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Targeting Telomerase via Its Key RNA/DNA Heteroduplex

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Telomerase is a promising "universal" anticancer target. It has been demonstrated that inhibition of telomerase leads to mortalization and death of previously immortal cell lines. We are interested in targeting telomerase by binding to the RNA/DNA duplex that forms during its catalytic cycle. The RNA strand of this duplex is a component of telomerase and acts as a template to direct the synthesis of the single-stranded DNA telomere. We have hypothesized that molecules that bind to this duplex will inhibit the enzyme by either preventing strand dissociation or by sufficiently distorting the substrate, thereby causing a misalignment of key catalytic residues. To test this hypothesis we have examined the activity of telomerase in the presence of a range of intercalating molecules, known for their broad duplex binding properties. Of the nine compounds we examined, four show promising lead activity in the low micromolar range. A kinetic analysis of the telomeric products suggests that these compounds do not act by stabilizing G-quartets, thereby supporting the telomeric RNA/DNA heteroduplex as the site of action. We anticipate using these lead compounds as the basis for combinatorial variation to increase the affinity and specificity for the target telomerase.

Key Words: telomerase; enzyme inhibitors; intercalators; heteroduplex.

INTRODUCTION

Telomerase is a potential universal anticancer target (*1*,*2*). Its activity is found in a large majority of cancer cells and immortal cell lines, while being absent in most normal somatic cells (*3*). In addition to this correlation, there are mechanistic reasons why cancerous cell require telomerase activity. When normal cells divide, the single-stranded overhangs of chromosome ends (telomeres) get progressively shorter with each round of division, due to the mechanism of DNA replication (*4*). When the telomeres reach a critical length, the chromosome becomes unstable, which can result in cell senescence and death. The telomerase present in cancer cells, however, is able to add multiple lengths of the telomeric sequence (TTAGGG in humans) to the telomere and in so doing prevent the cell from reaching this critical stage.

The three major strategies extant in the literature for inhibiting telomerase target

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the nucleic acid substrate and template of telomerase. In the first strategy, pursued by Hurley and coworkers, molecules which are able to specifically target the G-quartet structure formed by the telomeric substrate have been demonstrated to inhibit the enzyme, presumably by sequestering the substrate in a folded and inaccessible form (*5*). In the second strategy, anti-sense oligonucleotides (such as PNAs as used by Corey and coworkers) target the RNA template portion of telomerase and block the substrate access to it (*6,7*). In the third strategy, Blackburn and coworkers have used chain-terminators such as ddGTP to prevent further extension of telomeres (*8*). These strategies have proven effective. They have demonstrated the feasibility of specifically inhibiting telomerase, and, in addition, have demonstrated *in vivo* the mortalization of previously immortal cell lines via telomerase inhibition (*9*).

We are interested in inhibiting telomerase via a fourth strategy: by binding to the RNA/DNA duplex that forms during the catalytic cycle of telomerase. This hetero-duplex is a central feature of the telomerase mechanism (*4*) (Fig. 1). It forms during the original annealing of the substrate telomere to the template RNA and during its extension. Cech and coworkers have proposed that the length of this duplex is between 4 and 11 base-pairs (from kinetic results using *Euplotes* telomerase) (*10*). We hypothesized that molecules which bind this duplex may inhibit telomerase by one of two mechanisms: either stabilizing the duplex and preventing strand dissociation (a key step in the catalytic cycle of telomerase) or by sufficiently distorting the substrate duplex, and thereby causing misalignment of key catalytic groups.

This RNA/DNA duplex is an appealing target for several reasons: RNA/DNA duplexes are relatively rare structures in cells, especially when compared to DNA/DNA duplexes (sources of cellular RNA/DNA duplexes include those formed during transcription and in Okazaki fragments). Second, this target RNA/DNA duplex has a specific sequence (TTAGGG, the telomeric sequence), which makes it even more rare. The structural uniqueness of RNA/DNA duplexes combined with the rarity of the specific target sequence suggests that the telomeric duplex will be a highly specific therapeutic target, and allow its differentiation from more ubiquitous DNA/DNA duplexes. Finally and perhaps most importantly, because the RNA/DNA duplex is bound tightly by the protein portion of telomerase, molecules which can bind to this duplex will be able to access the unique protein surfaces of telomerase. RNA/DNA duplex binding molecules should therefore be able to act as platforms on which to introduce functionalities that can recognize specific telomerase protein surfaces, and in so doing increase the affinity and specificity of the compounds for telomerase.

MATERIALS AND METHODS

Preparation of HeLa Cell Extract

Homogenates containing telomerase activity were prepared using a method based on that of Kim *et al.* (*3*). Briefly, HeLa S3 cells obtained from the National Cell Culture Center were suspended in cold washing buffer (10 mM Hepes-KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, pH 7.5) and pelleted at 10,000*g* for 1 min at 4°C. The pellet was resuspended in cold lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM BME, 1 mM DTT, 0.5% CHAPS, 10% glycerol) and lysed for 60 min on ice. The suspension was then centrifuged at 100,000

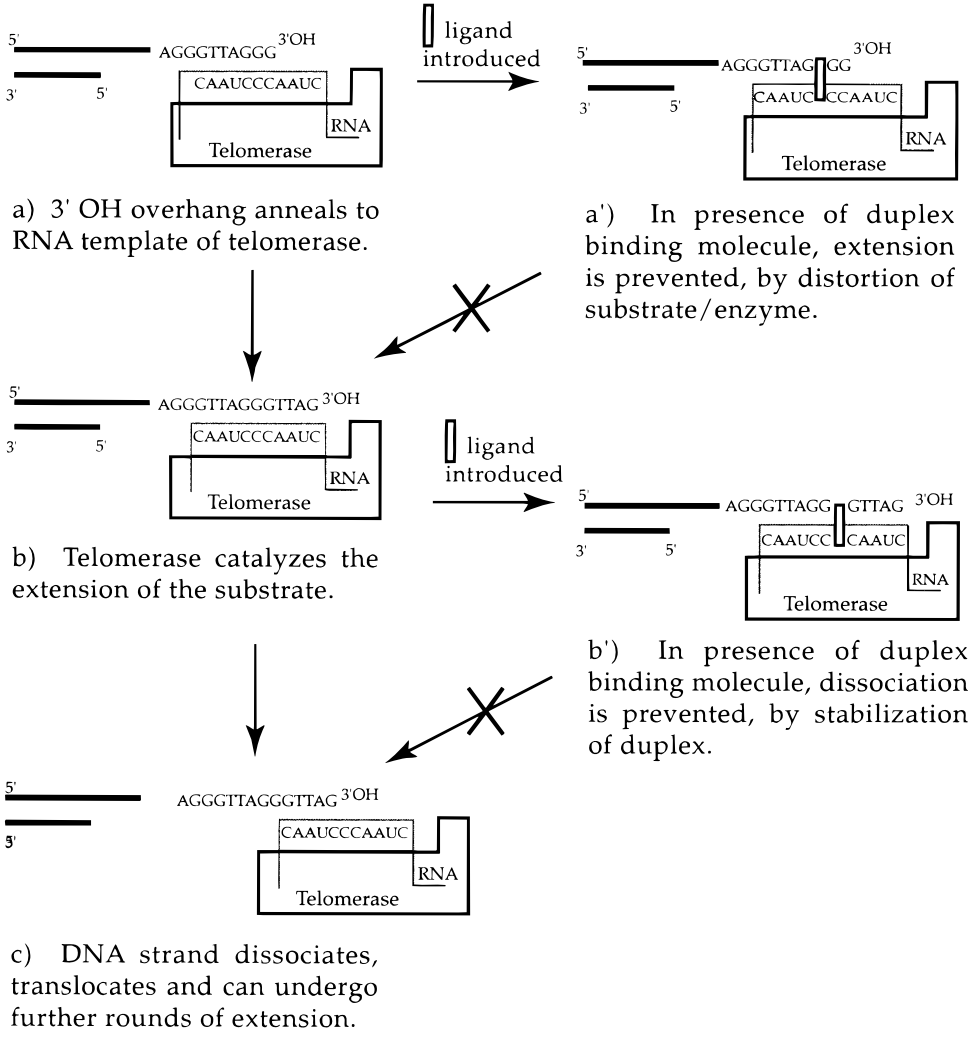


FIG. 1. Telomerase mechanism, showing possible mechanisms of action of heteroduplex binding inhibitors.

g for 1 h at 4°C, the supernatant removed and adjusted to 20% glycerol, aliquoted, and stored at −80°C.

Preparation of Duplex Binding Molecules

Duplex binding compounds were obtained from commercial sources. Approximately 1 mg of intercalator was dissolved in 500 μl H_2O), vortexed 20 min, and centrifuged for 20 min to remove particulate matter from solution. The supernatant was removed and the concentrations determined spectrophotometrically using extinction coefficients from the literature.

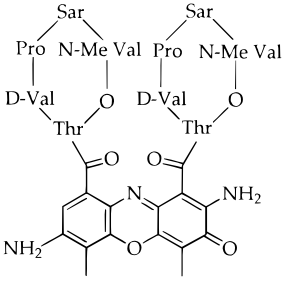
Assay Procedure

We have used the direct telomerase assay procedure of Sun *et al.* (*11*). All assays were performed in a 96-well teflon plate, as we have observed that intercalators adsorb to plastic surfaces, leading to differences in nominal and actual concentration values. Teflon plates greatly reduce this effect (data not shown). The assay mixture has a final makeup of 50 mM Tris-Oac, pH 7.2, 50 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM Spermidine, 1 mM dATP, 1 mM dTTP, 2.4 μM [α-^{32}P]dGTP, 1 μM biotinylated primer substrate oligo (5′biotinylated $(TTAGGG)_3$) 3.2 μl HeLa cell extract and the appropriate amount of test compound, in a final volume of 20 μl. This solution was incubated at 37°C for 1 h. After the reaction period, the mixture was transferred to eppendorf tubes and the reaction quenched by the addition of 20 μl prewashed magnetic dynabeads (Dynabeads M-280 Streptavidin in 10 mM Tris–HCl, 2 M KCl, pH 7.2). The reaction product was allowed to bind at room temperature, shaking for 30 min. To each tube was added 400 μl washing buffer (10 mM Tris–HCl, 1 M NaCl, pH 7.5), placed in a magnetic separator, and the liquid was removed. The beads were washed a total of 10 times by this method. After the washing step, 200 μl 5M guanidine HCl was added to the beads, heated at 90°C for 20 min, and the guanidine solution was removed. The oligo product was pelleted by the addition of tRNA and glycogen to the guanidine solution and adjusting to 75% EtOH, chilled for 30 min at −10°C, and centrifuged at 17,500*g* for 30 min. The supernatant was removed and the pellet allowed to air-dry. The pellet was dissolved in 2.5 μl fresh loading buffer (80% formamide, 1× TBE) and vortexed gently for 20 min. It was then denatured by heating at 90°C for 10 min and then cooled on ice. The sample was loaded onto a precast 8% polyacrylamide 7 M urea gel and electrophoresed for 45 min at 2000V. The gel was then exposed to a storage phosphor screen for 24–48 h and subsequently read on a phosphorimager.

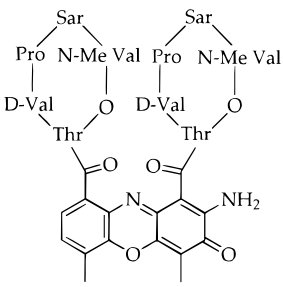
RESULTS AND DISCUSSION

To test the hypothesis that duplex binding molecules could inhibit telomerase, we assayed the enzyme in the presence of a range of intercalators, compounds with well established and broad affinity for a range of duplex structures (*12*). We selected nine compounds which represented a range of structural motifs: acridine based (e.g., rivanol), anthraquinone based (e.g., doxorubicin), phenanthridine based (e.g., ethidium bromide), peptide containing (e.g., actinomycin D) (Fig. 2). These compounds were initially assayed using a concentration of 50 μM. Compounds which reduced activity to $<$50% were subsequently assayed using a range of concentrations to determine IC_{50} values. Six concentrations were assayed, including a control point which contained no inhibitor.

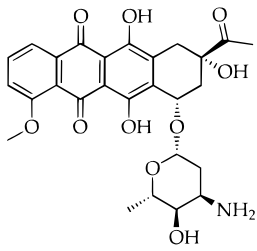
We utilized the magnetic bead assay of Sun *et al.* in which α ^{32}P-labeled dGTP is incorporated into a telomeric substrate (5′ biotinylated $(TTAGGG)_3$) (*11*). This addition is catalyzed by telomerase activity contained within HeLa cell homogenates. The product oligonucleotide containing incorporated ^{32}P was then isolated using streptavidin linked magnetic beads. The advantage of direct assays (such as that of Sun *et al.*) over PCR based telomerase assays is that there is no potential for interference by the assayed inhibitor with the DNA polymerase which is a key component of the PCR



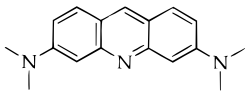
7-amino actinomycin D



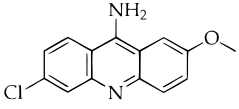
actinomycin D



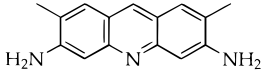
daunorubicin



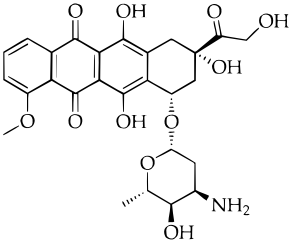
acridine orange



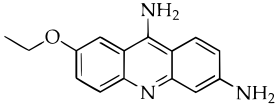
9-amino-6-chloro-2-methoxy acridine
(ACMA)



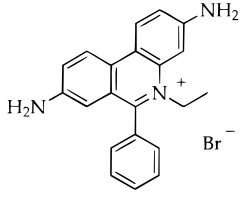
acridine yellow



doxorubicin



6,9-diamino-2-ethoxy acridine
(rivanol)



ethidium bromide

FIG. 2. Structures of compounds assayed.

reactions. Total telomerase activity was determined by quantifying the incorporated radioactivity in the whole lane. Sun *et al.* have shown that this whole lane signal is proportional to the amount of cell extract used (*11*).

The activity of telomerase in the presence of inhibitor was expressed as a proportion of the control activity, and therefore varied between 0 and 1. These proportions (p) were fit by nonlinear regression to the expression $p = 1/(1 + [I]/IC_{50})$, where $[I]$ was the experimental inhibitor concentration and IC_{50} was the fit parameter (concentration of inhibitor required to achieve 50% of uninhibited activity). Table 1 summarizes the data so determined. Standard error values and correlation coefficients are indicated. Four of the nine compounds showed promising lead activity in the low micromolar range. Ethidium bromide has an IC_{50} of 3.3 μM and rivanol has an IC_{50} of 8.2 μM.