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Telomere shortening and cell senescence induced by perylene derivatives in A549 human lung cancer cells

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ABSTRACT

Cancer cells evade replicative senescence by re-expressing telomerase, which maintains telomere length and hence chromosomal integrity. Telomerase inhibition would lead cancer cells to senesce and therefore prevent cancer cells from growing indefinitely. G-quadruplex ligands can attenuate telomerase activity by inducing G-quadruplex formation at the 3′-overhang of telomere and at the human telomerase reverse transcriptase (*hTERT*) promoter; the former prevents telomerase from accessing the telomere, and the latter acts as a transcriptional silencer. The present investigation found that perylene derivatives PM2 and PIPER induced G-quadruplex formation from both telomeric DNA and the *hTERT* promoter region *in vitro*. Further, TRAP assay showed that these compounds inhibited telomerase in a dose-dependent manner. When A549 human lung cancer cells were treated with these compounds, hTERT expression was down-regulated. Moreover, the crude protein extract from these treated cells exhibited less telomerase activity. In the long-term treatment of A549 lung cancer cells with sub-cytotoxic dose of these perylenes, telomere shortening, reduction of cell proliferation and tumorigenicity, and cell senescence were observed. The results of this study indicate that perylene derivatives warrant further consideration as effective agents for cancer therapy.

1. Introduction

Telomere is a specific nucleoprotein structure at the end of every eukaryotic chromosome.[1] It differentiates the linear chromosomes from other DNA double-strand breaks, preventing them from end-to-end fusion and DNA degradation.[2] Telomeric DNA is progressively shortened in each round of cellular division due to the end replication problem.[3] In higher eukaryotes such as vertebrates, telomeric DNA consists of several kilobases of the 6-bp (TTAGGG) tandem repeats.[4] These long telomeric repeats allow a somatic cell to replicate for a number of cycles before its telomeres are shortened to a critical length, which then triggers the cell to enter senescence.[5] In contrast to normal somatic cells, most cancer cells solve this replicative senescence problem by re-expressing telomerase to maintain their telomere length.[5,6] Therefore, telomerase inhibition will lead cancer cells to senesce, preventing cancer cells from growing indefinitely.

G-quadruplex ligands have been widely studied as telomerase inhibitors, notably by targeting the 3′-overhang of telomeres.[7,8] At the cellular level, however, G-quadruplex ligands can exert their anticancer activity by acting as transcriptional silencers in many oncogenes, including *c-Myc, c-Kit, Hif-1α, KRAS, PDGF-A, Rb, RET, VEGF*, and human telomerase reverse transcriptase (*hTERT*).[9] The hTERT core promoter between −21 and −90 is a G-rich sequence that contains three Sp1 binding sites. This G-rich sequence was found to form a unique end-to-end stacked G-quadruplex structure, in which one G-quadruplex, formed by four consecutive G-tracts, is linked to another G-quadruplex formed by two pairs of consecutive G-tracts separated by a 26-base loop in an *in vitro* experiment.[10] The G-quadruplex formation in this region can block the binding sites for Sp1 and act as transcriptional silencer.

Among the G-quadruplex ligands, perylene derivatives have been well characterized with regard to their ability to induce G-quadruplex formation on the telomeric sequence and the *hTERT* promoter sequence.[11,12] Previously, we showed that our new water-soluble N,9-disubstituted perylene monoimide, PM2, along with the prototypic perylenediamine derivative PIPER, induce G-quadruplex formation on the *VEGF* promoter sequence and acted as transcriptional silencer of *VEGF* gene in various cancer cell lines.[13] In this study, we investigated both PM2 and PIPER (Fig. 1) for their ability to induce G-quadruplex formation, from both telomeric DNA and the *hTERT* promoter region, and their

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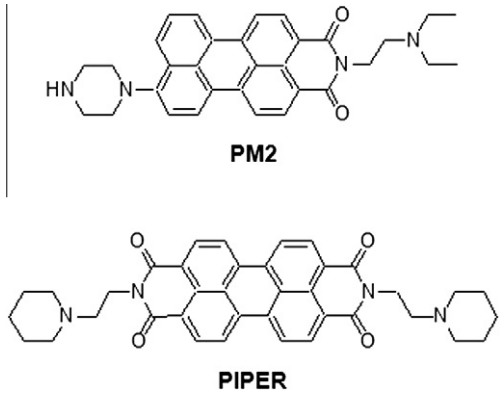

Figure 1. Structure of PM2 and PIPER.

telomerase inhibition *in vitro*. We then investigated whether these perylenes could down-regulate hTERT transcription in A549 lung cancer cells, which would lead to reduced telomerase activity in these treated cells. We also investigated the long-term influence of these compounds on telomere length, cell proliferation and tumorigenicity, and cell senescence in A549 lung cancer cells.

2. Results

2.1. Duplex/G-quadruplex competition assay

We employed the duplex/G-quadruplex competition assay to evaluate the G-quadruplex formation and binding specificity of PM2 and PIPER on the *hTERT* promoter sequences and the telomeric sequences. The *hTERT* core promoter sequence between −21 and −90 (Fig. 2A) was reported to form two end-to-end G-quadruplexes, in which one G-quadruplex was formed by the first four consecutive G-tracts (tract 1–4, sequence −23 to −39), and another G-quadruplex formed by two pairs of consecutive G-tracts separated by a 26-base loop (tract 5–6 and 11–12, sequence −47 to −90).[10] Therefore, we divided this core promoter into two parts: the hTERT1 sequence (−21 to −46, 26 bp) and the hTERT2 sequence (−47 to −90, 44 bp), and we tested these two sequences separately.

As illustrated in Figure 2B (hTERT1 sequence), 2C (hTERT2 sequence), 2D (telomeric sequence), without PM2 or PIPER, these oligonucleotides prefer to form duplex at the conditions used in the duplex/G-quadruplex competition assay. However, when the hTERT1 duplex or the telomeric duplex was incubated with either PM2 or PIPER, the G-rich strand was induced to form an intramolecular G-quadruplex (band M) in a dose-dependent manner (Fig. 2B and D). In general, PM2 induced intramolecular G-quadruplex better than PIPER, and has a preference toward the hTERT1 sequence to the telomeric sequence.

Figure 2C shows the results in which the hTERT2 duplex was employed. Intramolecular G-quadruplex formation was weakly detected when PM2 was used at 4 μM. At higher concentrations of both PM2 and PIPER, intramolecular G-quadruplex formation decreases in favor of intermolecular G-quadruplex (band T). This result differs from the other two sequences due to the length of the oligonucleotides and the number of G-tracts. Specifically, hTERT1 and the telomeric sequences are short (26 bp) and consist of four consecutive G-tracts; therefore, the induction to form intramolecular G-quadruplex is facile. In contrast, the hTERT2 sequence is long (44 bp) and consists of eight G-tracts; therefore, the competition to form one intramolecular G-quadruplex against the duplex is much more difficult. At higher concentrations of PM2 or to a lesser extent PIPER, the induction to form the parallel intermolecular G-quadruplex involving all G-tracts is more favorable.

2.2. Down-regulation of *hTERT* mRNA expression by PM2 and PIPER

Previously, we reported that both PM2 and PIPER could preferentially induce G-quadruplex formation on the *VEGF* promoter sequence, and these agents could down-regulate *VEGF* expression in the A549 lung cancer cells.[13] Moreover, these agents could down-regulate two other genes with G-quadruplex motif in their promoter, the *c-Myc* and *Bcl-2* gene, but not the other two constitutively expressed genes, *Max* and *GAPDH*, or an inducible gene, *Cox-2*, which has no G-quadruplex motif in its promoter.[13] In this study, we wished to examine whether these compounds could also down-regulate *hTERT* expression in the A549 lung cancer cells since the previous results showed that they could induce G-quadruplex formation on the *hTERT* promoter sequences. The A549 cells were first treated with PM2 or PIPER for 24 h before their mRNAs were isolated and converted into cDNAs by reverse transcriptase. The gene expression was then analyzed by semi-quantitative PCR (Fig. 3A) and real-time quantitative PCR (Fig. 3B) using *hTERT*-specific primers. The figures show that both PM2 and PIPER down-regulated *hTERT* mRNA expression in a dose-dependent manner. The real-time quantitative PCR results from three independent experiments (Fig. 3B) show that PM2 down-regulated the *hTERT* expression significantly more than PIPER. At a concentration of 6 μM, PM2 reduced *hTERT* mRNA to about 37% of the control, while PIPER reduced *hTERT* mRNA to about 68% of the control. The difference in the down-regulation of *hTERT* expression might reflect the preferential binding of PM2 to the *hTERT* promoter sequences observed in the duplex/G-quadruplex competition assay, especially the hTERT1 sequence (Fig. 2B).

2.3. Telomerase inhibition by PM2 and PIPER

A G-quadruplex ligand can attenuate telomerase activity either by preventing telomerase from accessing its substrate or by down-regulating *hTERT* expression at the *hTERT* promoter. To evaluate both modes of telomerase inhibition by our perylenes, we measured telomerase activity in the presence of various concentrations of either PM2 or PIPER in two different situations: (a) the direct effect on telomerase activity in a cell-free system, and (b) the cellular effect on telomerase activity due to the down-regulation of *hTERT* expression.

2.3.1. The direct effect on telomerase activity in a cell-free system

The TSG4 primer, which possesses the G-quadruplex motif, was first incubated with various concentrations of PM2 or PIPER at 37 °C for 3 h to allow G-quadruplex formation, before the telomerase extension mixture was added. After the extension reaction, the perylene was extracted from the reaction mixture by phenol/chloroform, and the telomerase products were then precipitated with ethanol. The precipitate was re-dissolved in the amplification reaction mixture, and the telomerase-extended products were amplified by PCR. The amplification products were then separated by electrophoresis, and the data were collected by a phosphoimager. As shown in Figure 4A, without incubation with the perylenes (Lane 0), telomerase extended the TSG4 primer in the typical 6-bp increment ladder. When the concentration of PM2 or PIPER was increased, the extended products decreased in a dose-dependent manner. Figure 4C shows the % telomerase activity plotted as a function of the concentration of the test compounds. The telomerase activity was determined from the cumulative fluorescence intensity of TRAP products quantified by ImageJ software. PM2 inhibits telomerase slightly better than PIPER, with an IC_{50} of 0.8 ± 0.1 μM, compared to that of PIPER at 1.4 ± 0.2 μM. From this experiment, we conclude that PM2 and PIPER directly inhibit

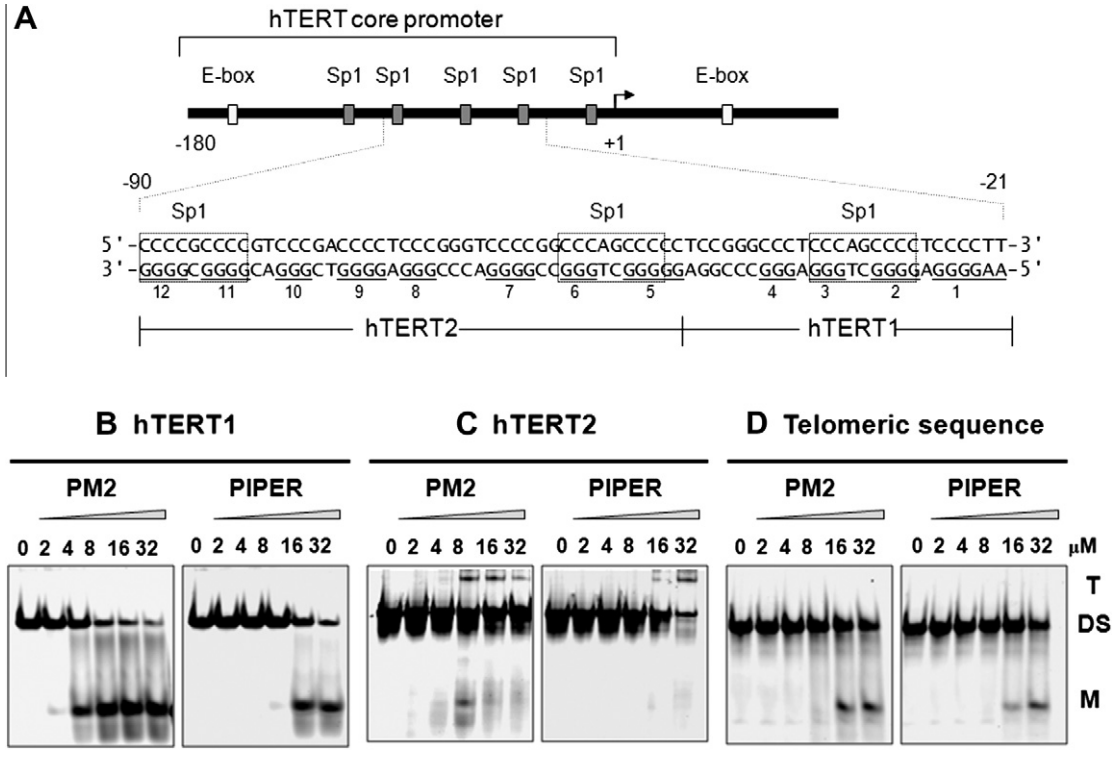

Figure 2. Duplex/G-quadruplex competition assay with *hTERT* promoter sequences and telomeric sequence. (A) The diagram and DNA sequence of the *hTERT* core promoter. The sequence between −21 and −90 contains 12 G-tracts having three or more guanines; each G-tract is underlined and numbered with Arabic number. The sequence is divided into two parts, hTERT1 and hTERT2, to be tested in this assay separately. (B–D) Gel data from the hTERT1, hTERT2, and telomeric sequence, respectively. The 20-μl reaction mixture consisting of FAM-labeled G-rich strand (2 μM), the complementary C-rich strand (2 μM), and the indicated concentration of either PM2 or PIPER in Tris–HCl buffer (pH 7.4) containing 100 mM KCl, was first denatured at 95 °C for 5 min before slowly cooled to 55 °C and incubated 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 and visualized with a Typhoon™ imaging system. DNA bands are indicated as double-stranded DNA (DS), monomeric G-quadruplex (M), tetrameric G-quadruplex (T).

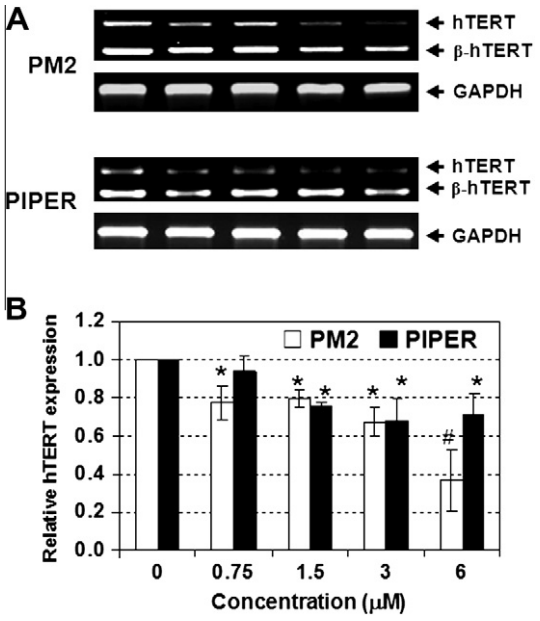

Figure 3. The effect of PM2 and PIPER on *hTERT* expression in A549 cells. The A549 cells were treated with the indicated concentration of either PM2 or PIPER for 24 h before their mRNAs were isolated and converted into cDNAs by reverse transcriptase. The gene expression was then analyzed by semi-quantitative PCR (A) and real-time quantitative PCR (B). Data were analyzed according to the standard curve method and normalized with *GAPDH* expression. Relative gene expression was presented as a bar graph; each bar represents mean ± SD from three independent experiments. Statistical significance: *P*-values are (*) $P < 0.05$ and (#) $P < 0.001$.

telomerase *in vitro* due to its ability to induce G-quadruplex DNA formation, primarily at the TSG4 primer, which prevents the access of telomerase to its substrate.

2.3.2. The cellular effect on telomerase activity due to the down-regulation of *hTERT* expression

The A549 cells were treated with either PM2 or PIPER for 24 h before the cells were lysed, and the crude cellular protein lysate was used as a source of telomerase in our fluorescent-modified TRAP assay. In this experiment, the MTS oligonucleotide, which possesses no G-quadruplex motif, was used as the primer for telomerase extension. Figure 4B shows that the telomerase activity (from the same amount of crude protein extract) diminished in a dose-dependent manner when A549 cells were treated with increasing concentrations of PM2 or PIPER. Figure 4D shows the % telomerase activity plotted as a function of the concentration of the test compounds from three independent experiments. The data indicate that PM2 reduces telomerase activity better than PIPER. The activity of the telomerase correlates well with the level of *hTERT* mRNA from our previous results, suggesting that the reduction in telomerase activity by PM2 or PIPER is likely due to the down-regulation of *hTERT* expression.

2.4. Effects of long-term treatment with subcytotoxic dose of PM2 and PIPER

Ideally, specific telomerase inhibitors prevent telomere maintenance in cancer cells without affecting other cellular mechanisms. The telomeres of these treated cancer cells should gradually shorten with each cell division, just like normal cells, albeit faster due to

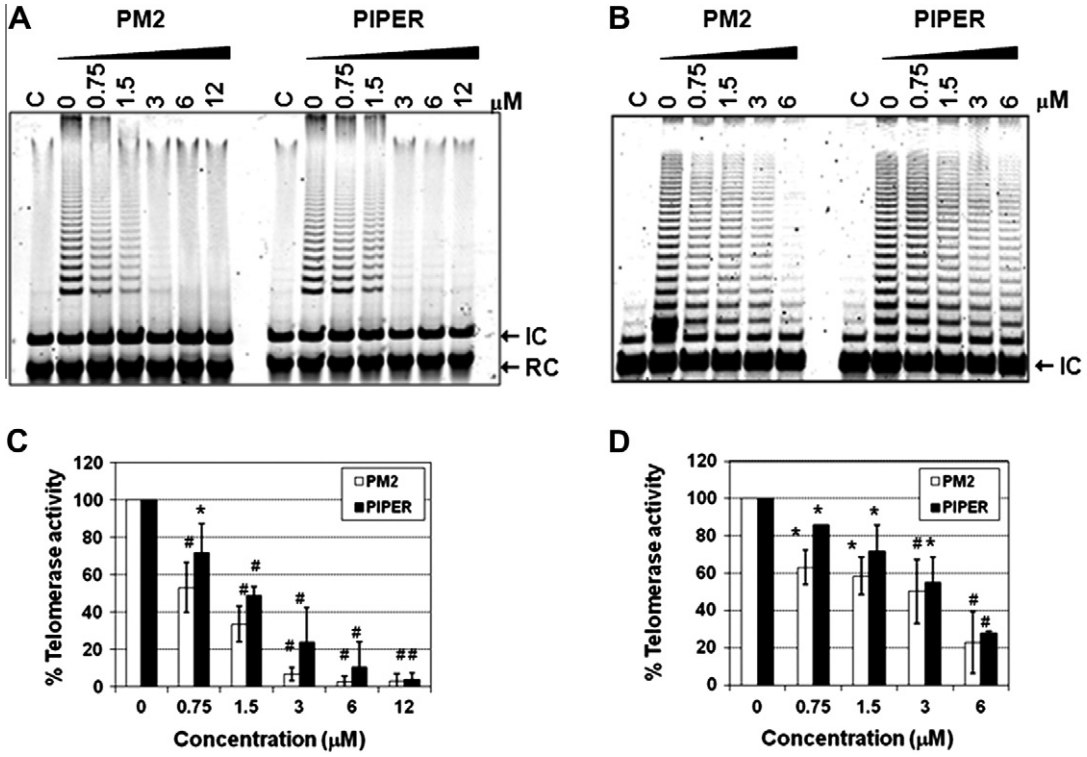

Figure 4. The effect of PM2 and PIPER on telomerase activity. (A) The direct effect on telomerase activity in a cell-free system. The TSG4 primer was first incubated with various concentrations of PM2 or PIPER at 37 °C for 2 h before the telomerase extension mixture was added. After the extension reaction, the perylene was extracted from the reaction mixture by phenol/chloroform, and the telomerase products were precipitated with ethanol. The telomerase-extended products were then amplified by PCR, and then separated by non-denaturing polyacrylamide gel electrophoresis. The data were collected by a phosphoimager. (B) The cellular effect on telomerase activity due to the down-regulation of *hTERT*. The A549 cells were incubated with either PM2 or PIPER for 24 h before the cells were lysed, and the crude cellular protein extract was used as a source of telomerase in the fluorescent-modified TRAP assay. MTS oligonucleotide was used as the primer for telomerase extension. The telomerase-extended products were amplified by PCR without phenol/chloroform extraction. The amplification products were then separated by electrophoresis. The data were collected by a phosphoimager. (C, D) Bar graph represents a plot of the % telomerase activity as function of the concentration of the test compounds from (A) and (B), respectively. The telomerase activity was determined from the cumulative fluorescence intensity of TRAP products quantified by ImageJ software. The graph represents mean telomerase activity ± SD from three independent experiments. Statistical significance: *P*-values are (*) $P < 0.05$ and (#) $P < 0.001$.

rapid cell division. Once telomeres shorten to a critical length when they can no longer maintain their functional telomere integrity, the chromosomal instability that follows will trigger growth arrest and cell senescence.[14] The cancer cells should show a reduction in proliferation potential and their clonogenicity. In the experiments described above, we showed that PM2 and PIPER reduce telomerase activity by direct telomerase inhibition and down-regulation of *hTERT* expression in A549 cancer cells. These effects should lead to telomere shortening and chromosome instability in these cancer cells after several cell divisions. As such, we investigated the long-term effects in the A549 cells treated with subcytotoxic doses of either PM2 or PIPER by evaluating (i) telomere length, (ii) cell proliferation, (iii) clonogenicity, and (iv) senescence-associated β-galactosidase activity. Previously, we reported that the IC_{50} values of PM2 and PIPER on A549 cells were 4.0 ± 0.1 μM and 52.4 ± 2.0 μM, respectively.[13] The sub-cytotoxic doses of PM2 and PIPER chosen here (0.4 and 0.8 μM) were found to allow >80% of the A549 cells to survive in the acute cytotoxicity assay. For this long-term treatment, the culture media was changed every 3 days, and the cells were counted and replated every 6 days. The remaining cells in each passage were collected to prepare genomic DNA for telomere length assay, or replated for colony-forming assay and senescence-associated β-galactosidase activity assay.

2.4.1. Effect on telomere length

Long-term treatment with subcytotoxic doses of PM2 and PIPER on A549 cells progressively shortened their telomeres with time. As shown in Figure 5A, the mean TRF length in the control group remained stable at 3.6 kb during the course of study (54 days). However, the mean TRF length from cells treated with 0.4 μM of PM2 decreased from 3.6 to 3.5 kb and 2.9 kb on day 24 and 54, respectively. The decrease in the mean TRF length is slightly more pronounced from cells treated with 0.8 μM of PM2, in which the length decreased to 3.2 kb on day 30. We collected no DNA samples beyond this point because the cell proliferation rapidly declined, and the cells were unable to proliferate after day 36 (see Fig. 6A). The same progressive telomere shortening was observed for the cells treated with the subcytotoxic doses of PIPER (Fig. 5B). Since PIPER is less toxic than PM2, we prolonged our treatment to the maximum of 78 days. The mean TRF length remained stable in the control group during the course of study. The mean TRF length from cells treated with 0.4 μM of PIPER decreased from 3.6 to 3.3 kb and 3.0 kb on day 30 and 78, respectively. The decrease in the mean TRF length is slightly more pronounced from cells treated with 0.8 μM of PIPER, in which the length decreased to 3.1 and 2.7 kb on day 30 and 78, respectively. From these results, we conclude that the telomere erosion observed in the A549 cells treated with either PM2 or PIPER is likely due to telomerase inhibition, which was already demonstrated by our previous *in vitro* experiments *(vide supra)*.

2.4.2. Effects on cell proliferation and cell senescence

The effect of long-term exposure to sub-cytotoxic doses of PM2 and PIPER on A549 cell proliferation and cell senescence depends on each individual perylene, the dosage amount, and the time. As

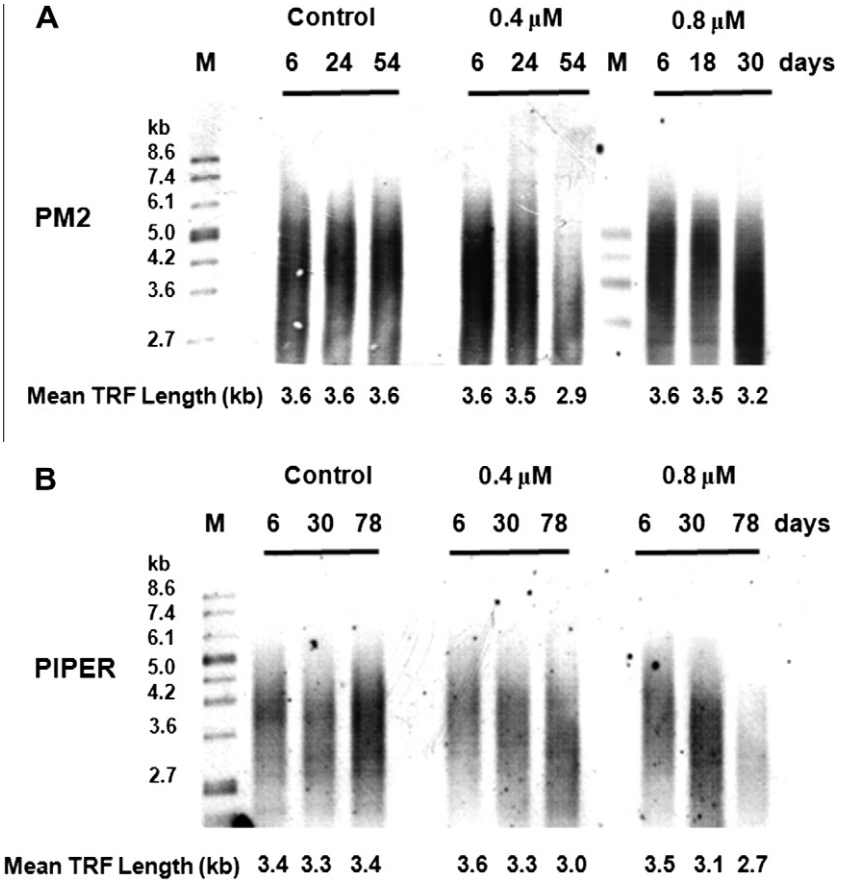

Figure 5. The effect of PM2 (A) and PIPER (B) on telomere length in A549 cells. The A549 cells were treated with the indicated sub-cytotoxic doses of PM2 or PIPER for the indicated days, with the cultured media changed every 3 days and the cells replated every 6 days. Genomic DNA was extracted, and telomere restriction fragments (TRF) were analyzed using the *Telo*TAGGG Telomere Length Assay kit. M represents molecular weight marker.

shown in Figure 6A, treatment with 0.4 μM PM2 had little effect on the population doubling of A549 cells up to 42 days of treatment, but after that, the population doubling declined slightly, with most cells remaining viable. At the higher dose of 0.8 μM PM2, the population doubling started to decline on day 18 and reached a plateau on day 36 when cell division was arrested. The senescence-associated β-galactosidase activity assay of cells treated with 0.4 μM PM2 for 54 days showed some cells with signs of senescence; namely, cell enlargement and β-galactosidase activity were observed (enlarged blue-stained cells, Fig. 6B). Treatment with 0.4 μM PIPER had little effect on the proliferation of A549 cells since the population doubling was almost identical to the control throughout the entire course of the study (78 days). In contrast, treatment with 0.8 μM PIPER led to a decline in population doubling of about 20% without affecting cell viability, suggesting an increase in the length of the cell cycle (Fig. 6A). The senescence-associated β-galactosidase activity assay performed on A549 cells collected from cells treated with 0.4 μM PIPER and 0.8 μM PIPER for 78 days revealed a few senescent cells as well (Fig. 6B).

2.4.3. Effect on clonogenicity

Long-term treatment with subcytotoxic doses of PM2 and PIPER reduced the clonogenicity of A549 cells, as evaluated by a colony-forming assay. The A549 cells from the long-term treatment with 0.4 or 0.8 μM of either PM2 or PIPER, as described above, were collected on the indicated day. Then, 2×10^3 cells were replated in a new tissue culture dish, and the cells were allowed to form colonies for 14 days. Figure 6C (top panels) shows the examples of the colony-forming assay after treatment with PM2 for 24 days or PIPER for 30 days; the colony formation was reduced in a dose-dependent manner. Figure 6C (lower panels) shows the bar graph plotted between the % colony formation (compared to control) and days of treatment, each bar represents mean ± SD from three independent experiments. When the A549 cells were treated with PM2 for 6 days, the % colony formation remained close to 100% at both doses. However, when the treatment with PM2 was prolonged to 24 days, the % colony formation decreased to 73% and 35% in sets treated with 0.4 and 0.8 μM PM2, respectively. When the treatment with 0.4 μM PM2 was prolonged to 54 days, the % colony formation decreased further to 60%. We have no data from the set treated with 0.8 μM PM2 for 54 days because the cells failed to proliferate beyond day 36. For PIPER, the % colony formation from the A549 cells treated with 0.4 μM and 0.8 μM PIPER for 6 days is 95% and 93%, respectively. When the treatment with 0.4 μM PIPER was prolonged to 30 days, the % colony formation remained the same at 96%, while the number decreased slightly to 89% in the sets treated with 0.8 μM PIPER. When the treatment with PIPER was prolonged to 78 days, the % colony formation decreased to 78% and 66% in the sets treated with 0.4 μM and 0.8 μM PIPER, respectively.

3. Discussion and conclusion

G-quadruplex ligands have been shown to inhibit telomerase by targeting the 3′-overhang of telomeres,[7,8] and by acting as transcriptional silencer at the *hTERT* promoter.[9] In this study, we have shown that both perylene derivatives, PM2 and PIPER, could induce G-quadruplex formation from both telomeric DNA and the *hTERT*

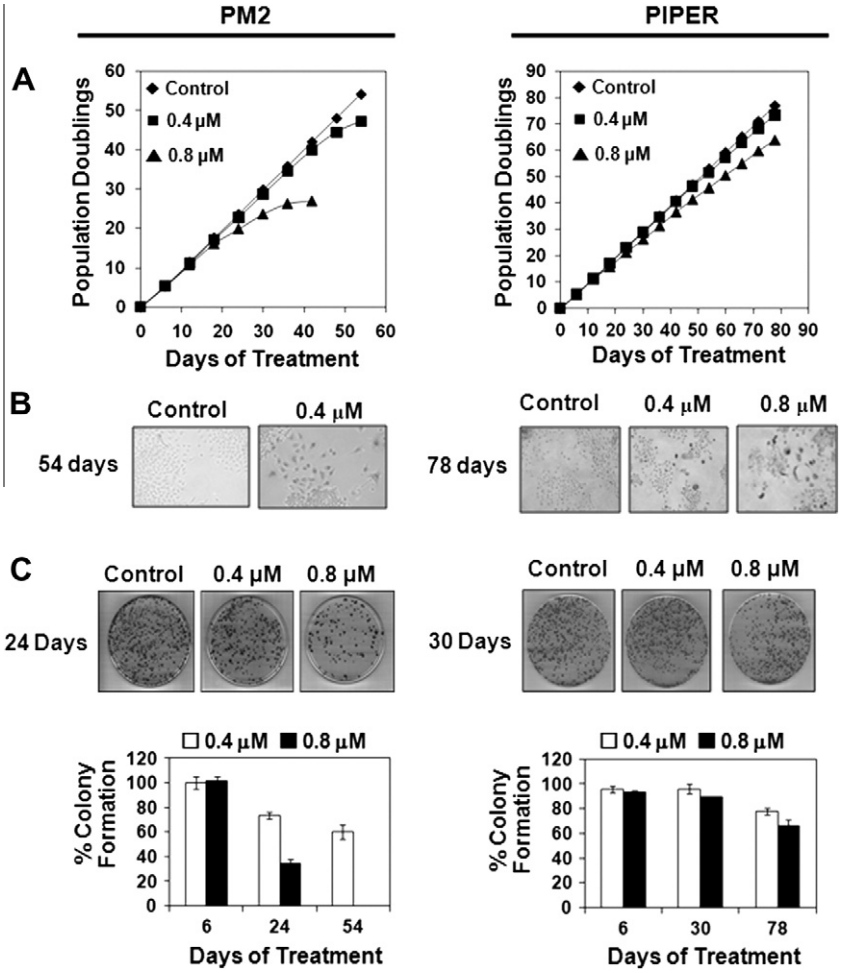

Figure 6. Effects of long-term treatment with subcytotoxic doses of PM2 and PIPER on cell proliferation (A), cell senescence (B), and clonogenicity (C). The A549 cells were treated in the absence or in the presence of the indicated sub-cytotoxic doses of PM2 or PIPER for the indicated days, with the cultured media changed every 3 days and the cells replated every 6 days. The following assays were performed in three independent experiments. (A) Cells from each passage were counted, and the growth curves between the cumulative numbers of population doublings were plotted against time. (B) Cells were collected on the indicated day and replated in a 24-well plate. The cells were allowed to grow for 48 h, fixed, stained with X-gal solution, and photographed under a fluorescence microscope. The morphological changes, including cell enlargement and β-galactosidase positive cells (blue stained cells), are indicatives of cell senescence. (C) Cells were collected on the indicated day, and 2×10^3 cells were replated in a new tissue culture dish. The cells were allowed to form colonies for 14 days and then stained with crystal violet (top panels). Each plate was then scanned by a phosphoimager, and the colonies were counted using ImageQuant TL software (Nonlinear Dynamics). In the bottom panels, the bar graph shows a plot of the % colony formation versus days of treatment.

promoter region, and they could inhibit telomerase *in vitro*. Further, treatment of A549 human lung cancer cells with these compounds down-regulates *hTERT* expression. The crude protein extract from these treated cells exhibits reduced telomerase activity, reflecting a diminished production of functional telomerase. In addition, long-term treatment of A549 cancer cells with sub-cytotoxic doses of PM2 and PIPER leads to telomere shortening, reduction of cell proliferation and tumorigenicity, and senescence. These behaviors correspond to the classic model of telomerase inhibition.[15]

Upon comparing the two perylene derivatives, PM2 was found to be more effective than PIPER in terms of G-quadruplex formation, down-regulation of *hTERT* mRNA expression, and telomerase inhibition (both direct telomerase inhibition and cellular telomerase production). Our previous study also found that PM2 was better than PIPER in terms of G-quadruplex binding affinity on the *VEGF* promoter sequence and the down-regulation of *VEGF* gene expression.[13] Since there are many factors that could affect these outcomes, we make no attempt to extrapolate these results. Nevertheless, PM2 is more water-soluble than PIPER, and there might consequently be more free molecules of PM2 available in solution at the equivalent concentrations tested.[13] PIPER is well-known to aggregate in aqueous solution, especially at elevated pH.[16,17] The selective binding of PM2 to the hTERT1 sequence, compared to that of the telomeric sequence, demonstrates that a G-quadruplex ligand can bind preferentially to one particular G-quadruplex form. Since the structures of monomeric G-quadruplexes are unsymmetrical and differ slightly from one sequence to another, the asymmetry of PM2 might afford a more favorable orientation and/or positioning for this G-quadruplex ligand.

PM2 also exerted its cytoxicity more potent than PIPER when it was exposed to A549 lung cancer cells. The IC_{50} of PM2 was much lower than PIPER, with a value of 4.0 ± 0.1 μM compared to 52.4 ± 2.0 μM for PIPER.[13] In the long-term treatment on A549 cells, PM2 reduced cell proliferation and clonogenicity, and induced cell-growth arrest better than PIPER at the same concentration. Prolonged treatment with sub-cytotoxic doses of both PM2 and PIPER resulted in telomere shortening, reflecting the telomerase inhibition by these compounds. The less acutely cytotoxic but equally or more effective analog of PM2 would be an ideal candidate for maintenance therapy, after surgery and conventional chemotherapy, to prevent recurrence of cancer.

In conclusion, we have shown that PM2 and PIPER could attenuate telomerase activity by inducing G-quadruplex formation at the 3′-overhang of telomere and down-regulating *hTERT* transcription. Long-term treatment of A549 cancer cells with sub-cytotoxic doses of PM2 and PIPER led to telomere shortening, reduction of cell proliferation and tumorigenicity, and senescence. All of these results support the role of these G-quadruplex ligands as telomerase inhibitors. The results presented here might promote further investigation of perylene derivatives as effective agents for cancer therapy.

4. Materials and methods

4.1. Materials

Unless mentioned, all materials were purchased from commercial suppliers and used without further purification. All oligonucleotides and fluorescence-tagged oligonucleotides were purchased from Pacific Science (Thailand). The DNA sequences for these oligonucleotides are listed in Supplementary data Table 1. The synthesis and characterization of PIPER and PM2 has been described elsewhere.[17,18]

4.2. Duplex/G-quadruplex competition assay

The 20-μl reaction mixture [consisting of the indicated concentration of a perylene derivative, 2 μM of FAM-labeled G-rich strand, and 2 μM of its complementary C-rich strand, in 10 mM Tris–HCl (pH 7.4) containing 100 mM KCl] was first heated at 95 °C for 5 min before slowly cooling to 55 °C and incubating 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.

4.3. Semi-quantitative RT-PCR analysis

A549 cells (3.0×10^5 cells) were first grown as a monolayer in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 mg/ml) on a 6-well tissue culture plate at 37 °C in a humidified CO_2 (5%) incubator for 24 h, before further incubating with the indicated concentration of either PM1 or PIPER for another 24 h. The culture medium was discarded, and the cells were washed twice with phosphate buffered saline (PBS) before the total RNA was isolated by Trizol® (Gibco). The concentration of total RNA was then estimated by spectrophotometry (OD. 260 nm). Total RNA (2.0 μg) was converted into cDNAs using oligo$(dT)_{18}$ primer and M-MuLV reverse transcriptase (Fermentas) according to the manufacturer's instruction. Subsequent PCR was carried out in a thermocycler using gene-specific primers. PCR products were electrophoretically separated on 1% agarose gel and visualized by ethidium bromide staining. The specific PCR cycle used for each gene was carefully chosen so that the intensity of the detected PCR product was proportional to the initial amount of cDNA in the reaction.

Quantitative real-time RT-PCR analysis. The A549 cells were treated in the same manner as in the semi-quantitative RT-PCR mentioned above. Real-time PCR reactions were carried out on the cDNAs using SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instruction. The PCR cycle was programmed as follows: one cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Data were analyzed according to the standard curve method and normalized by *GAPDH* expression. Relative gene expression was calculated from three independent experiments. The DNA sequences of primers are listed in Supplementary data Table 1.

4.4. Modified fluorescent TRAP assay

We investigated the effect of perylene derivatives on telomerase activity using two approaches: (a) the direct effect on telomerase activity in a cell-free system and (b) the cellular effect on telomerase activity. Our TRAP assay was performed according to a published protocol introduced by Szatmari and Aradi; this method retains the original length of telomerase products, and therefore the processivity of telomerase can be analyzed.[19] For the detection of the amplified telomerase products; however, we modified the method by using a fluorescent-tagged primer instead of radioactive nucleotide incorporation.

(a) The direct effect on telomerase activity in a cell-free system. First, 10 μl of 10× test compound was incubated with 80 μl telomerase reaction buffer [20 mM Tris–HCl (pH 7.4), 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.1 mg/ml bovine serum albumin, 0.005% Tween 20, 200 μM dNTPs, and 15 pmol TSG4 primer] at 37 °C for 2 h. Then, 10 μl of crude telomerase (50 ng) from the telomerase-transfected HEK293T cells, produced according to the published protocol,[20] were added, and the reaction mixture was allowed to incubate further at 30 °C for 30 min before the reaction was stopped by heating at 95 °C for 5 min. Then, 5 μl of 100 nM FAM-tagged RC duplex were added into the reaction mixture as a recovery control. The test compound was extracted twice with phenol–chloroform, and the DNA was precipitated with ethanol. The DNA pellet was resuspended in 10 μl of water, in which 5 μl of the resuspended DNA mixture were added into the 45 μl of amplification reaction mixture [2.5 units *Taq* DNA polymerase, 15 pmol RP-FAM primer, 0.25 pmol RPc3g, 0.01 pmol IC, and 7.5 pmol NT primers in 20 mM Tris–HCl (pH 7.4), 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.1 mg/ml bovine serum albumin, 0.005% Tween 20, 200 μM dNTPs], and PCR was performed in a thermocycler with the following conditions: the first three cycles: 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 90 s, followed by 25 cycles of: 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. The amplification products were electrophoretically separated in 8% non-denaturing polyacrylamide gel and visualized with a phosphoimaging system (Typhoon; Molecular Dynamics). The data were quantified by ImageJ software. The assay was performed in three independent experiments, and the $IC_{50} \pm SD$ value for telomerase inhibition was calculated for each perylene derivative.

(b) The cellular effect on telomerase activity. A549 cells were treated with the indicated concentrations of PM2 or PIPER for 24 h. The cells were lysed with 200 μl of CHAPS lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$ 0.1 mM EGTA, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol, protease inhibitor cocktail, and 200 unit/ml RNase inhibitor), and the supernatant was collected and quantified for protein concentration by Bradford assay (BioRad). The 6 μg of protein from this supernatant served as a source of telomerase. The following TRAP assay was performed in the same manner as in (a), but using MTS primer instead of TSG4 primer, and without the phenol–chloroform extraction and ethanol precipitation.

4.5. Long-term proliferation assay

Three sets of cell cultures were assayed simultaneously: the control group with only culture media and the two experimental groups with the indicated sub-cytotoxicity concentration (0.4 and 0.8 μM) of a perylene derivative supplemented in the culture

media. The A549 cells (1.5×10^5 cells) were first seeded onto a 75 cm^2 tissue culture flask in RPMI 1640 medium supplemented with 10% fetal bovine serum, in the absence or presence of the indicated sub-cytotoxicity dose of a perylene derivative. The culture media was changed every 3 days. After the A549 cells reached confluence every 6 days, the cells were trypsinized and counted using a hematocytometer. Then, the 1.5×10^5 cells were replated onto a new culture flask with fresh culture media. The number of population doublings was calculated by the equation: $n = (\log P_n - \log P_0)/\log 2$, where P_n is the number of cells after n doublings; P_0 is the initial seeding density. The cumulative number of population doublings was plotted against time. The assay was performed in three independent experiments. The remaining cells in each passage were collected to prepare genomic DNA for telomere length assay, or replated for colony-forming assay and senescence-associated β-galactosidase activity assay.

4.6. Telomere length assay

The average telomere lengths of A549 cells, in the absence and presence of a perylene derivative, were assayed using *Telo*TAGGG Telomere Length Assay kit (Roche Applied Science) according to the manufacturer's instruction. In brief, total DNA was isolated from A549 cells using DNAzol® (Invitrogen). The 2 μg of purified DNA was digested with *Rsa*I and *Hin*fI, electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane (Immobilon™-Ny, Millipore). The membrane was then washed, cross-linked, and hybridized with a DIG-labeled telomeric probe. After hybridization, the membrane was incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase. The telomeres were visualized as a smear on an X-ray film using a chemiluminescent system. The average TRF length was calculated according to the formula $\sum(OD_i)/\sum(OD_i/L_i)$, where OD_i indicates the absorbance at the position i, and L_i is the molecular weight marker at the same position.[21]

4.7. Colony-forming assays

The cultured A549 cells from the long-term proliferation assay were seeded at low density of 2×10^3 cells in a 10-cm dish to allow colony formation. Cells were fed with fresh growth media every 4–5 days. After 2 weeks, the colonies were stained with crystal violet. The plates were digitally scanned, and the colony number was then counted using the ImageQuant TL software (Nonlinear Dynamics). For accuracy, experiments giving less than 25 colonies in the controls were not counted.

4.8. Senescence-associated β-galactosidase activity assay

The cultured A549 cells from the long-term proliferation assay were replated in a 24-well plate and grown for 48 h. The media was then removed, and the cells were rinsed three times with PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde solution for 5 min at room temperature. The cells were washed again with PBS twice and stained overnight in the solution containing 1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$. The solution was removed the following day, the cells were rinsed with PBS, and the stained cells were viewed under a Nikon fluorescence microscope.

4.9. Statistical analysis

Difference in the mean value among the groups was determined using one-way analysis of variance (ANOVA), and differences between individual using a Student *t*-test by SPSS 16.0 software package (SPSS Inc). Values of $P < 0.05$ were considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.12.020.

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