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A 6-mer Photocontrolled Oligonucleotide as an Effective Telomerase Inhibitor

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Abstract: Telomerase is a relevant target for cancer therapy. A small 6-mer psoralen-containing oligonucleotide is shown to cause efficient photo-cross-linking to human telomere DNA by G-quadruplex formation. The small, photocontrolled oligonucleotide shows effective telomerase inhibition, both *in vitro* and *in vivo*, only when light irradiated. Data suggest that it is a promising agent for the treatment of cancer.

Introduction

Telomerase or its telomere DNA substrate has been suggested as a target for cancer therapy because telomerase is activated in 80−90% of human tumors.[1] The development of new approaches to targeting human telomeric DNA is thus an area of great interest because of the potential for discovering anticancer agents.[1]

Many small molecules are reported to inhibit telomere elongation by telomerase.[1] These molecules target and stabilize the G-quadruplex, a structure adopted by the 3′-overhang of telomere, and directly inhibit telomerase activity by blocking its access to telomere.[2] Several nucleoside analogues and oligonucleotides are also reported to be promising agents. For instance, azidothymidine (AZT) has the potential to target the telomeric end of chromosomes in cancer cells, promoting cell death.[1d] Recently, approaches for alkylating telomere duplex DNA or directly cleaving telomere single-strand DNA have been developed.[3]

These promising molecules, however, may have the undesired side effect of damaging normal cells because they cannot discriminate between normal and cancer cells. Photodynamic therapy is useful for minimizing the side effects, because the main effects appear only in the area and time of irradiation. Here we report that a small,photocontrolled 6-mer oligonucleotide shows effective telomerase inhibition both *in vitro* and *in vivo*. Such a small oligonucleotide should have high cellular uptake and minimal nonspecific binding with *in vivo* substrate.

Results and Discussion

Design of a Small, Photocontrolled 6-mer Oligonucleotide To Target Human Telomeric DNA by Dimeric G-Quadruplex Formation. For this photocontrolled approach to inhibit telomerase activity, we employed a photo-cross-linking reaction. Psoralen, a linear furocoumarin used for the treatment of skin diseases and cutaneous T-cell lymphoma,[4] was selected as a photo-cross-linking reagent because of its reaction selectivity with pyrimidine bases, especially thymine.[4c] Because human telomeric DNA contains thymine in its sequences, a 6-mer

(1) (a) Neidle, S.; Parkinson, G. *Nat. Drug Discovery* **2002**, *1*, 383–393. (b) Hurley, L. H. *Nat. Rev. Cancer* **2002**, *2*, 188–200. (c) Shay, J. W.; Wright, W. E. *Nat. Rev. Drug Discovery* **2006**, *5*, 577–584. (d) Mergny, J.-L.; Riou, J.-F.; Mailliet, P.; Teulade-Fichou, M. P.; Gilson, E. *Nucleic Acids Res.* **2002**, *30*, 839–865. (e) Kim, N. W.; Piatyszek, M. A.; Prowse, K. R.; Harley, C. B.; West, M. D.; Ho, P. L.; Coviello, G. M.; Wright, W. E.; Weinrich, S. L.; Shay, J. W. *Science* **1994**, *266*, 2011–2015.

(2) (a) Kim, M. Y.; Vankayalapati, H.; Shin-Ya, K.; Wierzba, K.; Hurley, L. H. *J. Am. Chem. Soc.* **2002**, *124*, 2098–2099. (b) Teulade-Fichou, M.-P.; Carrasco, C.; Guittat, L.; Bailly, C.; Alberti, P.; Mergny, J.-L.; David, A.; Lehn, J.-M.; Wilson, W. D. *J. Am. Chem. Soc.* **2003**, *125*, 4732–4740. (c) Jantos, K.; Rodriguez, R.; Ladame, S.; Shirude, P. S.; Balasubramanian, S. *J. Am. Chem. Soc.* **2006**, *128*, 13662–13663. (d) De Cian, A.; DeLemos, E.; Mergny, J.-L.; Teulade-Fichou, M.-P.; Monchaud, D. *J. Am. Chem. Soc.* **2007**, *129*, 1856–1857. (e) De Cian, A.; Cristofari, G.; Reichenbach, P.; De Lemos, E.; Monchaud, D.; Teulade-Fichou, M. P.; Shin-ya, K.; Lacroix, L.; Lingner, J.; Mergny, J.-L. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17347–17352. (f) Kieltyka, R.; Englebienne, P.; Fakhoury, J.; Autexier, C.; Moitessier, N.; Sleiman, H. F. *J. Am. Chem. Soc.* **2008**, *130*, 10040–10041. (g) Campbell, N. H.; Parkinson, G. N.; Reszka, A. P.; Neidle, S. *J. Am. Chem. Soc.* **2008**, *130*, 6722–6724. (h) Ma, D.-L.; Che, C.-M.; Yan, S.-C. *J. Am. Chem. Soc.* **2009**, *131*, 1835–1846. (i) Chen, Z.; Zheng, K. W.; Hao, Y. H.; Tan, Z. *J. Am. Chem. Soc.* **2009**, *131*, 10430–10438. (j) Yu, H. Q.; Zhang, D. H.; Gu, X. B.; Miyoshi, D.; Sugimoto, N. *Angew. Chem., Int. Ed.* **2008**, *47*, 9034–9038. (k) Palumbo, S. L.; Ebbinghaus, S. W.; Hurley, L. H. *J. Am. Chem. Soc.* **2009**, *131*, 10878–10891.

(3) (a) Sasaki, S.; Bando, T.; Minoshima, M.; Shimizu, T.; Shinohara, K.; Takaoka, T.; Sugiyama, H. *J. Am. Chem. Soc.* **2006**, *128*, 12162–12168. (b) Xu, Y.; Suzuki, Y.; Lonnberg, T.; Komiyama, M. *J. Am. Chem. Soc.* **2009**, *131*, 2871–2874.

(4) (a) Parrish, J. A.; Stern, F. S.; Pathak, M. A.; Fitzpatric, J. B. Photochemotherapy of skin diseases. *The Science of Photomedicine*; Plenum Press: New York, 1982; pp 595−624. (b) Knobler, R. M.; Honigsmann, H.; Edelson, R. L. Psoralen phototherapies. In *Psoralen DNA Photobiology*;CRC Press: Boca Raton, FL, 1988; Vol. II, pp 117−134. (c) Sigurdsson, S. T.; Rink, S. M.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 12633–12634.

(5) (a) Xu, Y.; Noguchi, Y.; Sugiyama, H. *Bioorg. Med. Chem.* **2006**, *14*, 5584–5591. (b) Matsugami, A.; Xu, Y.; Noguchi, Y.; Sugiyama, H.; Katahira, M. *FEBS J.* **2007**, *274*, 3545–3556. (c) Luu, K. N.; Phan, A. T.; Kuryavyi, V.; Lacroix, L.; Patel, D. *J. Am. Chem. Soc.* **2006**, *128*, 9963–9970. (d) Ambrus, A.; Chen, D.; Dai, J. X.; Bialis, T.; Jones, R. A.; Yang, D. Z. *Nucleic Acids Res.* **2006**, *34*, 2723–2735. (e) Zhang, N.; Phan, A. T.; Patel, D. J. *J. Am. Chem. Soc.* **2005**, *127*, 17277–14285. (f) Xu, Y.; Sato, H.; Sannohe, Y.; Shinohara, K.; Sugiyama, H. *J. Am. Chem. Soc.* **2008**, *130*, 16470–16471.

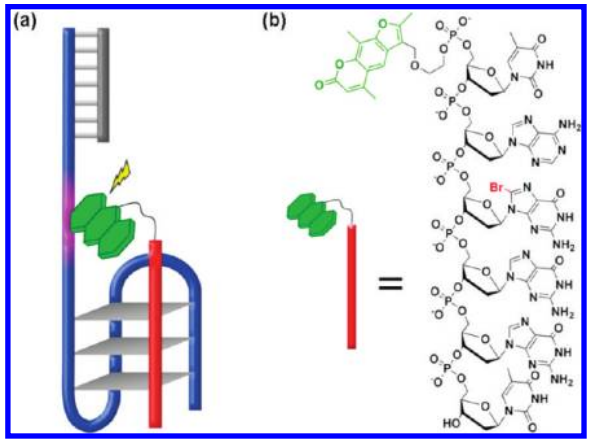

Figure 1. (a) Representation of photo-cross-linking of human telomeric DNA by a small, photocontrolled 6-mer oligonucleotide. Dimeric G-quadruplex formation directs the 6-mer photo-cross-linking reagent to telomere DNA to give a photocontrolled adduct upon irradiation at 365 nm.[5e] (b) Structure of a 6-mer oligonucleotide with a photo-cross-linking reagent, psoralen, at the 5′-end (psoralen-5′-TA^{Br}GGGT). Red bromine indicates the substitution of dG with syn-preferring 8-bromoguanosine (^{Br}G). We previously demonstrated that proper substitutions of dG with ^{Br}G stabilize the G-quadruplex structure.[5f]

oligonucleotide bearing psoralen should produce a photoadduct with the telomere target upon irradiation at 365 nm (Figure 1b). The resulting photocontrolled adduct is expected to inhibit telomere elongation from telomerase. To direct psoralen to the target sequence, we developed novel approach on the basis of previous studies.[5e] Patel et al. showed that a dimeric G-quadruplex with a new topology was formed by three-repeat and single-repeat human telomeric sequences.[5e] We and others reported the same topology containing the (3+1) G-tetrad core for human telomeric G-quadruplex on the basis of circular dichroism (CD) and NMR studies.[5a−d] Our recent studies also demonstrated that such a (3+1) G-quadruplex can stabilize T-loop structure.[5f] This (3+1) dimeric G-quadruplex structure suggests how a segment of 6-mer G-tract can bind to three G-tracts.[5e] On the basis of these pioneering works, we formed a G-quadruplex between the 6-mer psoralen-conjugated oligonucleotide and telomere target sequence (Figure 1a). Formation of a stable G-quadruplex between the 6-mer oligonucleotide and the target sequence is a key step in the photo-cross-linking reaction. For this purpose, proper substitution of dG with ^{Br}G was carried out to stabilize, or "freeze" (Figure 1b), the G-quadruplex structure formed by 6-mer oligonucleotide and target telomere DNA.[5e]

Characterization of the Psoralen-Conjugated 6-mer Oligonucleotide. Psoralen-conjugated 6-mer oligonucleotide with ^{Br}G (Pso-ODN-1 in Table 1) was confirmed, by CD spectroscopy, to form a hybrid G-quadruplex with a telomere DNA substrate (a positive band at 290 nm, with weak negative peaks near 255 and 235 nm; Figure 2a).[6] The ^{Br}G substitution in the Pso-ODN-1 results in a significant increase in thermal stability as compared to no substitution of the ^{Br}G in the oligonucleotide. According to CD melting experiments, the T_m value of G-quadruplexes formed by the Pso-ODN-1 with ^{Br}G was increased by 5.8 °C over that of the oligonucleotide without ^{Br}G (Figure 2b).

(6) (a) Petraccone, L.; Trent, J. O.; Chaires, J. B. *J. Am. Chem. Soc.* **2008**, *130*, 16530–16532. Dash, J.; Shirude, P. S.; Hsu, S. T.; Balasubramanian, S. *J. Am. Chem. Soc.* **2008**, *130*, 15950–15956. (b) Rosenzweig, B. A.; Ross, N. T.; Tagore, D. M.; Jayawickramarajah, J.; Saraogi, I.; Hamilton, A. D. *J. Am. Chem. Soc.* **2009**, *131*, 5020–5021.

Photo-cross-linking between Pso-ODN-1 and human telomeric DNA was studied on a 5′-end FAM-labeled 40-mer DNA substrate containing a three-repeat telomere sequence (T-1 in Table 1). In the presence of 200 mM KCl, a solution of Pso-ODN-1 and T-1 was irradiated for 10 min at 365 nm and analyzed by polyacrylamide gel electrophoresis (PAGE) (Figure 3a). Without irradiation, no photoadduct appears (lane 1). With irradiation, a new band with slower mobility appears (lane 2). To further confirm psoralen-DNA cross-linking in terms of photoreversibility, we further irradiated the reaction solution of lane 2 at 254 nm. The new band at lane 2 disappears, and the original T-1 band reappears (lane 3). The photoreversibility suggests that the new band in lane 2 is the photoadduct between Pso-ODN-1 and T-1. To confirm whether photo-cross-linking is initiated by G-quadruplex formation, 5′-end FAM-labeled 40-mer DNA that does not contain telomere sequence (T-2) was prepared. Irradiation of the mixture of Pso-ODN-1 and T-2 in the presence of 200 mM KCl does not produce photoadduct at all (lane 4). To further verify that G-quadruplex formation induces the photo-cross-linking, more rigorous investigations were performed to examine the cross-linking activity by using mutated DNA substrates (small changes in T-1) and 6-mer oligonucleotide without ^{Br}G (Supporting Information, Figures S1 and S2). We prepared six kinds of control DNA substrates in which dG residues in the three-repeat telomere sequence of T-1 substrate were substituted with dTs. In ^{1}T-1, ^{2}T-1, and ^{3}T-1 substrates, three dG residues in different positions were substituted with dTs. In ^{4}T-1, ^{5}T-1, and ^{6}T-1, only one dG residue was substituted with dT. Irradiation of the mixture of Pso-ODN-1 and these substrates (^{1}T-1 through ^{6}T-1) in the presence of 200 mM KCl does not produce photoadduct at all (Figure S1). Moreover, the cross-linking reaction activity of 6-mer oligonucleotide with ^{Br}G (Pso-ODN-1) is higher than that of 6-mer oligonucleotide without ^{Br}G (Pso-ODN-1′) (Figure S2). These results strongly indicate that G-quadruplex formation directing the psoralen to telomere DNA is necessary for the photo-cross-linking.

We next examined the time course of photo-cross-linking (Figure 3b,c). For Pso-ODN-1 and telomeric T-1, the reaction saturates at only 10 min irradiation to give about 60% photoadduct (Figure 3c). For Pso-ODN-1 and nontelomeric T-2, the reaction does not produce photoadduct even at 30 min irradiation. These results indicate that photo-cross-linking by the small 6-mer oligonucleotide is quite selective and effective for telomere DNA, as well as controllable by switching the light on/off. PAGE analysis of the photo-cross-linking reaction indicated that the yield of photoadduct saturates at 2 equiv of Pso-ODN-1 per T-1 (Supporting Information, Figure S3).

Furthermore, we found that Pso-ODN-1 produces the photoadduct with the four-repeat-long telomere substrate (T-3) that has been known to form intramolecular G-quadruplex (Supporting Information, Figure S4). This suggests that Pso-ODN-1 can overcome the hindered secondary structure formed on DNA substrate targets to form photoadduct. We noted that the four-repeat telomere substrate had a lower activity compared with three telomeric repeats. It is known that human telomeric DNA can form an intramolecular G-quadruplex via a four-repeat telomere sequence. Such a secondary structure formed on DNA substrate may hinder the localization of probe to target DNA. Stabilization of the dimeric (3+1) G-quadruplex formed by Pso-ODN-1 and

Table 1. Oligonucleotides Used in This Study

name	sequence (5′−3′)[a]
Pso-ODN-1	Pso-TA^{Br}GGGT
Pso-ODN-2	Pso-TA^{Br}GGGT-Palm
Pso-ODN-3	Pso-TTTTTT-Palm
Pso-ODN-1′	Pso-TAGGGT
T-1	FAM-CAATTAGAATCAGGAATGGCATTAGGGTTAGGGTTAGGGT
T-2	FAM-CAATTAGAATCAGGAATGGCAGTGCAGATGTCGACCTAAG
T-3	FAM-CAATTAGAATCAGGAATGGCA(TTAGGG)$_4$
^{1}T-1	FAM-CAATTAGAATCAGGAATGGCATTATTTTTAGGGTTAGGGT
^{2}T-1	FAM-CAATTAGAATCAGGAATGGCATTAGGGTTATTTTTAGGGT
^{3}T-1	FAM-CAATTAGAATCAGGAATGGCATTAGGGTTAGGGTTATTTT
^{4}T-1	FAM-CAATTAGAATCAGGAATGGCATTAGGGTTATGGTTAGGGT
^{5}T-1	FAM-CAATTAGAATCAGGAATGGCATTAGGGTTAGTGTTAGGGT
^{6}T-1	FAM-CAATTAGAATCAGGAATGGCATTAGGGTTAGGTTTAGGGT

[a] Abbreviations: Pso, psoralen; FAM, fluorescein; Palm, palmitic acid.

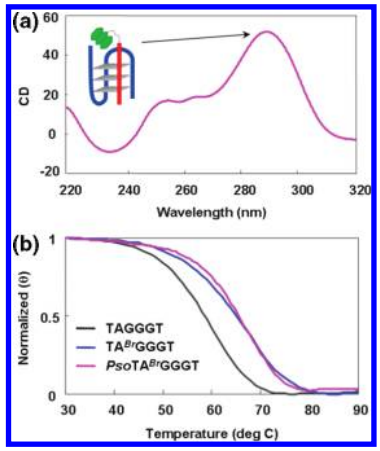

Figure 2. (a) CD spectrum of mixed Pso-ODN-1 d(Pso-TA^{Br}GGGT) (10 μM) and 16-mer telomere DNA d(GGGTTAGGGTTAGGGT) (10 μM) in the presence of 200 mM KCl at 20 °C. (b) CD melting profile of a mixture of 6-mer DNA (10 μM) and 16-mer telomere DNA d(GGGTTAGGGTTAGGGT) (10 μM) in the presence of 200 mM KCl, measured at 290 nm: black, TAGGGT, T_m = 59.3 °C; blue, TA^{Br}GGGT, T_m = 65.1 °C; pink, Pso-ODN-1, T_m = 65.4 °C.

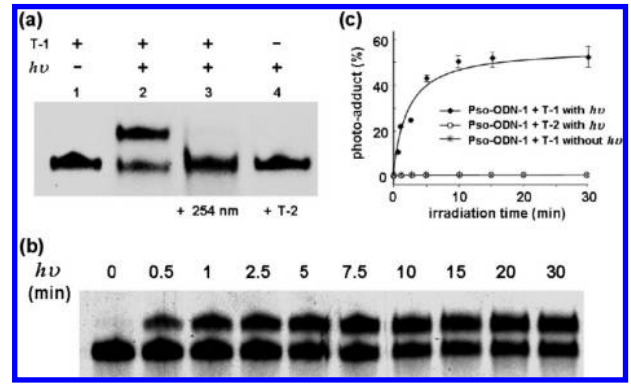

Figure 3. (a) Denaturing PAGE analysis of photo-cross-linking of human telomere DNA. Lane 1: 5′-end FAM-labeled 40-mer DNA containing a three-repeat telomere sequence at the 3′-end (T-1) with Pso-ODN-1, before irradiation at 365 nm. Lane 2: T-1 with Pso-ODN-1 irradiated for 10 min. Lane 3: further 254 nm irradiation for 10 min of the reaction solution of lane 2. Lane 4: 5′-end FAM-labeled 40-mer DNA (T-2) that does not contain telomere sequence with Pso-ODN-1 irradiated at 365 nm for 10 min. [target] = 1 μM, [Pso-ODN] = 2 μM, [HEPES pH 7.0] = 5 mM, [KCl] = 200 mM, 20 °C. (b) PAGE analysis of photo-cross-linking reaction of Pso-ODN-1 and T1 for different irradiation times: 0, 0.5, 1, 2.5, 5, 7.5, 10, 15, 20, and 30 min. (c) Time course of the photo-cross-linking reaction. Pso-ODN-1 and T-1 with irradiation (◆), Pso-ODN-1 and T-2 with irradiation (○), and Pso-ODN-1 and T-1 without irradiation (*). Experiments were performed in triplicate.

target sequence will increase the activity of the photo-cross-linking reaction. A further approach for stabilizing the dimeric G-quadruplex by incorporating a G-quadruplex-stabilizing molecule (such as TMPyP4) into the 3′ end of Pso-ODN-1 is currently under investigation in our laboratory.

It is noted that Pso-ODN-1 may form DNA/RNA hybrids with RNAs. Thus, we next examined the T_m of DNA/RNA formed by Pso-ODN-1 and a complementary RNA and whether photo-cross-linking occurred with such a RNA species. It was found that the DNA/RNA has a very low thermal stability (T_m < 20 °C, even lower than body temperature) and is not capable of cross-linking (Supporting Information, Figure S5). The result suggests that such oligonucleotide conjugates are less damaging in RNA molecules, avoiding unintended toxic/off-target effects for cellular application.

Inhibition of Telomerase Activity by the Small, Photocontrolled 6-mer Inhibitor. Once efficient photo-cross-linking was established, we tested whether Pso-ODN-1 inhibits telomerase activity in a modified telomeric repeat amplification protocol (TRAP) assay (Figure 4). This assay has been widely used to provide qualitative and quantitative estimates of telomerase activity as a means to identify telomerase inhibitors. Light irradiation for 10 min using Pso-ODN-1 at concentrations of 150, 300, and 600 nM leads to the disappearance of PCR products in gel (Figure 4), suggesting that the inhibition effect on telomerase activity increases with increasing concentration

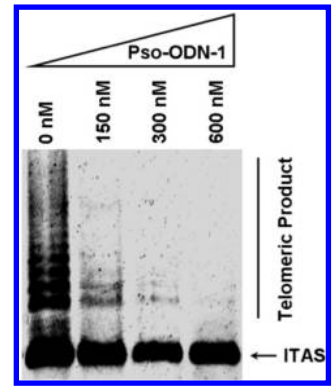

Figure 4. Telomerase inhibition by Pso-ODN-1. The gel shows the effect of increasing concentrations of Pso-ODN-1 on telomerase activity. With light irradiation for 10 min, the small oligonucleotide leads to the disappearance of PCR products. The position of the internal standard (ITAS) is indicated. [Pso-ODN-1] = 0, 150, 300, and 600 nM.

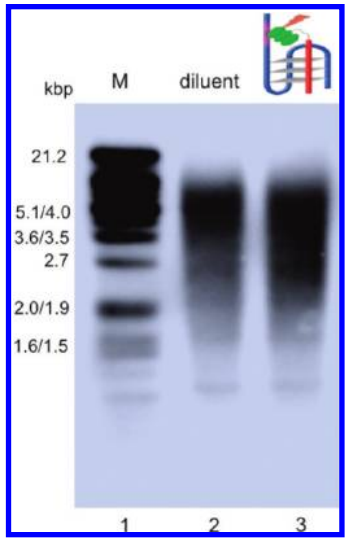

Figure 5. Effect on telomere length of treatment with Pso-ODN-2 in cancer cells. Hela cells were preincubated with Pso-ODN-2 (50 nM) for 12 h at 37 °C and then irradiated for 10 min at 365 nm. The resulting cells were incubated for 1 week. Diluent- and Pso-ODN-2-treated cells were collected for the analyses of TRF lengths. M is the marker lane.

of Pso-ODN-1. This assay clearly shows that Pso-ODN-1 is a potent inhibitor of telomerase, with an activity in the nanomolar scale.

Next, we further examined the effects of Pso-ODN-1 on telomere lengths in cancer cells. To enhance cellular uptake of Pso-ODN-1, a long alkyl chain, palmitic acid ($CH_3(CH_2)_{14}$-COOH), was bound at the 3′-end of Pso-ODN-2 by amide coupling (Supporting Information, Scheme S1).[7] We treated Hela cells with or without Pso-ODN-2 (50 nM) for 1 week. As shown in Figure 5, the faster migrating band in lane 3 (Pso-ODN-2-treated sample) extends lower in comparison to lane 2 (the diluent-treated sample). This result suggested that treatment with Pso-ODN-2 induces significant telomere shortening in cancer cells compared with the untreated control. The effective telomere shortening in cancer cell implies that the 6-mer oligonucleotide bearing psoralen causes efficient photo-cross-linking to human telomere DNA by G-quadruplex formation.

Having confirmed that Pso-ODN-2 effectively inhibits telomerase activity by G-quadruplex formation, we next evaluated its efficacy in living cells. If this psoralen-conjugated oligonucleotide can cause photo-cross-linking to telomere DNA upon irradiation, the resulting photoadduct may prevent telomerase from telomere elongation, leading to apoptosis in cancer cells.[1,7] Cancer cells (Hela cells) were preincubated with or without Pso-ODN-2 for 12 h at 37 °C and then irradiated for 10 min at 365 nm. The resulting cells were incubated and imaged at 0, 24, 48, and 72 h (Figure 6 and Supporting Information, Figure S6). With both Pso-ODN-2 and irradiation, significant morphologic changes were observed at 72 h (Figure 6a,e). In contrast, neither Pso-ODN-2 without irradiation nor the diluent with irradiation causes observable morphologic changes (Figure 6b,f; Figure 6d,h). To confirm that the effective changes with Pso-ODN-2 arise from photo-cross-linking in the G-quadruplex formed between Pso-ODN-2 and telomere target, oligonucleotide without telomere sequence (Pso-ODN-3) was prepared and used to treat cells. No significant morphologic changes are observed (Figure 6c,g). The live and dead cells at 72 h were stained and visualized with a nuclear counterstain to confirm the efficacy of the small oligonucleotide. Intense red fluorescence from the dead cells is evident in Figure 6i (as compared with Figure 6j−l), revealing that the photocontrolled oligonucleotides caused cell death. About 50% of the cells are confirmed to be dead (Supporting Information, Figure S7). These results strongly suggest that G-quadruplex formation and subsequent photo-cross-linking to telomere DNA causes inhibition of telomere elongation and triggers cell death.

To offer insight into whether the small, photocontrolled 6-mer inhibitor has specific effects on cancer cells, we treated normal human cells with Pso-ODN-2. Normal human dermal fibroblast, which had no telomerase activity, were preincubated with Pso-ODN-2 for 12 h at 37 °C and then irradiated for 10 min at 365 nm. The resulting cells were incubated and imaged at 72 h. We did not observe any significant morphologic changes (Figure 7). The cells at 72 h were stained and visualized with a nuclear counterstain. Only green fluorescence was observed (Figure 7). The results confirm that the small 6-mer inhibitor has no effect on normal human cells.

The effects of Pso-ODN-2 on normal and cancer cells were further investigated by the β-galactosidase staining (SA-β-Gal) assay. The senescence-associated β-galactosidase has been used as an indicator for cellular senescence.[8] Hela cells and normal human dermal fibroblast were treated with Pso-ODN-2, diluent, or Pso-ODN-3 as a control for 12 h and then irradiated for 10 min at 365 nm. The resulting cells were assessed for SA-β-Gal activity at 72 h. Pso-ODN-2-treated cancer cells increased in SA-β-Gal activity and exhibited senescent morphology (Figure 8a). In contrast, Pso-ODN-2-treated normal cells did not induce senescent morphology (Figure 8d), similar to diluent- or control Pso-ODN-3-treated cells (Figure 8c,f; Figure 8b,e). These results offer preliminary information about the ability of Pso-ODN-2 to alter telomere maintenance and cell growth in cancer but not in normal cells.

Next we used a clonogenic assay to reveal the effects of Pso-ODN-2 on normal and cancer cells.[8] This assay is frequently used to determine the effect of specific agents on the survival and proliferation of cells.[8] Hela cells and normal human dermal fibroblast were treated with Pso-ODN-2, diluent, or Pso-ODN-3 for 12 h and then irradiated for 10 min at 365 nm. Equal numbers of cells were seeded into culture dishes in triplicate and then incubated in complete medium for 72 h. The dishes were then stained with methylene blue (Figure 9). The clonogenic capacity of cancer cells pretreated with Pso-ODN-2 was suppressed ($14.0 \pm 2.9\%$ of diluent-treated control; $P < 0.01$) (Figure 9a) compared that of cancer cells pretreated with with Pso-ODN-3 or diluent (Figure 9b,c). In contrast, no significant changes are observed for normal human dermal fibroblast treated with Pso-ODN-2 (Figure 9d), similar to the cells treated with Pso-ODN-3 or diluent (Figure 9e,f). These results further suggest

(7) (a) Rezler, E. M.; Bearss, D. J.; Hurley, L. H. *Curr. Opin. Pharmacol.* **2002**, *2*, 415–423. (b) Shay, J. W.; Zou, Y.; Hiyama, E.; Wright, W. E. *Hum. Mol. Genet.* **2001**, *10*, 677–685. (c) Pendino, F.; Tarkanyi, I.; Dudognon, C.; Hillion, J.; Lanotte, M.; Aradi, J.; Segal-Bendirdjian, E. *Curr. Cancer Drug Targets* **2006**, *6*, 147–180. (d) Dikmen, Z. G.; Gellert, G. C.; Jackson, S.; Gryaznov, S.; Tressler, R.; Dogan, P.; Wright, W. E.; Shay, J. W. *Cancer Res.* **2005**, *65*, 7866–7873. (e) Pennarun, G.; Granotier, C.; Gauthier, L. R.; Gomez, D.; Hoffschir, F.; Mandine, E.; Riou, J.-F.; Mergny, J.-L.; Mailliet, P.; Boussin, F. D. *Oncogene* **2005**, *24*, 2917–2928.

(8) (a) Smogoorzewska, A.; de Lange, T. *EMBO J.* **2002**, *21*, 4338–4348. (b) Li, G. Z.; Eller, M. S.; Hanna, K.; Gilchrest, B. *Exp. Cell Res.* **2004**, *301*, 189–200. (c) Hochreiter, A. E.; Xiao, H. L.; Goldblatt, E. M.; Gryaznove, S. M.; Miller, S. B.; Slaedge, G. W.; Herbert, B. S. *Clin. Cancer Res.* **2006**, *12*, 3184–3192.

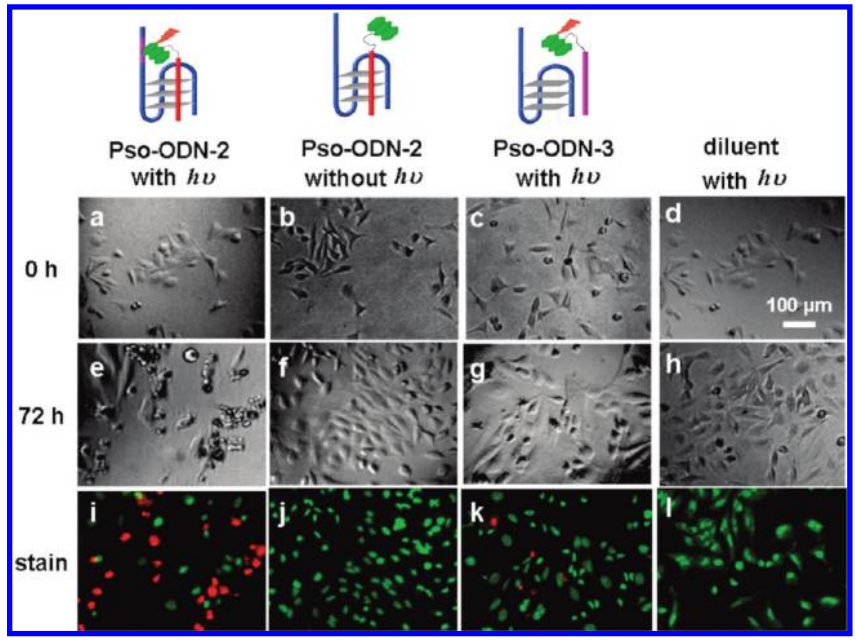

Figure 6. (a−h) Morphologic changes of Hela cells during the treatment of photocontrolled oligonucleotides for 72 h. Cells were preincubated with Pso-ODN-2 (50 nM), Pso-ODN-3 (50 nM), or diluent alone for 12 h at 37 °C and then irradiated for 10 min at 365 nm. The resulting cells were incubated and imaged at 0 and 72 h. (i−l) Dead and live cells at 72 h were stained with a nuclear counterstain and visualized as fluorescence. Dead cells were stained with PI and fluoresced red; live cells were stained with SYTO 9 and fluoresced green.

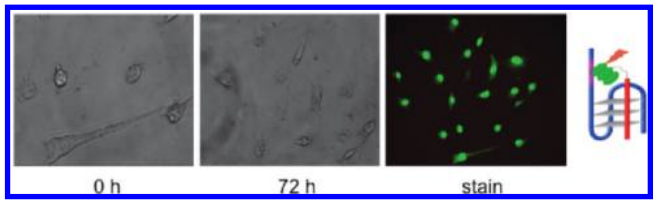

Figure 7. Morphologic changes of normal human dermal fibroblast during the treatment of photocontrolled oligonucleotides for 72 h. Cells were preincubated with Pso-ODN-2 (50 nM) for 12 h at 37 °C and then irradiated for 10 min at 365 nm. The resulting cells were incubated and imaged at 72 h. Dead and live cells at 72 h were stained with a nuclear counterstain and visualized as fluorescence. Dead cells were stained with PI and fluoresced red; live cells were stained with SYTO 9 and fluoresced green.

that Pso-ODN-2 induces an inhibition response in the survival and proliferation of cancer cells but has no effect on normal human cells.

In conclusion, we demonstrated that the formation of G-quadruplex between a small 6-mer oligonucleotide with a photo-cross-linking reagent and telomere target causes efficient photo-cross-linking to human telomere DNA upon light irradiation. The small, photocontrolled oligonucleotide shows effective telomerase inhibition, both *in vitro* and *in vivo*, only when light-irradiated. Doses of both psoralen oligonucleotide and light irradiation trigger death of cancer cells. Because the main effect on the target is controllable by switching the light on/off, the small, photocontrolled oligonucleotide can minimize side effects by irradiation of only cancer cells, which alleviates the main problem with present-day anticancer agents. Such a small molecule should have high cellular uptake and small, nonspecific binding with RNA in cells. *In vitro* and *in vivo* data suggest that this small, photocontrolled oligonucleotide is a promising agent for the treatment of cancer.

Experimental Section

The oligonucleotides prepared and used in this study are listed in Table 1(see Scheme S1 in the Supporting Information).

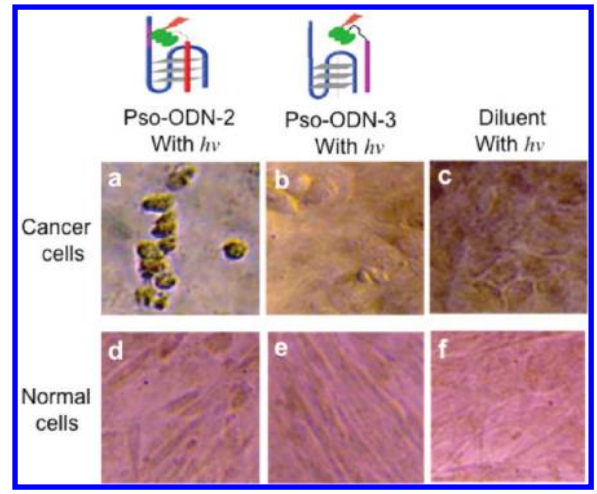

Figure 8. Pso-ODN-2-induced senescent morphology and SA-β-Gal expression in normal and cancer cells. Hela cells (a−c) and normal human dermal fibroblast (d−f) were preincubated with Pso-ODN-2, Pso-ODN-3, or diluent alone for 12 h at 37 °C and then irradiated for 10 min at 365 nm. The resulting cells were stained and assayed for SA-β-Gal activity at 72 h.

General. Nucleoside phosphoramidite, psoralen C2 phosphoramidite, and 8-Br-dG-CE phosphoramidite were purchased from Glen Research. Water was deionized by a Millipore water purification system and sterilized by an autoclave. UV irradiation at $300 < \lambda < 400$ nm was achieved with a UV spot light source (Hamamatsu Photonics, 200 W) and UV-D36C filter (Asahi Technoglass) at 0.1 W cm^{-2} min^{-1}.

Synthesis of Pso-ODN-2 and Pso-ODN-3. Oligonucleotides were prepared by the conventional phosphoamidite method on controlled pore glass (CPG) supports (1 μmol) using an Applied Biosystems 3400 DNA synthesizer. Psoralene-5′-TA^{Br}GGG-3′ or psoralene-5′-TTTTTT-3′ was synthesized on 1 μmol scale 3′-amino-modifier C7 CPG by the conventional solid-phase synthesis (see Scheme S1). The CPG was incubated with 20% piperidine for 20 min at room temperature, followed by washing with dimethylformamide (DMF). The resulting CPG was incubated with the mixture

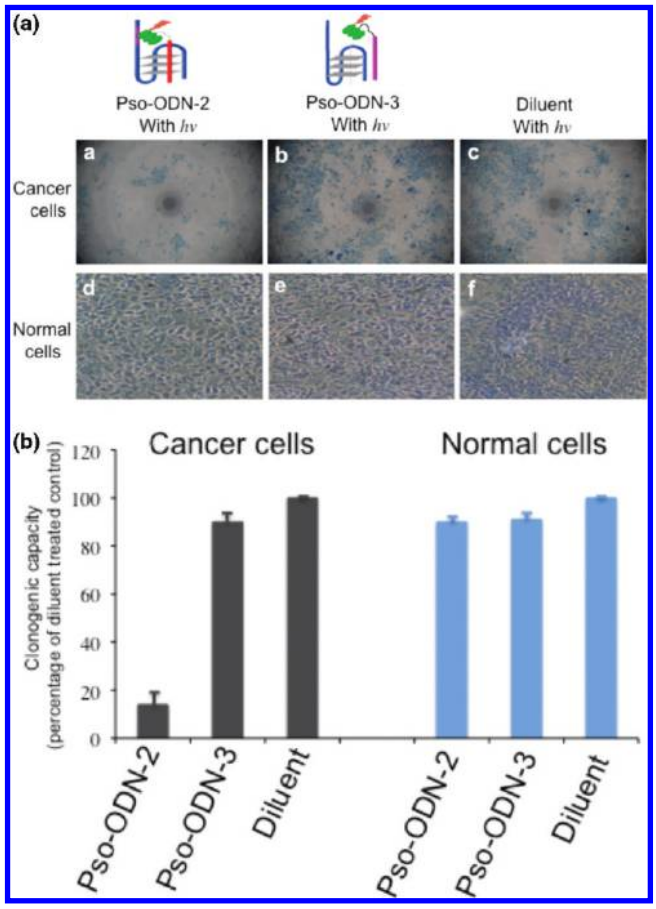

Figure 9. Effect of Pso-ODN-2 on clonogenic capacity of Hela cells and normal human dermal fibroblast. Cells were treated with Pso-ODN-2, Pso-ODN-3, or diluent for 12 h at 37 °C and then irradiated for 10 min at 365 nm. The resulting cells were incubated and assayed (clonogenic assay) at 72 h. (a) Appearance of stained dishes. Colonies of cells were stained as described in the Experimental Section. Methylene blue was used as an indicator to determine if a cell is alive or not. (b) Quantification of clonogenic capacity. Colonies of triplicate cultures shown above were counted and plotted as percentage of diluent-treated control.

of palmitic acid (51.3 mg, 200 μmol), DCC (41.3 mg, 200 μmol), and DMAP (24.4 mg, 200 μmol) in DMF (1 mL). After being stirred for 12 h at room temperature, the solution was removed, and the resulting CPG was washed with DMF, water, and methanol (three times each). After incubation of the CPG with methylamine (50%) in ammonia at 65 °C for 10 min, the resulting supernatant was collected and evaporated to dryness. The dried pellet was diluted with water (1 mL) and then purified with RP-HPLC to give the desired product. The concentrations of oligonucleotides were determined by Nanodrop. All oligonucleotides in this study were characterized by MALDI-TOF MS (*m*/*z*) as follows: Pso-ODN-1, 2289.9 (calcd 2289.4); T-1, 13056 (calcd 13056); T-2, 12939 (calcd 12939); Pso-ODN-2, 2430.5 (calcd 2430.6); Pso-ODN-3, 2571.5 (calcd 2572.7).

Reversed-Phase HPLC Purification. Purification of oligonucleotides was carried out by reversed-phase HPLC using a Merck LiChrospher RP-18e column. The following protocol was used: run time, 40 min; flow rate, 0.5 mL min^{-1}; binary system. Gradient (time in min (% buffer B)): 0 (0), 40 (100). Elution buffers: (A) 0.1 M ammonium formate; (B) 0.1 M ammonium formate with 50% acetonitrile. Elution of oligonucleotides was monitored by ultraviolet absorption at 270 nm.

CD Measurements and Analysis of CD Melting Profile. CD spectra were measured using a Jasco model J-725 CD spectrophotometer. The spectra were recorded using a 1 cm path-length cell. In CD melting studies, diluted samples were equilibrated at room temperature for several hours to obtain equilibrium spectra. The melting curves were obtained by monitoring a 290 nm CD band. Solutions for CD spectra were prepared as 0.3 mL samples at 10 μM strand concentration in the presence of 200 mM KCl and 5 mM HEPES buffer, pH 7.0.

UV Irradiation. After psoralen-conjugated oligonucleotide and target DNA formed the G-quadruplex, UVA irradiation of the solution was done at 0.1 W cm^{-2} min^{-1} at room temperature. The resulting mixture was dissolved in loading buffer and analyzed by 10% denaturing PAGE, followed by visualization with GelStar.

TRAP Assay. Telomerase activity in the presence of Pso-ODN-1 was evaluated by the stretch PCR method with Telo-Chaser system (Toyobo) according to the manufacturer's instruction. Briefly, Hela cells were collected by centrifugation at 1000*g* for 5 min at 4 °C and washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3). Cells were then treated with lysis solution to give a concentration of 2.5×10^3 cells/μg. The protein concentration of the cell extract was determined by using Bio-Rad protein assay dye reagent concentrate. Cell extracts containing 1 μg of total protein (~2500 cells) and Pso-ODN-1 (at final concentrations of 150, 300, and 600 nM) were added to 40 μL of the telomerase extension mixture and incubated at 37 °C for 10 min with UVA irradiation. Following the purification steps, 30 cycles of PCR were performed, consisting of denaturing at 95 °C for 30 s, annealing at 68 °C for 30 s, and extension at 72 °C for 45 s. The PCR products were eletrophoresed in a 10% native PAGE, followed by visualization with SYBR Green.

Cell Culture and Morphologic Analysis. Hela cells were seeded in a 96-well plate and incubated at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, 1.5 mL) containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Neonatal normal human dermal fibroblasts (NHDF-Ad) were purchased from Lonza Walkersville, Inc. Pso-ODN-2 or Pso-ODN-3 (10 μL, 1 μM) in PBS was mixed with cell medium (90 μL) in plate and preincubated for 12 h. Irradiation at 365 nm was then achieved for 10 min. The cells were imaged at 0, 24, 48, and 72 h. After incubation for 72 h, staining of nuclear DNA in dead and live cells was carried out using PI (Wako Pure Chemical Industries) and SYTO 9 (Molecular Probe). The cells were incubated with PI or SYTO 9 in PBS (0.5 μM) for 10 min at 37 °C. The excitation and the absorbance filter was 546/12 nm and 600/40 nm for PI, or 480/40 and 527/30 for SYTO 9. The viable and dead cells were counted using the Trypan blue dye exclusion method.

Telomere Length Assay. Genomic DNA was extracted from cell pellets (1×10^6) using DNeasy blood and tissue kit (QIAGEN) in the presence of RNaseA and proteinase K. Genomic DNA was digested with a two-enzyme mix (1 unit/μg each of *Hin*fI and *Rsa*I), and the digested DNA was separated on a 0.8% agarose gel in 0.5× TBE buffer. The terminal restriction fragment (TRF) gel was depurined for 5 min in 0.25 mol/L HCl, denatured for 30 min in 0.5 mol/L NaOH and 1.5 mol/L NaCl, neutralized for 30 min in 0.5 mol/L Tris-HCl and 1.5 mol/L NaCl, pH 7.5, rinsed with 20× SSC buffer (3 mol/L NaCl and 0.3 mol/L sodium citrate, pH 7.0) for 10 min, and transferred on Whatman 3MM paper with 20× SSC for 16 h at room temperature. The blot was cross-linked for 1 min with 254 nm UV, washed and dried, and then prehybridized in DIG Easy Hyb (Roche) at 42 °C for 30 min. The blot was probed with a DIG telomere probe (Roche) for 3 h at 42 °C in DIG Easy Hyb. The blot was washed with 2× SSC and 0.1% SDS for 10 min at room temperature, 0.2× SSC and 0.1% SDS for 30 min at 50 °C, and washing buffer for 2 min at room temperature. The blot was then blocked for 30 min with blocking buffer, treated with anti-DIG-AP in maleic acid buffer for 30 min, washed for 30 min with washing buffer, and equilibrated with detection buffer

(all buffers are produced by Roche). After CDP-Star (Roche) was added on the blot and incubated for 5 min, chemiluminescence was detected using a LAS-4000EPUVmini instrument (FUJIFILM).

***β*-Galactosidase Staining (SA-*β*-Gal) Assay.** Pso-ODN-2 or Pso-ODN-3 (20 μL, 1 μM) in PBS was mixed with cell medium (180 μL) in-plate and preincubated for 12 h. Irradiation at 365 nm was then done for 10 min. Cells were cultured in 5% CO_2 at 37 °C for 72 h. The cells were washed in PBS, fixed for 15 min (room temperature) in 4% formaldehyde in PBS, washed, and incubated at 37 °C with cell staining working solution from the β-galactosidase staining kit (Mirus). Staining was evident in 16 h.

Clonogenic Assay. Equal numbers of cells were seeded into 96-well plates in triplicate and treated with Pso-ODN-2 (50 nM), Pso-ODN-3 (50 nM), or diluent alone for 12 h. Irradiation at 365 nm was then achieved for 10 min. The cells were incubated in complete medium for 72 h. The cell colonies were stained for 10 min in 4% (w/v) methylene blue solution in PBS, washed once again with water, and then counted.

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Supporting Information Available: PAGE experiments (Figures S1−S4), T_m and UV irradiation (Figure S5), and cell treatment experiments (Figures S6 and S7); solid-phase synthesis of oligonucleotides (Scheme S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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