A Novel Telomerase Template Antagonist (GRN163) as a Potential Anticancer Agent

Akira Asai, Yuko Oshima, Yoshihiro Yamamoto, Taka-aki Uochi, Hideaki Kusaka, Shiro Akinaga, Yoshinori Yamashita, Krzysztaa Pongracz, Ronald Puzan, Ellen Wunder, Mieczyslaw Piatsz, Shihong Li, Allison C. Chin, Calvin B. Harley, and Sergei Gryaznov


ABSTRACT

Telomerase, the enzyme responsible for proliferative immortality, is expressed in essentially all cancer cells, but not in most normal human cells. Thus, specific telomerase inhibition is potentially a universal anticancer therapy with few side effects. We designed N3′–PS thio-phosphoramide (NPS) oligonucleotides as telomerase template antagonists and found that their ability to form stable duplexes with the telomerase RNA subunit was the key factor for antitumor activity. In biochemical assays 11–13-mer NPS oligonucleotides demonstrated sequence- and dose-dependent inhibition of telomerase with IC_{50} values < 1 nM. Optimization of the sequence, length, and bioavailability resulted in the selection of a 13-mer NPS oligonucleotide, GRN163, as a drug development candidate. GRN163 inhibited telomerase in a cell-free assay at 45 ± 7 pM, and in various tumor cell lines at ~1 nM and approximately 0.3–1.0 μM in the presence and absence of carriers, respectively. GRN163 was competitive with telomeric primer binding, primarily because of hybridization to human telomerase RNA (hTR) component. Tumor cells treated with GRN163 in culture underwent telomere shortening, followed by cellular senescence or apoptosis after a period of time that generally correlated with initial telomere length. In a flank DU145 (prostate cancer) xenograft model, parenterally administered GRN163 caused suppression of tumor growth with no discernible gross toxicity. These data demonstrate that GRN163 has significant potential for additional development as an anticancer agent.

INTRODUCTION

Human telomerase is a ribonucleoprotein reverse transcriptase containing essential RNA (hTR) and protein (hTERT) subunits (1, 2). The enzyme is responsible primarily for maintaining the length of telomeres at chromosomal ends in immortal cells, using the 3′ overhang of the telomere as a primer for de novo synthesis of d(GTGGTAG)_{n} repeats. Protective and antiapoptotic roles of telomerase possibly involve mechanisms independent of telomere length have also been suggested recently (3, 4). There are two key differences in telomere biology between normal somatic cells and tumor cells. First, immortal tumor cells in culture and the vast majority of primary tumors express telomerase constitutively, whereas normal somatic cells and tissues either lack telomerase or express only low or transient levels in certain stem or committed progenitor cell compartments (5, 6). As expected, the germ-line cells in reproductive tissues, yet are more acid resistant and display a limited nonspecific charge. This article must therefore be hereby marked in accordance with July 13, 2017. © 2003 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on July 13, 2017. © 2003 American Association for Cancer Research.
TELOMERAZE INHIBITOR: GRN163

vivo efficacy data with a drug development candidate, GRN163. Our results show that GRN163 is an efficient and sequence-specific telomerase inhibitor with good intracellular bioavailability even in the absence of trans-membrane carriers, that tumor cells treated with GRN163 progress to apoptosis in a telomere length-dependent manner, and that tumor growth in vivo can be suppressed with parenterally delivered GRN163. We conclude that NPS oligonucleotides are attractive candidates for additional development as anticancer agents.

MATERIALS AND METHODS

Chemicals. NP and NPS oligonucleotides (listed in Table 1) were prepared on 1 µmol scale using ABI 394 synthesizers as described previously (29, 30), except that step-wise sulfurization during synthesis of NPS compounds was done with 0.1 M phenylacetyl disulfide in CH2CN/2.6-lutidine (9:1, v/v) for 5 min. Additionally, acetic anhydride was replaced by iso-butynic anhydride in the capping reagent. The 3'-aminonucleoside-containing 5'-phosphorimidates and CPG-based solid supports were purchased from Annovis Inc. (now Transgenic Inc). Oligonucleotide compounds were analyzed and purified, if needed, by ion exchange or reversed-phase high-performance liquid chromatography, desalted by gel filtration on NAP-10 columns (Pharmacia), and characterized by 31P NMR, mass spectrometry, and by PAGE. Purity of compounds was at least 80% as judged by PAGE. PS oligonucleotides were purchased from GENSET. CDDP was purchased from Sigma.

Cells. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO2. Human kidney carcinoma Caki-1 cells (JCRB) were cultured in McCoy’s 5A medium, 10% FBS, 1% penicillin/streptomycin solution (PS). Human epidermoid carcinoma A431 cells (JCRB) were cultured in DMEM, 10% FBS, each 1% PS. Human lung carcinoma A549 cells (American Type Culture Collection) were cultured in DMEM, 10% FBS, 1% PS. Human prostate carcinoma DU145 cells (American Type Culture Collection) were cultured in MEM, 10% FBS, 1% PS, and 1% sodium pyruvate. All of the media were purchased from Life Technologies, Inc.

Standard (PCR-based) Cell-free Telomerase Assay. Telomerase activity was measured in crude cellular extracts using TRAP (5) with a TRAPEZE XL Telomerase Detection kit (INTERGEN) or TRAPEZE XL Telomerase Detection kit (INTERGEN) and a Gene Amp PCR System 9700. TRAPEZE PCR products were resolved in 12.5% polyacrylamide gels. After electrophoresis, gels were stained with SYBR Green I and scanned by FluorImager SI (Molecular Dynamics). TRAPEZE XL PCR products were diluted twice with diethyl pyrocarbonate water and transferred to a 96-well black plate. Fluorescein (495 nm/516 nm) and Texas Red (600 nm/620 nm) were used for telomerase products and internal controls, respectively.

Direct (Non-PCR-based) Cell-free Telomerase Assay (*FlashPlate* Assay). To reduce background and increase sensitivity of detecting telomerase, we prepared stock solutions of affinity-purified human telomerase from hTERT-transduced 293 cells (31). Telomerase inhibition IC50 values for compounds were determined by adding various concentrations of test material to the telomerase extract and initiating reactions by addition of a 5'-end biotin-labeled telomerase substrate primer (5), dATP, dGTP, and TTP in reaction buffer. After incubation for 90 min at 37°C, reactions were stopped with SSC/EDTA solution [3X SSC, 10 mM EDTA final concentration, where 1X SSC is 150 mM NaCl and 15 mM sodium citrate (pH 7.0)]. Aliquots of reaction mixtures were transferred to 96-well Streptavidin FlashPlate Plus plates (Perkin-Elmer), and the 5'-biotinylated telomerase activity products were captured during 2-h incubation at room temperature. Wells were washed with 2X SSC, and a hybridization solution containing 4 µM 32P-labeled oligonucleotide probe complementary to 3.5 telomeric repeats was added to the wells and incubated for 4 h at room temperature. Unbound probe was removed, and the plate was washed with a solution of 2X SSC, 10 mM EDTA, and 0.1% SDS. The amount of probe annealed to the captured products was measured by scintillation counting in a TriLux Microbeta 96-well plate counter (Wallac). Telomerase activity in extracts preincubated with serial, semi-log dilutions of tested compounds was expressed as percentage of control (vehicle added) and plotted as a function of inhibitor concentration. IC50 values were calculated from curve-fitting equations.

Cell-based Telomerase Assay. Cells were seeded on 48-well plates (1.0 × 105/well) and preincubated for 24 h at 37°C in a humidified atmosphere of 5% CO2 before exposure to various dilutions of test compounds for 72 h. After washing with PBS, crude cell extracts were prepared with CHAPS lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 0.1 mM benzamide, 5 mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol] and total protein concentration determined with standard reagents. Using equivalent total protein amounts, telomerase activity in each extract was determined by TRAP as described above for the standard cell-free assay, the telomerase products quantified and normalized to the internal standard, and IC50 values calculated from curve-fitting equations.

Short-Term Cell Viability Assay. Cells were seeded on 96-well plates (1.0 × 105/well) and preincubated for 24 h at 37°C in a humidified atmosphere of 5% CO2 before exposure to different dilutions of test compounds for 72 h. After washing with PBS, 50 µl of XTT reagents (Cell Proliferation kit II; Roche Diagnostics) was added to each well, the cells were incubated for 3 h at 37°C, and absorbance (455 nm/650 nm) was measured to estimate cell viability.

Telomere Length Assay. Cells were seeded on six-well plates (1.0 × 105/well) in 2 ml of medium with various concentrations of test compounds. Cells were counted and 1.0 × 105 passaged every 72–96 h. Residual cells at each passage were stored at −80°C. At completion of each experiment, genomic DNA was extracted from cell pellets using DNeasy Tissue kit (Qiagen) and digested with restriction enzymes RsaI (5 units/µl µg DNA) and HinfI (5 units/µl µg DNA), followed by ethanol precipitation. The digested DNA fragments were separated on 0.7% agarose gel, transferred to a nylon membrane, and cross-linked by UV irradiation. The membrane was incubated with (C3,T3)2-FITC PNA, and the hybridized probe was detected with ECF signal amplification (Amersham Pharmacia Biotech), using a FluorImager SI (Molecular Dynamics). The mean TRF length was calculated as described previously (1).

FACS Analysis. Cells were seeded on 12-well plates (1.5 × 105/well) and preincubated for 24 h at 37°C in a humidified atmosphere of 5% CO2 before treatment with different dilutions of test compounds for 72 h. Cells were washed with PBS, trypsinized, collected, and then incubated in RNase (0.1% NP40 and 0.25 mg/ml RNase A) for 30 min at 37°C. PI solution (500 µg/ml PI and 1.0% NP40) was added and the cells were incubated for ~20 min at 4°C. Flow cytometry analysis was performed with FACSscan (Becton Dickinson).
from approximately 14–19 mg/kg/day (group 3). Tumor size was measured twice a week with calipers, and tumor volume was calculated using the following formula: tumor volume = [length × width × width]/2. The tumor xenograft in group 3 animal failed to grow after 1 week and, thus, was excluded from analysis, although the lack of growth could represent the effects of 7 days of treatment with GRN163. One animal in group 1 and 2 animals in group 3 died shortly after implantation of pumps at day 0 or day 28, apparently because of surgical complications, and were also excluded from analysis. Statistical analysis was done on all of the nonexcluded tumors by a t test comparison of individual growth rates (log tumor volume versus time) in the treated animals versus the control animals.

**TUNEL Assay.** Cells (3.0 × 10^6) were seeded into a 10-cm dish and preincubated for 24 h at 37°C in a humidified atmosphere of 5% CO2 before treatment with different dilutions of test compounds for 72 h. After washing with PBS, cells were trypsinized and counted. About 1–2 × 10^6 cells were suspended in PBS and fixed with 1% formaldehyde in PBS solution at 4°C, followed by incubation on ice for 15 min. After washing with ice-cold PBS, cells were labeled and stained with ApopTag Fluorescein Direct in situ Apoptosis Detection kit (INTERGEN). Flow cytometry analysis was performed with EPICS ELITE flow cytometer (Coulter Hialeah).

**Gel-Shift Analysis of Native Telomerase Complexes.** Purified telomerase (0.5 finol) was incubated with 32P end-labeled d(TTAGGG)_3 primer (2 nM), GRN163 or GRN137227, in the presence of a random 30-nucleotide long unlabeled (cold) oligonucleotide (200 nM). Half of the samples were treated with proteinase K (100 μg/ml; 0.2% SDS) for 10 min at 37°C before analysis on a native gel. Complexes were separated on a composite acrylamide (3.5%, 1.59% bis-acrylamide)-agarose (0.5%) gel containing 50 mM Tris and 50 mM glycine. The gel (14 cm in length × 1.5 cm) was run at 200 V at 4°C until the 1.5 cm) was run at 200 V at 4°C until the xylene cyanol dye had migrated 10 cm. The gel was dried and analyzed with a PhosphorImager (Molecular Dynamics).

**RESULTS AND DISCUSSION**

**NPS Oligonucleotides Are Potent and Sequence-specific Inhibitors of Telomerase.** The sugar-phosphate backbone structures of the PS, N3’→P5’ NP, and N3’→P5’ NPS oligonucleotides used in this study are shown in Fig. 1. All of the tested NP and NPS oligonucleotides contained a 3’-amino group, and PS counterparts contained a 3’-hydroxyl. We compared the effects on human telomerase of a few these isosequential oligonucleotides targeting the template region of hTR using the conventional PCR-based cell-free TRAP assay (Fig. 2). The oligonucleotide sequence 5’-TAGGGTTAGACAA-3’ is complementary to a 13 nucleotide-long region overlapping and extending four nucleotides beyond the 5’ boundary of the template region of hTR. The NPS (GRN163) and NP (GRN135930) versions of this sequence were 100–500-fold more potent than the isosequential PS (GRN139868) oligonucleotide in inhibiting telomerase in this assay (IC50 values of 0.14, 0.64, and 62 nM, respectively), perhaps because of improved base-pairing of the NP oligonucleotides compared with PSSs (27). The greater potency of the NPS over the NP compound may be attributable to stabilizing interactions between the oligonucleotide backbone soft nucleophile sulfur and hTERT amino acids adjacent to the template region of hTR. Mismatched (GRN137227) or sense-oriented (GRN138924) NPS oligonucleotides of the same nucleotide composition were inactive or only modestly active at the highest concentration tested in this study (100 nM; Fig. 2A).

PCR-based telomerase assays are not optimal for determining accurate IC50 values, because subtle effects of compounds on primer binding and Taq polymerase during the amplification process may dramatically alter apparent telomerase inhibitory activity. For this reason, we conducted a more thorough analysis of the NPS oligonucleotides using a relatively new non-PCR-based “Flashplate” assay (Ref. 32; also outlined in “Materials and Methods”), with a highly purified telomerase extract (Table 1). In these studies, representing many analyses on multiple preparations of different oligonucleotides, we found that the most potent 13-mer NPS oligonucleotides (GRN163 and GRN137940) inhibited telomerase by 50% at 40–50 pm (Table 1, Expts. 1 and 5). Slightly shorter NPS oligonucleotides (11- and 12-mers derived from GRN163 via elimination of one or two 3’-terminal nucleosides) also targeting the template region showed higher IC50 values, but were still in the sub-nanomolar range (Table 1, Expts. 6, 7, and 9). A single telomere repeat oligonucleotide NPS 6-mer was essentially inactive in the assays used (Table 1, Expt. 11).

The IC50 values for the mismatch and exact sense versions of GRN163 against purified telomerase were ~2,000- and 20,000-fold higher, respectively, than that for GRN163, indicating a high level of sequence specificity and minimal nonspecific inhibitory activity of the oligonucleotides with NPS backbone chemistry (Table 1, Expts. 1–3). This conclusion was supported by the 300-fold reduction in activity of the mismatch 11-mer (Table 1, Expts. 7 and 8), the lack of activity of NPS oligothymyidylate (Table 1, Expt. 10) toward telomerase, and the lack of activity of GRN163 at concentrations up to 30 μM toward DNA polymerase from Escherichia coli, T3 RNA polymerase, HIV-1 reverse transcriptase, and calf thymus topoisomerase I (data not shown).

Interestingly, the all-NPS duplex of GRN163 with its complementary strand was a relatively potent telomerase inhibitor (Table 1, Expt. 4), suggesting that binding of a telomere-like NPS duplex to the enzyme is able to block telomerase binding to its primer-substrate. Alternatively, dissociation of the duplex during the assay may lead to the release of GRN163, and consequently, the observed telomerase inhibitory effect. However, the low-nanomolar telomerase inhibition by the duplex was seen even when the complementary (A,C-rich) strand was added in excess (~2-fold) to ensure that minimal amount of single stranded GRN163 was available for binding (data not shown). Additional work is required to elucidate the precise mechanism of telomerase inhibition by this NPS duplex.

---

Fig. 1. General structure of oligonucleotides used in this study. A, PS; B, N3’→P5’ NP; C, N3’→P5’ NPS.
The NP version of GRN163, which lacks the soft nucleophilic sulfur atoms (or thio-phosphate groups) in the backbone, was ~15-fold less effective than GRN163 in inhibiting telomerase in the direct telomerase assay (Table 1, Expts. 1 and 12), although it showed a similar level of sequence specificity (>1500-fold for the same set of mismatches; Table 1, Expts. 12 and 13 versus Expts. 1 and 2). This suggests that the increased potency of NPS oligonucleotides by addition of sulfur to the backbone is not offset by any reduction in sequence specificity. Although somewhat less potent than GRN163, the NP version of GRN163 was ~100-fold more potent at inhibiting telomerase activity than the isosequential PS oligonucleotide (Fig. 2A). We selected GRN163 as the development candidate because of its high potency and specificity, as well as because the sulfur in the backbone substantially increased bioavailability (cellular uptake) of the oligonucleotide, as shown below.

**GRN163 Is a Potent Inhibitor of Telomerase in Tumor Cell Cultures.** The effect of GRN163 on telomerase activity in various tumor cells was studied initially in a short-term cell-based TRAP assay (Fig. 2, B and C; Table 2). When Caki-1 renal carcinoma cells were exposed to GRN163 (without uptake enhancers) at 3, 10, and 30 μM for 3 days, telomerase activity was barely detectable in the cellular extracts (Fig. 2B). The partial mismatch and complementary analogues of GRN163 were essentially inactive at the same concentrations (Fig. 2B). The inhibition of telomerase by GRN163 in cells, as opposed to carry over of the compound after cellular extraction, was confirmed by demonstrating that GRN163 blocked extension of an oligonucleotide telomeric substrate introduced into tumor cells (data not shown). Similar effects of GRN163 at these concentrations were seen in tumor cell lines A549, A431, and DU145 (Fig. 2C). Additionally, activity of GRN163 in several other cell lines was evaluated. The source of these tumor cell lines together with their initial telomere length (estimated by TRF length), and the IC₅₀ values for GRN163 with and without cellular uptake enhancers summarized in Table 2.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>TRF (kbp)</th>
<th>IC₅₀ (carrier)</th>
<th>IC₅₀ (+ carrier)</th>
<th>p53 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>HME50-SE/breast epithelium</td>
<td>4.5</td>
<td>0.5</td>
<td>0.0008²</td>
<td>wt</td>
</tr>
<tr>
<td>Caki-1/renal carcinoma</td>
<td>2.3</td>
<td>0.3</td>
<td>&lt;0.001; 0.01⁵</td>
<td>wt</td>
</tr>
<tr>
<td>DU145/prostate adenocarcinoma</td>
<td>2.8</td>
<td>0.5</td>
<td>n.d.⁵</td>
<td>mu</td>
</tr>
<tr>
<td>A431/epidermoid carcinoma</td>
<td>4.5</td>
<td>1.0</td>
<td>&lt;0.001⁶</td>
<td>mu</td>
</tr>
<tr>
<td>HT-3/cervix carcinoma</td>
<td>3.0</td>
<td>1.0</td>
<td>n.d.</td>
<td>wt</td>
</tr>
<tr>
<td>ACHN/renal adenocarcinoma</td>
<td>2.1</td>
<td>0.3</td>
<td>0.001⁶</td>
<td>wt</td>
</tr>
<tr>
<td>A549/ging carcinoma</td>
<td>6.5</td>
<td>0.5</td>
<td>n.d.</td>
<td>wt</td>
</tr>
<tr>
<td>SW620/colon adenocarcinoma</td>
<td>2.6</td>
<td>0.5</td>
<td>n.d.</td>
<td>mu</td>
</tr>
<tr>
<td>786-O/renal adenocarcinoma</td>
<td>3.4</td>
<td>0.3</td>
<td>n.d.</td>
<td>mu</td>
</tr>
<tr>
<td>K562/leukemia</td>
<td>7.3</td>
<td>1.0</td>
<td>n.d.</td>
<td>mu</td>
</tr>
</tbody>
</table>

* wt and mu correspond to wild-type and mutant p53, respectively.

² and ³ Carriers are Lipofectamine, Lipofectamine 2000, or FuGene6.

⁴ Carriers are: MPG peptide, where MPG is a “cage-forming” 27-amino acid-long peptide (Ref. 37).

⁵ n.d., not determined.

Table 2 Telomerase inhibition (IC₅₀ values) by NPS oligonucleotide GRN163 in tumor cell lines

---

Fig. 2. A, telomerase inhibition by NPS, NP, and PS oligonucleotides (sequences and GRN numbers are in Table 1) in a PCR-based cell-free assay. Compounds were incubated with CHAPS extract from A431 cells for 30 min. An aliquot of the mixture (50 ng of total protein) was subjected to TRAP analysis (5, 17). The amplified products were resolved by PAGE and stained with SYBR Green I. Lanes 1, 8, 12, 17, and 24, no inhibitor added. Lanes 16 and 31, no extract added. GRN163, GRN135930 (930), and GRN139868 (868) were added at 0.001, 0.01, 1, 10, and 100 nM for Lanes 2–7, 18–23, and 25–30, respectively. GRN139824 (924) and GRN137227 (227) were added at 1, 10, and 100 nM for Lanes 9–11 and 13–15, respectively. B, telomerase inhibition by NPS oligonucleotides in human Caki-1 tumor cells. Cells were treated with NPS oligonucleotides for 72 h. A CHAPS extract was prepared and equivalent amounts of protein (50 ng) were subjected to a PCR-based telomerase (TRAP) assay. In Lanes 1 and 15, no extract was added, and in Lanes 2 and 12, no inhibitor was added. GRN163, 924, and 227 were added to cells at 3, 10, and 30 μM to Lanes 3–5, 6–8, and 9–11, respectively. C, A549, A431, and DU145 human tumor cells were cultured and telomerase assays performed as in B. In Lanes 1 and 14 no extract was added. GRN163 was added to cells at 0, 3, 10 and 30 μM (Lanes 2–5, 6–9, and 10–13, respectively).
Effects of GRN163 on Short-Term Cell Viability. The effects of GRN163, and its partial mismatch (GRN137227) and complement (GRN138924) control analogues on viability of four tumor cell lines of differing telomere lengths were studied with a metabolic (XTT) assay performed after cells were continuously exposed to oligonucleotides for 3 days (Fig. 3, A–C). No uptake enhancers were used for any of the compounds tested in these experiments. Exposure to GRN163 induced a 50% decrease in viability (at day 3) of Caki-1 and DU145 cells at 20 nM and 20 nM, respectively, but had no effect on viability of A431 or A549 cells at any concentration up to 30 nM during this timeframe (Fig. 3A), although telomerase activity was essentially eliminated in all four of these cell lines by 10–30 nM of GRN163 (Fig. 2, B and C). The specific cytotoxicity of GRN163 observed in Caki-1 and DU145 cells is not dependent on functional p53, because DU145 express mutant p53 and A549 cells (which were not sensitive to GRN163 within the 3-day exposure of this experiment) express wild-type p53 (Table 2). It appears that death of Caki-1 and DU145 cells resulting from short-term exposure to GRN163 was because of critical telomere loss, as these cells have significantly shorter TRF lengths, whereas having minimal effect on average TRF length.

The obtained data suggest that the cells with critically short TRFs respond to GRN163 administration much faster than cells with longer telomeres. However, the sensitivity of these cancer cell lines to GRN163 may be associated with not only their telomere length, but potentially also with other yet unknown functions of telomerase in those cells.

Neither the partial mismatch nor the complement versions of GRN163 (GRN137227 and 138924) had noticeable effects on cell viability (10%) of any of the cell lines at any time point or concentration tested, again suggesting that the effect of GRN163 is sequence specific and that the NPS backbone chemistry is not acutely toxic to cells (Fig. 3, B and C). By comparison, the relatively nonspecific cis-platinum DNA cross-linking compound CDDP was toxic to all four of the tumor cell lines with an LD50 of 10–10 M (Fig. 3D), similar to its LD50 in normal cells (data not shown). The mechanism of cell death induced by GRN163 in Caki-1 cells was consistent with apoptosis, as determined by FACS analysis and TUNEL labeling (Fig. 4, A and B). The partial mismatch compound GRN137227 had no effect on FACS DNA content or TUNEL staining. In other experiments with HT-3 tumor cells, exposure to 10 M GRN163 for 3 weeks induced cell death and up-regulated caspase activity 5-fold compared with control or GRN137227-treated cells, which retained proliferative capacity (data not shown).

To additionally evaluate a correlation between telomere length and cellular senescence caused by GRN163, we used a kidney cancer derived cell line 786-O with telomeres of intermediate length (TRF 3.4 kb; Table 2). Exposure of these cells to GRN163 for 3 days resulted in a dose-dependent telomerase inhibition with an IC50 value

---

Fig. 3. Effect of NPS oligonucleotides on tumor cells survival during short-term (72 h) treatment. Cells were treated with different doses of oligonucleotides GRN163 (A), GRN138924 (B), GRN137227 (C), and the cis-platinum compound CDDP (D) on viability of Caki-1 (●), DU145 (▲), A431 (□) and A549 (○) cells, as determined by XTT assay (“Materials and Methods”).

---

Effects of GRN163 on Short-Term Cell Viability. The effects of GRN163, and its partial mismatch (GRN137227) and complement (GRN138924) control analogues on viability of four tumor cell lines of differing telomere lengths were studied with a metabolic (XTT) assay performed after cells were continuously exposed to oligonucleotides for 3 days (Fig. 3, A–C). No uptake enhancers were used for any of the compounds tested in these experiments. Exposure to GRN163 induced a 50% decrease in viability (at day 3) of Caki-1 and DU145 cells at 20 nM and 20 nM, respectively, but had no effect on viability of A431 or A549 cells at any concentration up to 30 nM during this timeframe (Fig. 3A), although telomerase activity was essentially eliminated in all four of these cell lines by 10–30 nM of GRN163 (Fig. 2, B and C). The specific cytotoxicity of GRN163 observed in Caki-1 and DU145 cells is not dependent on functional p53, because DU145 express mutant p53 and A549 cells (which were not sensitive to GRN163 within the 3-day exposure of this experiment) express wild-type p53 (Table 2). It appears that death of Caki-1 and DU145 cells resulting from short-term exposure to GRN163 was because of critical telomere loss, as these cells have significantly shorter TRF lengths, whereas having minimal effect on average TRF length.

The obtained data suggest that the cells with critically short TRFs respond to GRN163 administration much faster than cells with longer telomeres. However, the sensitivity of these cancer cell lines to GRN163 may be associated with not only their telomere length, but potentially also with other yet unknown functions of telomerase in those cells.

Neither the partial mismatch nor the complement versions of GRN163 (GRN137227 and 138924) had noticeable effects on cell viability (10%) of any of the cell lines at any time point or concentration tested, again suggesting that the effect of GRN163 is sequence specific and that the NPS backbone chemistry is not acutely toxic to cells (Fig. 3, B and C). By comparison, the relatively nonspecific cis-platinum DNA cross-linking compound CDDP was toxic to all four of the tumor cell lines with an LD50 of 10–10 M (Fig. 3D), similar to its LD50 in normal cells (data not shown). The mechanism of cell death induced by GRN163 in Caki-1 cells was consistent with apoptosis, as determined by FACS analysis and TUNEL labeling (Fig. 4, A and B). The partial mismatch compound GRN137227 had no effect on FACS DNA content or TUNEL staining. In other experiments with HT-3 tumor cells, exposure to 10 M GRN163 for 3 weeks induced cell death and up-regulated caspase activity 5-fold compared with control or GRN137227-treated cells, which retained proliferative capacity (data not shown).

To additionally evaluate a correlation between telomere length and cellular senescence caused by GRN163, we used a kidney cancer derived cell line 786-O with telomeres of intermediate length (TRF 3.4 kb; Table 2). Exposure of these cells to GRN163 for 3 days resulted in a dose-dependent telomerase inhibition with an IC50 value
were treated with 3 μM GRN163 or GRN137227 (227). Cells were fixed and stained with PI 72 h after compound treatment, after by FACS analysis as described in “Materials and Methods.” Lanes: 1, marker; 2, treatment without oligonucleotides; 3, treatment with 30 μM GRN163 for 24 days; 4, first treatment with 30 μM GRN163 for 75 days, then withdrawal of GRN163 and cell growth for additional 30 days; 5, treatment with 30 μM GRN227 for 105 days. Mean TRF lengths are as follows: Lanes 2, 7.1 kb; Lane 3, 2.8 kb; Lane 4, 5.9 kb; Lane 5, 8.3 kb.

Telomere Loss and Cell Death after Long-Term Exposure of A431 and K562 Cells to GRN163. A431 epidermoid tumor cells have an average TRF length of ~4.5 kb. Treatment with 30 μM GRN163 resulted in nearly complete inhibition of telomerase activity within 3 days (Fig. 2C) and a relatively rapid loss in the mean TRF length to 2.6 kb by day 24 (~80 bp/day or ~100 bp/doubling; Fig. 4C). No significant change in TRF length was seen with administration of the mismatch control oligonucleotide GRN137227 by day 24. However, despite a mean telomere length at day 24 similar to those of the Caki-1 and DU145 cells, which underwent rapid proliferative crisis within 3 days of GRN163 treatment, the A431 cell population survived for many additional weeks, albeit with a decline in growth rate past day 30 (data not shown). The rate of telomere loss in A431 cells after the first few weeks of GRN163 treatment was also reduced, as the mean TRF length fell to only 2.1 kb by day 64 (Fig. 4C), and cell crisis for the population as a whole only occurred after >5 months of exposure to GRN163 at 30 μM (Fig. 7; data from two parallel experiments are shown). At the time of cell crisis (day 162) telomeres were shortened to an average length of ~1.8 kb. PBS carrier rather than a mismatched oligonucleotide was used as a control for these experiments.

The change in growth rate and relatively slow rate of telomere loss after 20–30 days, together with the long time for complete proliferative crisis, was in marked contrast to the rapid effects seen with Caki-1 and DU145 cells, and may reflect shifts in the A431 cell population because of survival advantage of cells with longer telomeres, less telomerase inhibition, and/or inherently slower rates of telomere loss. The initial TRF length distribution for A431 cells ranged from 2 kb to ~8 kb and was still relatively broad at day 64 (Fig. 4C), but it is unknown how much this reflects differences in nontelomeric DNA content of individual TRFs, or telomere length variation within or between cells. Nevertheless, these factors may be relevant to the clinical application of telomerase inhibitors for some cancer patients, and point to the potential need in a subset of patients to maintain consistent exposure to effective concentrations of the inhibitor for several months to achieve effective killing of the entire tumor cell population. Alternatively, based on the data suggesting an antiapoptotic role of telomerase expression, it is possible that shorter durations of treatment may be effective if telomerase inhibition is combined with standard cytotoxic therapies (4, 23).

The potential need for long-term treatment is underscored by the observation that, for at least some tumor cell types in culture, withdrawal of a telomerase inhibitor results in regrowth of telomeres (24). We found that, in the long-term A431 cultures, telomere length recovered to pretreatment length if GRN163 was not included in the medium at each passage (data not shown). For K562 leukemia cells (initial TRF length of 7.3 kb), treatment with 30 μM of GRN163 for 105 days resulted in shortening of telomeres to an average TRF length of 2.8 kb, whereas no TRF shortening was seen with either mismatch control GRN137227 or buffer (Fig. 4D). However, treatment with GRN163 for 75 days followed by 30 days of growth without the drug resulted in the apparent recovery of TRF length to 5.9 kbp. These results again indicate the specific effect of GRN163 as a telomerase inhibitor and not as an agent having general irreversible cytotoxic effects. However, these findings also suggest that optimal treatment of some cancer patients with telomerase inhibitors as stand-alone therapy may require somewhat long-term treatment, if tumor cell death is achieved only through the mechanism of critical telomere loss. This approach would require a low general toxicity profile for the telomerase inhibitor to be used. Studies aimed at thorough evaluation of toxicity of GRN163 in vivo in several animal models are currently in progress, and the results will be reported in due course (preliminary data indicating very low toxicity of GRN163 in vivo in mice and rats).
were recently presented at a Special AACR Meeting on the Role of Telomerase and Telomeres in Cancer, December 7–11, 2002, San Francisco, CA). Alternatively, combinations of telomerase inhibitors and other anticancer compounds could, potentially, achieve tumor reduction more rapidly.

**GRN163 Forms a Stable Complex with Telomerase and Competitively Blocks Telomeric Primer Binding.** To investigate the mechanism of action of GRN163, highly purified cellular extract containing native telomerase (31) was preincubated with 5'-terminus 32P-labeled phosphodiester telomeric primer d(TTAGGG), the NPS oligonucleotides GRN163, or the mismatch control GRN137227. Products resulting from these interactions, as well as the complex formed by the NPS oligonucleotides with synthetically prepared hTR, were analyzed by electrophoresis on a polyacrylamide gel under nondenaturing conditions (Fig. 8). Portions of the reaction mixtures were also pretreated with proteinase K to remove the protein components from the potential complexes before electrophoresis. The gel-shift profiles demonstrate that GRN163 forms a very stable complex with native telomerase, primarily via duplex generation with the complementary site in the hTR template region. Treatment with proteinase K does not destroy the product of this oligonucleotide-RNA interaction, and only changes its molecular weight (Fig. 8, Lanes 2 and 6). The resultant deproteinized complex, which should contain only nucleic acid components, migrates in the gel similarly to the one formed by GRN163 with synthetic hTR molecule alone (Fig. 8, Lanes 6 and 9). In contrast, proteinase K treatment of complexes formed by telomerase with the d(TTAGGG) primer results in their abrogation (Fig. 8, Lanes 1 and 5). Mismatched oligonucleotide GRN137227 formed a significantly less stable complex with telomerase as well as with hTR alone (Fig. 8, Lanes 3 and 10). This correlates well with the melting temperature value (~20°C) for the duplex formed by the mismatch compound with the RNA strand under close-to-physiological buffer conditions (data not shown). These data indicate that whereas both hTERT and hTR interactions are likely involved in both phosphodiester d(TTAGGG), primer and GRN163 complexes with telomerase, the natural substrate primarily interacts with hTERT protein, whereas the NPS oligonucleotide GRN163 primarily interacts with hTR. Binding of the d(TTAGGG), primer and GRN163 to telomerase are mutually exclusive: either oligonucleotide added first blocks the other from binding (data not shown). The complex formed by the telomeric primer shows multiple size distributions (Fig. 8, Lane 1) consistent with the notion that the active or substrate-associated state of telomerase is in equilibrium between mono- and multimERIC forms (34).

**GRN163 forms a very stable complex with telomerase through tight association with hTR, and it is unlikely to activate hTR degradation in cells by RNase-H:** NPS oligonucleotides have the same C3'-endonucleoside sugar ring puckering as the parental NP oligonucleotides, and it is known that the latter do not activate RNase-H and, consequently, do not degrade their RNA target (35). We assessed the ability of GRN163 to activate RNase-H using a model duplex system comprising a 15-mer.

---

![Fig. 5. Effect of NPS oligonucleotides on telomerase activity and cell survival for 786-O (kidney cancer) cell line during long-term (20–30 days) exposure. Left, dose-dependent inhibition of telomerase (cell-based TRAP) by GRN163 (after 72 h of treatment) and lack of inhibition by mismatch control GRN137227 (227). Total cell protein production is shown as . Right, dose-dependent inhibition of 786-O cell proliferation (population doublings, PD) by prolonged treatment with 10 μM or 30 μM GRN163 and GRN137227. Results from two parallel experiments are shown.](image)

![Fig. 6. Effects of long-term treatment (4 weeks) of normal BJ fibroblasts, which lack telomerase activity, with 10 μM or 50 μM GRN163 in PBS; PBS alone was used as a control.](image)
natural phosphodiester RNA strand 5'-UUUGUCUAACCCUAA, which is isosequential to the hTR template region, and GRN163, (both strands at approximately 0.5–1 μM), in the presence of commercially available RNase-H. The RNA component of the duplex was not hydrolyzed by RNase-H, as was demonstrated by ion exchange high-performance liquid chromatography analysis (data not shown). In addition, in A431 cells treated with 30 μM of GRN163 for 3 days, no reduction in the level of hTR was observed by reverse transcription-PCR analysis, whereas telomerase activity was significantly inhibited, as was noted previously (Fig. 2C; data not shown). Thus, we conclude that based on the elucidated and described mechanism of action, GRN163 can be classified as a competitive inhibitor of telomeric DNA primer/substrate binding to the telomerase active site.

Antitumor Effects of Parenterally Administered GRN163 in Vivo. To evaluate whether GRN163 can suppress tumor growth in vivo, we analyzed the effects of this oligonucleotide using a DU145 human prostate carcinoma cells xenograft model. Earlier studies had shown that GRN163 could suppress tumor growth when injected intratumorally (data not shown). This, combined with the relatively good antitelomerase activity/bioavailability of GRN163 in cell cultures (even without lipid carriers) led us to test whether it could suppress growth of ectopic, flank DU145 tumors when administered parenterally. The results summarized in Fig. 9 show that GRN163 (formulated in water), systemically infused at the dose of ~10 or 20 mg/kg/day by an osmotic pump implanted in the i.p. cavity suppresses DU145 tumor growth over an 8-week period. Because of the small numbers of animals in each group, combined with some animals dying after pump implantation surgery, treatment groups were combined for statistical comparison to the control group: GRN163 decreased the growth rate of the tumors (0.020/day control versus 0.012/day treated; P < 0.058). Importantly, there was no significant difference, nor any trend toward a change, in overall growth of animals between the treatment groups and the control group, indicating that GRN163 had no gross toxicity to mice over an 8-week infusion period (data not shown).
SUMMARY

The sugar-phosphate backbone composition of NPS oligonucleotides confers a highly stable, sequence-dependent binding of these molecules to their RNA targets. The soft nucleophilic sulfur atoms in their backbone provide for additional protein stabilizing effects when the target complex is a ribonucleoprotein and also allow greater bioavailability. In vitro in the absence of uptake enhancers, in comparison with other classes of oligonucleotides. Thus, we were able to develop a relatively short 13-mer NPS oligonucleotide inhibitor of human telomerase, GRN163, which exhibited low-to-mid pM potency in cell-free systems, low- or sub-pM potency in tumor cells without uptake enhancers, and low-nM potency in tumor cells with uptake enhancers. GRN163 triggered tumor cell death by apoptosis in vitro in a telomere length-dependent but p53-independent manner, acting from within a few days (for certain cell lines with very short telomeres) to within a few months (in cells having longer and more heterogeneous telomerizes). In vivo experiments conducted with DU145 human prostatic tumor xenografts in nude mice showed that GRN163 (administered parenterally in water over 8 weeks) reduced the tumor growth rate. Additional in vivo studies with GRN163 in other human tumor xenografts are currently in progress. There was no apparent nonspecific toxicity of GRN163 or other NPS oligonucleotides observed in a variety of tumor cell lines or in several normal (telomerase-negative) cells tested in culture. However, longer-term studies with human cell types, including stem or progenitor cells with detectable telomerase activity, and animal studies are required to better understand the potential toxicity of this compound. Given the unique expression of telomerase primarily in immortal tumor cells, and the knowledge from telomerase activity, and animal studies are required to better understand the potential toxicity of this compound. Given the unique expression of telomerase primarily in immortal tumor cells, and the knowledge from telomerase activity, and animal studies are required to better understand the potential toxicity of this compound.

ACKNOWLEDGMENTS

We thank Miki Nomura, Ted Chu, and Queenie Lam for their contributions and help with cellular assays, David B. Karpf for critical input on the manuscript, and Melissa Fletcher for expert graphical assistance.

REFERENCES

A Novel Telomerase Template Antagonist (GRN163) as a Potential Anticancer Agent

Akira Asai, Yuko Oshima, Yoshihiro Yamamoto, et al.


Updated version
Access the most recent version of this article at:
[http://cancerres.aacrjournals.org/content/63/14/3931](http://cancerres.aacrjournals.org/content/63/14/3931)

Cited articles
This article cites 36 articles, 18 of which you can access for free at:
[http://cancerres.aacrjournals.org/content/63/14/3931.full#ref-list-1](http://cancerres.aacrjournals.org/content/63/14/3931.full#ref-list-1)

Citing articles
This article has been cited by 21 HighWire-hosted articles. Access the articles at:
[http://cancerres.aacrjournals.org/content/63/14/3931.full#related-urls](http://cancerres.aacrjournals.org/content/63/14/3931.full#related-urls)

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.