The samples were then rapidly cooled to 4 °C before the products were separated by electrophoresis in 16% non-denaturing polyacrylamide gels supplemented with 50 mM KCl (4 °C, 350 V, 10 h) and visualized with a Typhoon™ imaging system. DNA bands are indicated as double-stranded DNA (DS) or monomeric G-quadruplex (M).

(Sigma–Aldrich) in 10 mM Tris–HCl (pH 7.4) supplemented with 100 mM KCl (Fig. 4). We monitored the absorbance at time 0 (4A), 8 (4B), and 72 h (4C) after incubation with the DNA substrate. In the absence of DNA, both PIPER and PM 1 aggregated with time (with PIPER aggregating faster than PM 1), while PM 2 remained soluble in the solution even after 72 h. In contrast, in the presence of either VEGF quadruplex or salmon sperm DNA, all of the perylene derivatives remained soluble, and the absorbance increased with time. Comparing among the three perylene derivatives: PIPER, the least soluble compound, interacted with VEGF quadruplex the fastest; while PM 2, the most soluble compound, interacted with VEGF quadruplex the slowest.

Together with the results in Figures 2 and 3, we conclude that these three perylene derivatives can bind to both duplex and G-quadruplex, but likely have a slight preference toward G-quadruplex. The G-quadruplex selectivity of these perylene derivatives follows the order PIPER > PM 1 > PM 2.

Next, we investigated whether these compounds could down-regulate VEGF gene expression in living cancer cells. We employed A549 lung cancer cells as our model. To establish guidelines for choosing the appropriate range of concentration in our subsequent experiments, we first determined the cytotoxicity of our test compounds in A549 cells using the Sulforhodamine B (SRB) colorimetric assay.[13] The 50% growth inhibitory concentration (IC_{50}) was determined from the dose-response relationship between the compound concentration and the percentage of growth inhibition (see Supplementary data S1 and the corresponding Fig. S1.1). The IC_{50} values measured for $TmPyP_4$, PIPER, PM1, and PM2, were: 43.1 ± 1.3 μM, 52.4 ± 2.0 μM, 3.2 ± 0.3 μM, and 4.0 ± 0.1 μM, respectively. The data represent the mean value ± standard deviation of three independent experiments performed in triplicate.

To assess the expression level of the VEGF gene, we measured the VEGF mRNA level using semi-quantitative RT-PCR (see Supplementary data S2), and VEGF protein level using Western blot analysis. In semi-quantitative RT-PCR experiments, the A549 lung cancer cells were treated with various concentrations of a test compound for 24 h before the total RNA was extracted and the mRNAs were converted to cDNAs by reverse transcriptase. The cDNAs were then amplified using gene-specific primers and a well-chosen PCR cycle for each particular gene so that the intensity of the detected PCR products correlated with the amount of the cDNAs in the samples (see Supplementary data Fig. S2). For the Western blot analysis, the A549 lung cancer cells were treated in the same manner as above. The cellular proteins from the treated cells were extracted and separated by an SDS-PAGE before the VEGF protein was probed using rabbit polyclonal VEGF antibody (Santa Cruz Biotechnology).

Figure 5A shows the gel images of the semi-quantitative RT-PCR results. The 540-bp PCR product (V165) represents the full-length VEGF165 mRNA, and the 410-bp PCR product represents the VEGF121 variant mRNA. The GAPDH (glycerol-dehyde 3-phosphate dehydrogenase) mRNA was also monitored as internal control. The bands were quantified by scan densitometer, and the values of both VEGF bands were normalized to that of GAPDH before each value was plotted against the concentration of each compound. Figure 5B summarizes the quantitative data from three separate RT-PCR experiments. As illustrated by Figure 5A and B, the RT-PCR products of the VEGF gene decreased in a concentration-dependent manner, while the RT-PCR products of GAPDH, the control house-keeping gene, did not. The results suggest that these perylene derivatives repressed VEGF transcription. PM2, in particular, is the most effective compound in the down-regulation of VEGF expression. In contrast, the RT-PCR results from cells treated with $TmPyP_4$ show little decrease in VEGF mRNA expression, even at a concentration of 50 μM.

Figure 5C provides the results from Western blot analysis, which show that A549 cells treated with PM2 and PIPER led to a clear decrease in the VEGF protein in a concentration-dependent manner, while the other compounds had no apparent effect on the VEGF protein. The results from Western blot analysis correlate well with the results from the quantitative RT-PCR experiments. When the RT-PCR product from VEGF gene was reduced significantly, as in the case of PM2 and PIPER, the reduction in VEGF protein was also clearly observed. It is to be noted here that the results do not suggest that PIPER is more effective than PM1 since the concentrations used in these experiments were different (the maximum doses were chosen based on the IC_{50} of the compounds). However, the results suggest that PM2, among all the perylene derivatives tested here, is the most effective in suppressing VEGF gene expression, and these perylene derivatives are more effective than $TmPyP_4$.

Altogether, the results show that these perylene derivatives induce G-quadruplex formation from the VEGF promoter sequence, and they preferentially bind to G-quadruplex DNA. In living A549 lung cancer cells, these compounds down-regulate VEGF gene expression in a concentration-dependent manner. The level of gene regulation correlates well with the ability of the compounds to induce G-quadruplex formation in the duplex/G-quadruplex competition assay (Fig. 3), suggesting that the mechanism of action is likely from the G-quadruplex-mediated obstruction of transcription machinery. The perylene derivatives are also superior to $TmPyP_4$ in terms of VEGF down-regulation, despite the effective G-quadruplex induction by $TmPyP_4$ on the VEGF promoter sequence in the DNA polymerase stop assay (Fig. 2). The reason for the difference might lie in the fact that $TmPyP_4$ prefers to bind to duplex DNA and thus fails to induce G-quadruplex formation in the presence of the complementary strand. We therefore recommend using the duplex/G-quadruplex competition assay, rather than the DNA polymerase stop assay, for accurately predicting the outcome of gene suppression by G-quadruplex ligands.

Among the three perylene derivatives: PM1, the N-substituted perylene monoimide; PM2, the N,9-disubstituted perylene monoimide; and PIPER, the prototypic perylene diimide, PM2 appears to be the best in terms of G-quadruplex binding affinity on the VEGF promoter sequence and the down-regulation of VEGF gene expression. Since there are many factors that can affect the outcomes in Figures 3–5, we make no attempt to rationalize why PM2 is better than the others, and further investigation is required to fully interpret the data presented here. Nevertheless, an obvious feature is the presence of the piperazine side-chain in PM2 compared to

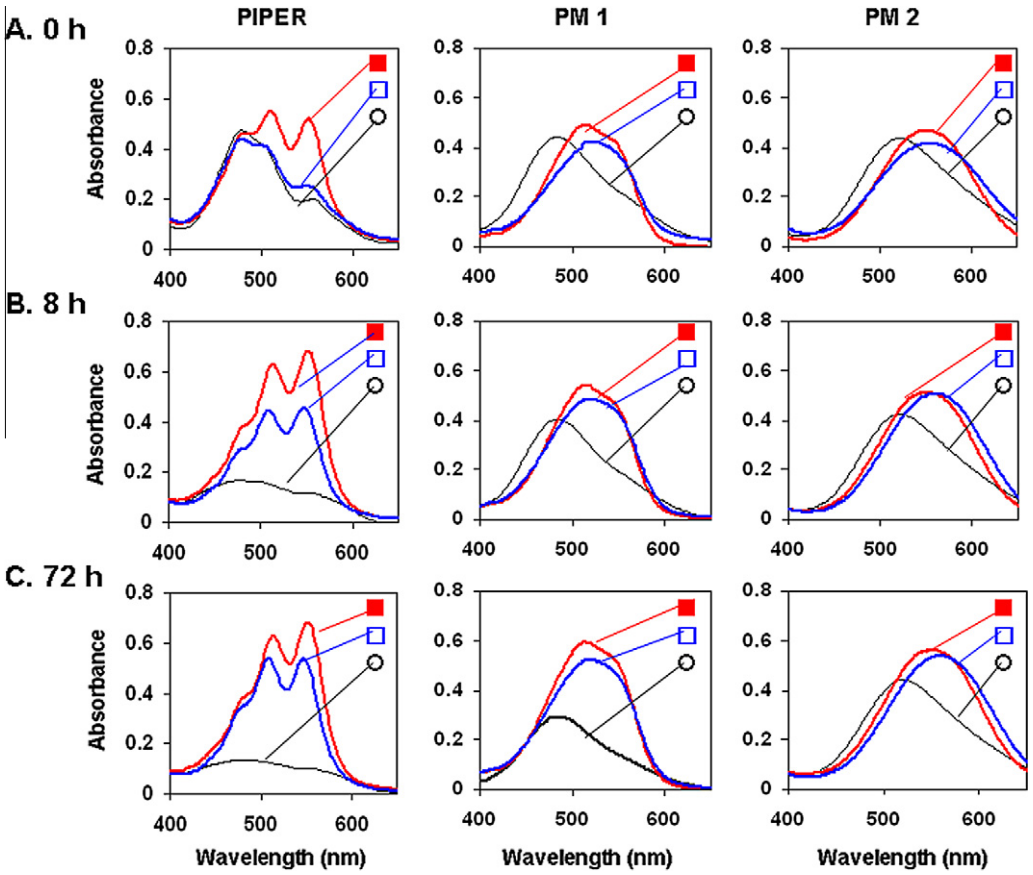

Figure 4. Visible absorbance spectra of perylene derivatives (40 μM) in the absence (○) and presence of the preformed VEGF quadruplex (20 μM V32G, ■), or the salmon sperm DNA duplex (equivalent absorbance to that of VEGF quadruplex, □), in 10 mM Tris–HCl (pH 7.4) buffer supplemented with 100 mM KCl, after incubation with the DNAs for: 0 h (A), 8 h (B), or 72 h (C).

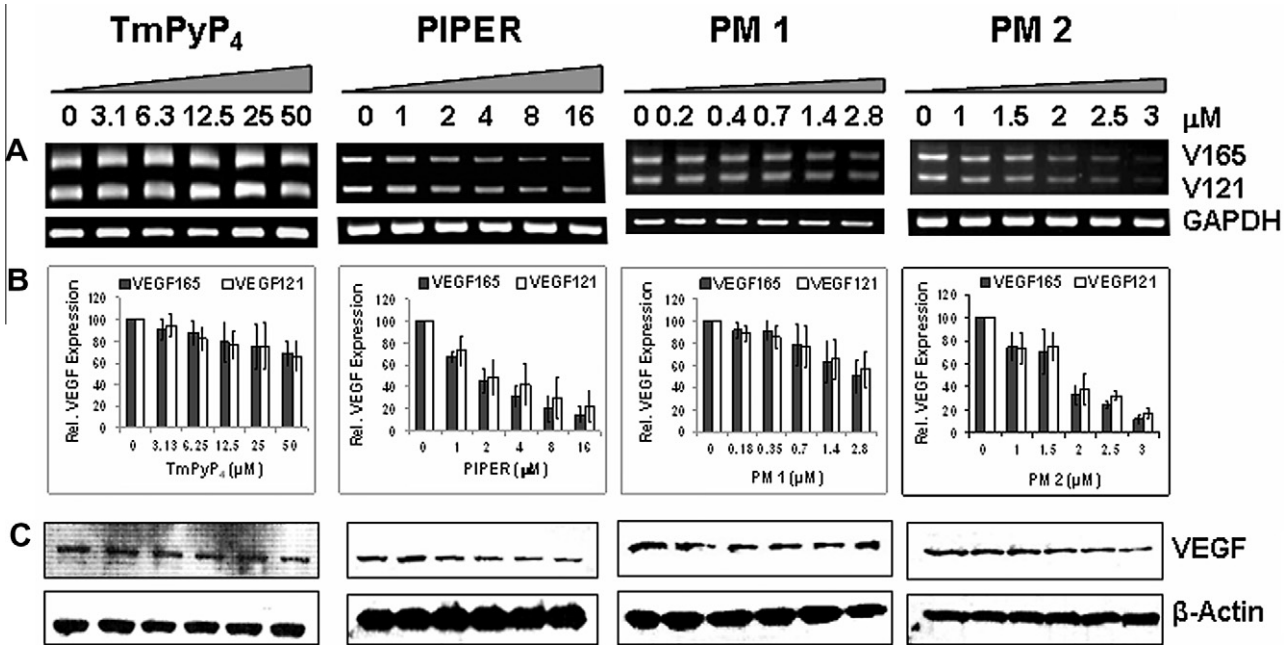

Figure 5. Down-regulation of VEGF gene in A549 lung cancer cells. A549 cells (3.0×10^5 cells) were treated with various concentrations of test compound for 24 h before the RNA or protein was extracted from the cell lysates. (A) Semi-quantitative RT-PCR. The mRNAs of treated cells were converted to cDNAs and amplified by PCR using gene-specific primers. The 540-bp PCR products (V165) represent the full-length VEGF165 mRNA, and the 410-bp PCR products represent the VEGF121 variant mRNA. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA was monitored as an internal control. (B) Graphs represent the semi-quantitative RT-PCR results from three separate experiments. Both VEGF bands were quantified by scan densitometry, and the values were normalized to that of GAPDH. (C) Western blot analysis. The cell lysates of treated cells were subjected to Western blotting using rabbit polyclonal VEGF antibody (Santa Cruz Biotechnology), followed by HRP-conjugated goat polyclonal anti-rabbit IgG (Abcam). The internal loading control, β-actin, was detected by HRP-conjugated mouse monoclonal anti β-actin (Abcam).

PM1, which can plausibly increase the binding affinity via electrostatic interactions with the phosphate backbone of G-quadruplex DNA. The asymmetry of PM2 might also account for the binding affinity to this particular G-quadruplex DNA, as it may orientate the molecule to a more favorable position. Since monomeric G-quadruplexes are unsymmetrical, and G-quadruplexes from different sequences are likely to vary, the asymmetry of PM2 could serve as a new platform for perylene modification in order to find a specific ligand for a specific G-quadruplex.

The role of G-quadruplex in the regulation of several genes has been either demonstrated or predicted.[14] For example, G-quadruplex formation in the insulin-linked polymorphic region upstream of the insulin gene was observed to enhance transcription.[15] On the other hand, G-quadruplex motifs on several oncogenes are found to be silencer elements, as in case of c-Myc[16] and KRAS,[17] including cancer-promoting genes such as PDGF[18] and VEGF.[7,8] The G-quadruplex motif has also found in the promoter of an anti-apoptotic gene Bcl-2.[19] To demonstrate that our perylene derivatives act as transcriptional silencers by inducing G-quadruplex formation at the promoter and are not VEGF-specific suppressors, we employed semi-quantitative RT-PCR to investigate the transcriptional expression of two other genes with G-quadruplex motif in their promoter: the c-Myc and Bcl-2, together with two constitutively expressed genes: Max and GAPDH, and an inducible gene, Cox-2, in A549 cells. These studies found that the transcriptional expression of all three genes with G-quadruplex motif in their promoter (VEGF, c-Myc, and Bcl-2) was suppressed in a dose-dependent manner by all three perylene derivatives, while the expression of the other three genes were not affected (see Supplementary data Fig. S3). The effects of PM 2 as a VEGF gene suppressor are also not cell-type specific, as we found that the compound suppressed VEGF gene expression in three other human cancer cell lines: the breast cancer cell line MCF-7, the colon cancer cell line HCT-15, and the cervical cancer cell line HeLa (see Supplementary data Fig. S4).

In conclusion, we have shown that our perylene derivatives are able to suppress VEGF transcription in human cancer cells, providing the foundation for the rational design and development of new perylene-based anti-angiogenesis agents for cancer therapy.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.089.

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