Telomerase Inhibition, Telomere Shortening, and Decreased Cell Proliferation by Cell Permeable 2′-*O*-Methoxyethyl Oligonucleotides

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Abstract: Telomerase is an attractive target for chemotherapy. Testing this hypothesis will require potent inhibitors with favorable pharmacokinetic properties. We report that 2′-methoxyethyl oligonucleotides complementary to the telomerase RNA component diffuse across cell membranes without the need for cationic carrier lipid, inhibit telomerase, and cause telomeres to shorten. The ability of antitelomerase oligomers to enter cells without the need to add lipid will simplify preclinical studies and may suggest advantages for clinical use.

Introduction. One oligonucleotide is an approved drug and three more are currently being tested in phase III clinical trials.[1] Toxicity in humans is low, in vivo stability is high, and the cost of scale-up synthesis has been reduced to approximately $200 per gram. These favorable properties suggest that it may be possible to develop oligonucleotides for many other targets.

One promising target is telomerase, a ribonucleoprotein that maintains telomere length.[2] Telomerase activity is expressed in tumor cells but not in most somatic cells, raising the attractive possibility that it could be a valuable target for drugs designed to treat many different types of cancer.[3]

Validating this hypothesis requires development of potent inhibitors. This is a challenging task. Telomeres in human tumors are hundreds to thousands of bases long and shorten at 50−100 bases per population, doubling in the absence of telomerase.[4,5] Therefore, it is expected that there will be a delay between initiation of treatment and observation of antiproliferative effects. This delay may not be an obstacle to clinical use for patients with life-threatening cancers who are accustomed to chronic therapies, but it does complicate drug development.

Inhibitors successfully tested to date include agents that interact with G-quadruplex structures, naphthalene derivatives, and oligonucleotides.[6−11] Oligonucleotides are well suited to be telomerase inhibitors because the template sequence of the RNA component of telomerase must bind the telomere to function. This sequence is therefore accessible to hybridization and functionally critical. These features have been exploited to develop potent telomerase inhibitors based on peptide nucleic acid, 2′-*O*-alkyl-substituted RNA, and phosphoramidate DNA.[12−16]

One widely noted obstacle to the development of oligonucleotide drugs is the inability of oligonucleotides to efficiently enter cultured cells in the absence of lipid carrier molecules.[17] Here, we report that 2′-methoxyethyl (MOE) RNA oligonucleotides can enter cultured cancer cells, cause telomeres to shorten, and reduce cell proliferation. The ability of anti-telomerase oligonucleotides to spontaneously enter cells and affect cell growth is further evidence that telomerase is an advantageous target for the development of oligonucleotide drugs.

Table 1. Oligonucleotide Sequences[a]

ISIS 24691	CAGUUAGGGUUAG
ISIS 125628	CAGUUAGAAUUAG
ISIS 113749	CUUCUCagttagggtUAGAC
ISIS 113750	CUUCUCagttgaattUAUAC
ZC-1	cagttagggttag
ISIS 263924	CAGGGAGTTTTAC
ISIS 263925	CATTGAGGGAGTT
ISIS 125625	CAGUUAGGGUUAG

[a] Bases are listed 5′ to 3′. Underlined bases containing phosphorothioate linkages. Uppercase bases are MOE, and lower case bases are DNA.

Hypothesis. Gryaznov and colleagues noted that a phosphoramidate oligonucleotide directed to the template sequence of human telomerase could be added to cells and inhibit telomerase.[16] This surprising result contradicted the common assumption that oligonucleotides cannot readily enter cells without the aid of cationic lipid.[17]

One explanation for their finding is that some feature of the anti-telomerase oligonucleotide renders it better able to penetrate cells and to bind to telomerase than is the case for traditional antisense oligomers that are designed to bind mRNA. An alternative explanation is that the oligomer adheres to the outside of the cell and binds telomerase after the cell is lysed for the telomere amplification repeat protocol (TRAP), the assay used to measure telomerase activity.[18]

To rule out the latter possibility and rigorously test the hypothesis that anti-telomerase oligomers can freely enter cells, it is necessary not only to examine inhibition of telomerase but also to examine phenotypes directly related to inhibition of cellular telomerase. Two such phenotypes are reduced telomere size and reduced cell proliferation.

Inhibition of Telomerase Activity by 2′-MOE Oligonucleotides. We chose to employ 2′-MOE oligonucleotides for our studies. 2′-MOE oligomers have been demonstrated to exhibit decreased immune stimulation, increased binding affinity, good pharmacokinetics, some oral bioavailability, and increased antisense efficacy.[19−22] 2′-MOE oligomers are currently being tested in one clinical trial and their scale-up synthesis has been optimized, important practical advantages for introduction of anti-telomerase therapy into the clinic.

We had previously shown that 13 and 12-base 2′-MOE oligonucleotides were potent inhibitors of telomerase when assayed in cell extract or delivered into cells using lipid but had not shown any effects on telomere length or cell proliferation.[14] We now directly added 13-base oligomer ISIS 24691 (Table 1) to DU145 (prostate cancer), LNCaP (prostate cancer), A549 (small cell lung cancer), and MCF-7 (breast cancer) cells. Cells were

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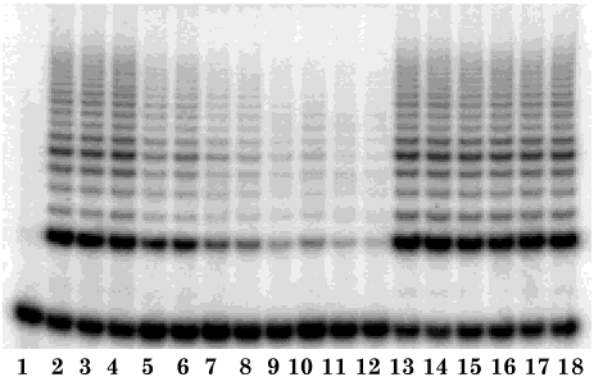

Figure 1. Effect on telomerase activity of direct addition of complementary ISIS 24691 or mismatch containing ISIS 125628 to DU145 cells: (lane 1) lysis buffer only; (lanes 2−4) no oligomer added; (lanes 5 and 6) 1.25 μM 24691; (lanes 7 and 8) 2.5 μM 24691; (lanes 9 and 10) 5 μM 24691; (lanes 11 and 12) 10 μM 24691; (lanes 13 and 14) 2.5 μM 125628; (lanes 15 and 16) 5 μM 125628; (lanes 17 and 18) 10 μM 125628.

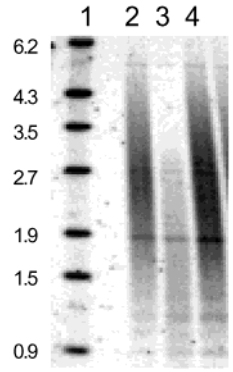

Figure 2. Effect of direct addition of ISIS 24691 on telomere length: (lane 1) molecular weight markers; (lane 2) untreated cells; (lane 3) cells treated with ISIS 24691; (lane 4) cells treated with mismatch-containing oligonucleotide 125628.

treated with oligomer for 72−96 h, carefully washed to remove surface-bound oligomer, lysed, and assayed by TRAP.

In all cell lines telomerase activity was inhibited by the fully complementary oligomer 24691 but not by the control ISIS 125628 (Figure 1). IC_{50} values for inhibition by 24691 in DU145, LNCaP, A549, and MCF-7 cells was 1, 0.3, 0.3, and 1 μM, respectively. For comparison, when using lipid, we normally use 100 nM oligomer to transfect DU145 cells.

ZC-1, a first-generation oligonucleotide containing phosphorothioate-modified DNA, did not inhibit telomerase upon direct addition to DU145 cells nor did ISIS 125625, a 2′-methoxyethyl RNA oligomer that was only partially substituted with PS linkages. These findings indicated that both non-DNA bases and PS linkages are necessary for uptake and inhibition. Scrambled control oligomers ISIS 263924 and ISIS 263924 that contain three consecutive guanines that are known to be critical determinants for inhibition by fully complementary oligomers also did not inhibit telomerase activity upon direct addition to DU145 cells.

For LNCaP cells, addition of lipid caused cells to detach. Therefore, the ability to directly add oligonucleotide and avoid using lipid allows experiments with this cell line that would have been difficult or impossible otherwise.

We also tested a 20-base chimeric 2′-MOE-DNA anti-telomerase oligonucleotide (Table 1) in A549 cells to test whether the unusually short (13-base) size of ISIS 24691 was responsible for uptake. The 20-base oligomer, whose design is typical of standard chimeric antisense oligomers, inhibited telomerase with an IC_{50} value of 0.25 μM, similar to that for ISIS 24691. This finding suggests that size does not play an essential role in spontaneous uptake.

Reduction of Telomere Length upon Direct Addition of ISIS 24691. One of the hallmarks of telomerase inhibition is the reduction of telomere length. We treated DU145 cells with ISIS 24691 and the analogous mismatch containing oligonucleotide for 56 days. Cells were harvested and genomic DNA was prepared for telomere length analysis using the telomere restriction fragment length (TRF) assay. The TRF assay is a modified Southern assay in which telomere repeats are probed with radioactively labeled oligonucleotide.[23]

DU145 cells were obtained directly from the American Type Culture Collection (ATCC) and possessed initial TRF values of 2.3 kB. TRF values are not equivalent to telomere length because the fragments include subtelomeric DNA. After 45 days, the measured TRF value of match-treated cells had been reduced to 1.7 kB (Figure 2). TRF values for cells that were not treated with any oligomer or with the mismatch-containing oligomer were not reduced.

The same amount of genomic DNA was added to all lanes. The signal in the TRF lane corresponding to match-treated cells is so faint because very little telomeric DNA remains for the probe to hybridize to. The TRF determination was repeated three times, with the same calculated result.

Reduction of Cell Proliferation upon Direct Addition of ISIS 24691. For chemotherapy, the goal of telomerase inhibition is reduced cell proliferation. As noted above, the fact that telomeres in cancer cells are hundreds or thousands of bases long suggests that telomerase inhibition will require weeks prior to an antiproliferative effect being observed.

As described above, we treated DU145 cells with ISIS 24691 and the analogous mismatch oligomer for 56 days. Cell numbers were periodically monitored. No immediate antiproliferative effects were observed, suggesting that direct addition of oligonucleotide is not toxic to cultured cells (Figure 3). After about 14 days, cell proliferation began to slow in cultures treated with match oligonucleotide 24691 but not in cultures treated with mismatch oligomer ISIS 125628 or in those that were untreated. After 8 weeks, cell growth was reduced 3-fold. These observations (no immediate effect on proliferation but a definite slowing over time) are consistent with our previous observations of cells transfected with lipid−oligonucleotide complexes.

Conclusions. We have demonstrated that addition of anti-telomerase oligonucleotides directly to cells causes telomerase inhibition, telomere shortening, and decreased cell proliferation. In four different cell lines, concentrations in the nanomolar to low micrimolar range are sufficient for telomerase inhibition.

Efficient and spontaneous uptake runs counter to prevailing dogma in the antisense field that lipid is required to achieve cellular effects. Our results also

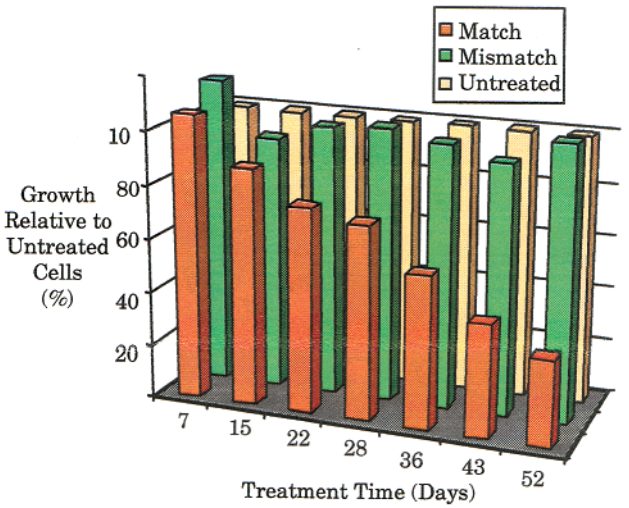

Figure 3. Decreased cell proliferation upon direct addition of ISIS 24691 compared to addition of mismatch containing ISIS 124628 and untreated cells.

directly contradict a previous study with anti-telomerase DNA oligonucleotides that contain phosphorothioate linkages, which reports that inhibition requires use of lipid.[24]

One explanation for our results is that 2′-MOE or phosphoramidate oligonucleotides that target the template region of telomerase are so potent that they do not require an optimized delivery system. Telomerase is not a typical antisense target because the RNA template is a part of an enzyme active site rather than a sequence with mRNA. It is highly accessible and functionally necessary, making it an ideal target for low concentrations of oligomer.

Spontaneous uptake of active anti-telomerase oligomers by cells has important implications for the development of antitelomerase therapeutics. These advantages may outweigh the disadvantage of requiring more oligonucleotide than would be needed for lipid-mediated transfection. Preclinical studies will be facilitated because toxicity due to lipid addition will be avoided and experimental protocols will be simplified. Cell lines, like LNCaP, that have interesting properties but that are difficult to transfect will become experimentally accessible. Another, more speculative advantage, is that similar improved intracellular efficacy may be observed in vivo. This is an exciting possibility, but it will need to be validated by careful animal studies.

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Supporting Information Available: Experimental information. This material is available free of charge via the Internet at http://pubs.acs.org.

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