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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

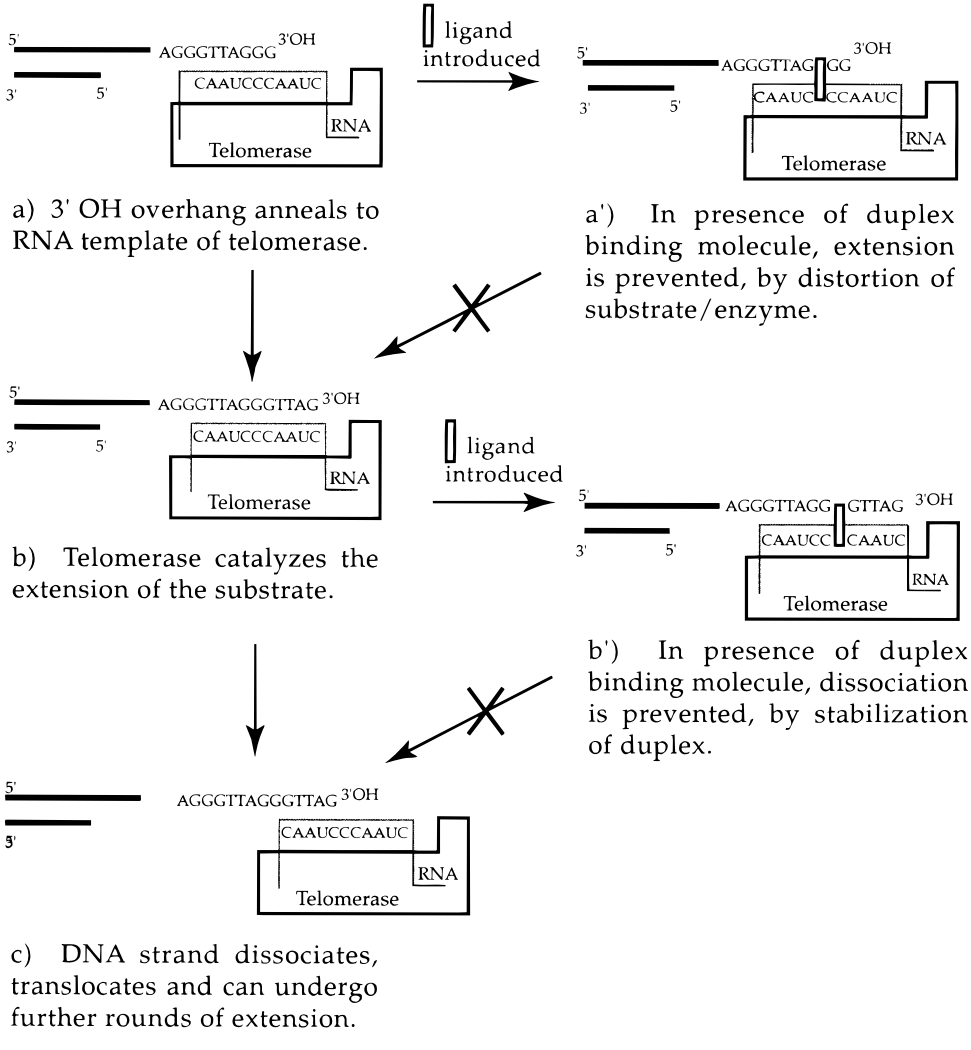


FIG. 1. Telomerase mechanism, showing possible mechanisms of action of heteroduplex binding inhibitors.

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.

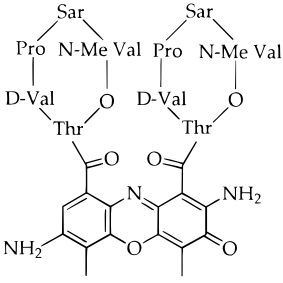
Assay Procedure

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.

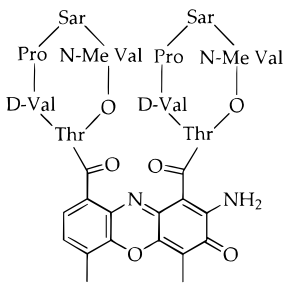
RESULTS AND DISCUSSION

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.

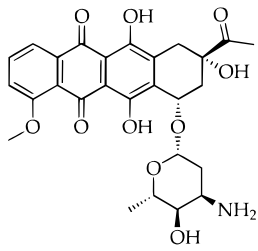
We utilized the magnetic bead assay of Sun *et al.* in which α ^{32}P-labeled dGTP is incorporated into a telomeric substrate (5′ biotinylated $(TTAGGG)_3$) (*11*). This addition is catalyzed by telomerase activity contained within HeLa cell homogenates. The product oligonucleotide containing incorporated ^{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



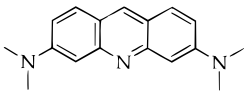
7-amino actinomycin D



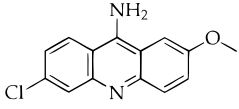
actinomycin D



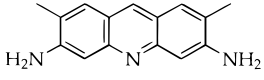
daunorubicin



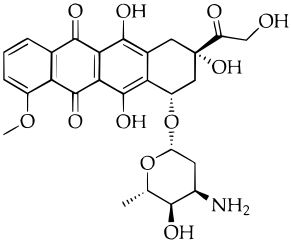
acridine orange



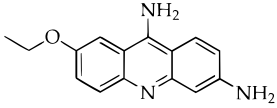
9-amino-6-chloro-2-methoxy acridine
(ACMA)



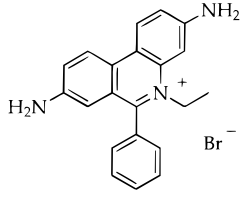
acridine yellow



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.

TABLE 1

Results of Telomerase Assay

Compound	IC_{50} (μM)	Standard error	*R*
Ethidium bromide	3.3	0.84	0.97
Rivanol	8.2	0.75	1.00
Acridine orange	12.2	1.4	0.99
Acridine yellow	21.7	6.5	0.95
7-Amino actinomycin D	[87]		
Actinomycin D	[99]		
ACMA	[>100]		
Doxorubicin	[>100]		
Daunorubicin	[>100]		

Note. IC_{50} values reported in micromolar (μM) units. Quantities given in brackets are estimates based on single point.

A typical gel for a telomerase inhibition analysis is shown in Fig. 3. The classical clustering of telomerase products with a six-base separation is readily apparent. The entire lane, which represents total telomerase extension products, can be quantitated as an indicator of total telomerase activity. In addition, the effect of the inhibitor on each round of telomeric extension can be assessed. In this case, quantitation of radioactive incorporation is made for each cluster individually. The results of this quantitation can then be plotted in the same manner as the whole lane results (Fig. 4 illustrates this using rivanol). It has been observed that compounds that inhibit telomerase by binding to the G-quartet inhibit at the cluster VI level (and higher) and not at the IV and V levels (*5*). In these cases the synthesis of clusters IV and V is unaffected by the inhibitors. This has been hypothesized as being due to the G-quartet binding molecule stabilizing the folded telomeric substrate, which can only happen after the cluster V product has been synthesized and released. This folded and stabilized substrate is then unable to bind to the template RNA effectively, which reduces synthesis of cluster VI. A detailed discussion of this mechanism may be found in the cited reference (*5*).

What is clear from our results is that there is no such cluster VI-specific inhibition. The IC_{50} values are similar at all clusters (see Table 2), suggesting that these compounds do not act by specific interaction with G-quartets. Even at the cluster IV level we observe inhibition. This is a level at which a full quartet has not yet been synthesized, supporting the model that these compounds do not act by binding to a substrate intramolecular G-quartet. This in turn is consistent with our hypothesis that these compounds act at the level of the RNA/DNA duplex of telomerase. What we observe is a small decrease in IC_{50} with each cluster (Fig. 4; Table 2). This is what may be expected from an inhibitor that is acting during each round of addition, such as an RNA/DNA binding molecule. Longer species have had to undergo multiple extensions, each of which may be inhibited by the duplex binding molecule. The inhibitory effect would be expected to be cumulative and indeed that is what is observed. All of the effective inhibitors we examined show this effect, although with ethidium this variation of IC_{50} with product cluster is very slight (Fig. 5; Table 3).

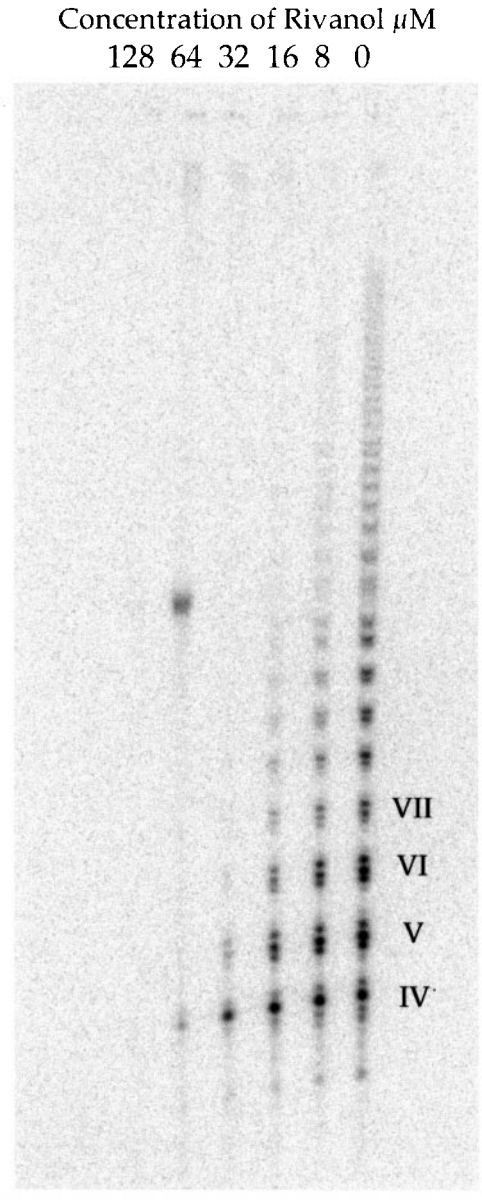


FIG. 3. Gel analysis of telomerase activity in presence of rivanol. 5′ Biotinylated three-repeat $(TTAGGG)_3$ substrate used.

We have shown that the examined inhibitors exert their action even at the cluster IV stage, a point at which a full G-quartet has not been synthesized. To further demonstrate that these compounds do not act by binding and stabilizing substrate G-quartets, we have examined the effect of rivanol on telomerase activity using a two

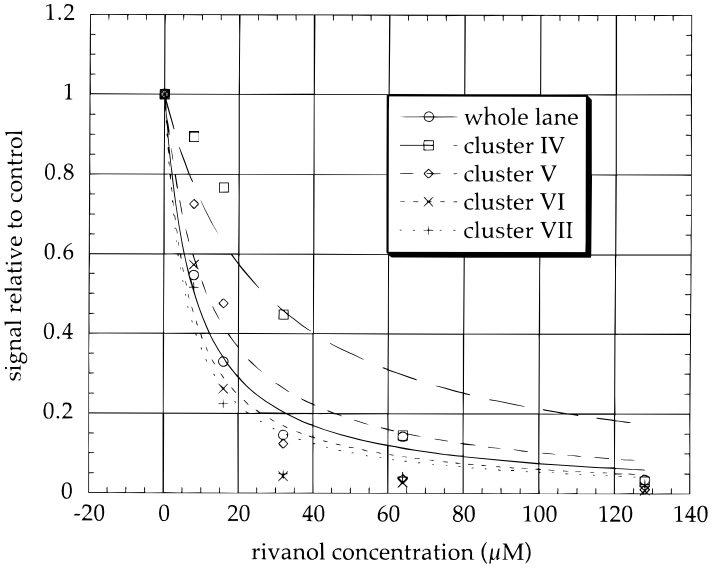


FIG. 4. Plot of telomerase activity in the presence of varying rivanol concentrations (whole lane reaction products and individual product clusters plotted).

repeat substrate (i.e., $(TTAGGG)_2$). The result of this experiment, which was performed in an identical manner to the previously described studies, is shown in Fig. 6. This shows that rivanol inhibits at all product clusters (whole lane $IC_{50} = 1.1\ \mu M$). The first cluster represents the extension of the 12-mer substrate to a 16-mer, or 2 1/2 repeats of the telomeric sequence. Again, the compound inhibits at a point where formation of an intra-molecular G-quartet is impossible. This is significant because the intramolecular G-quartet formed by the substrate is the experimentally observed target of G-quartet specific inhibitors of telomerase. It is clearly not possible that the compounds we have tested are acting at this site. It is of interest to note that the synthesis of telomeric products stalls after the synthesis of two product clusters, something that does not happen when the three-repeat substrate oligonucleotide is used.

Two possible sites of action of the compounds we have examined are the RNA/

TABLE 2

Telomerase Inhibition by Rivanol: Examination of Inhibition at Individual Clusters of Telomeric Products as Well as Whole Lane

Cluster quantitated	IC_{50} μM rivanol	Standard error	*R*
IV	27.0	7.3	0.95
V	11.5	3.0	0.96
VI	6.5	1.6	0.98
VII	5.7	1.1	0.98
Whole lane	8.2	0.75	1.00

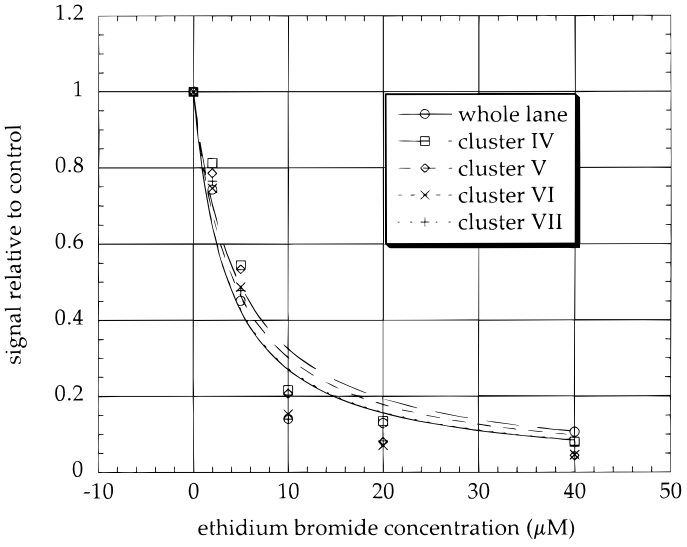


FIG. 5. Plot of telomerase activity in the presence of varying ethidium concentrations (whole lane reaction products and individual product clusters plotted).

DNA duplex that forms during telomerase's catalytic cycle, and the single-stranded RNA template strand of telomerase. While our data indicates that the mechanism of action is not mediated via a substrate G-quartet it does not differentiate between these other two possible mechanisms. Data by Ren and Chaires supports the concept that the action of these compounds is mediated by interaction with the RNA/DNA duplex (*13*). These authors examined the simultaneous competition of individual nucleic acid binding molecules with ten different nucleic acid species (which were isolated in individual dialysis chambers simultaneously in contact with a solution containing the nucleic acid binding molecule being examined). These nucleic acid species included single-stranded RNA, double-stranded RNA, double-stranded DNA, RNA/DNA duplex, and DNA quadruplex. Three of the compounds tested by Ren and Chaires were compounds that we tested against telomerase: ethidium, actinomycin D, and

TABLE 3

Telomerase Inhibition by Ethidium: Examination of Inhibition at Individual Clusters of Telomeric Products as Well as Whole Lane

Cluster quantitated	IC_{50} μM Et.Br	Standard error	*R*
IV	4.8	0.87	0.98
V	4.3	0.87	0.98
VI	3.7	0.75	0.98
VII	3.8	0.79	0.98
Whole lane	3.7	0.66	0.98

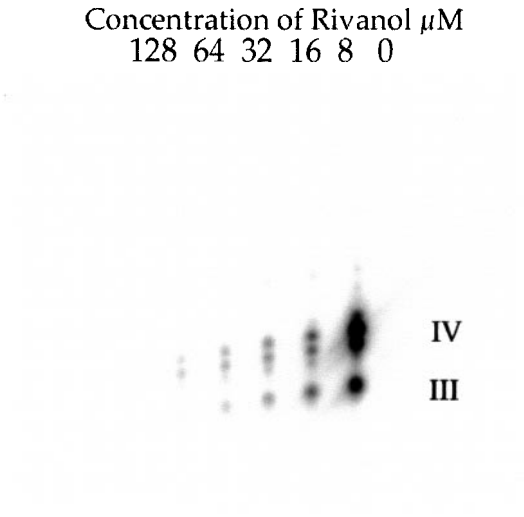


FIG. 6. Gel analysis of telomerase activity in presence of rivanol. 5′ Biotinylated two-repeat $(TTAGGG)_2$ substrate used.

daunomycin. Ren and Chaires observed that ethidium, our most potent telomerase inhibitor, when simultaneously presented with the 10 different nucleic acid species, bound with the greatest preference to RNA/DNA duplex and little or not at all to the quadraplex and single-stranded nucleic acid species. Furthermore, actinomycin D and daunomycin, two molecules that we demonstrated had little efficacy against telomerase showed little or no binding to RNA/DNA duplex and preferentially bound to DNA/DNA duplex. This strong correlation of our telomerase inhibition results with the ability of the compounds to preferentially bind RNA/DNA duplex further support this duplex as a possible site of action of the compounds we investigated.

CONCLUSIONS

We have demonstrated that simple duplex binding molecules are effective inhibitors of telomerase. This inhibition is not ubiquitous among the tested molecules but is influenced by the structural motif presented. Our results confirm that these compounds do not inhibit by G-quartet stabilization, and they presumably do not act as anti-sense or chain terminating inhibitors. A reasonable mechanism of action is that of binding to the key heteroduplex formed during telomerase's catalytic cycle. Other potential mechanisms include binding to the nontemplate duplex portions of the telomeric RNA. The RNA/DNA duplex as the target of these compounds is supported by the work of Ren and Chaires, which shows a strong correlation between affinity for RNA/DNA duplex of a given compound with our observed efficacy of the compound against telomerase.

While the compounds we tested are probably not specific enough to act as therapeutics, they are ideally positioned to be modified to become so. This is because the likely target of interaction, the telomerase RNA/DNA duplex or the telomerase RNA

template strand are both structures that are tightly associated with the protein portion of telomerase. Both the single-stranded template RNA and the RNA/DNA heteroduplex are tightly bound by the telomeric protein subunits. The significance of this is that molecules that are bound to either of these nucleic acid structures will be in close proximity to unique telomerase protein surfaces. These ligands can then act as platforms on which to introduce functionalities which can interact with these unique proximal protein surfaces. The aim of introducing these interactions is to increase both the affinity and specificity of interaction with telomerase. We should note that compounds that specifically bind to G-quartet folded substrates may not have an equivalent advantage in that it is not known that the G-quartet folded substrate is in a close and regular orientation relative to telomerase protein surfaces.

We are using the highest affinity lead compounds as the basis for combinatorial library design. This variation in structure will introduce elements into the lead molecules which may interact both with the target duplex as well as with the protein portions of the enzyme. The purpose of introducing these interactions is to increase the affinity and specificity of the compounds for telomerase over the more ubiquitous nucleic acid sites (e.g., duplex DNA) present in cells. In addition we are developing affinity methods based on the target heteroduplex that allow the identification of high affinity molecules from a combinatorially derived mixture. The combination of combinatorial variation and affinity selection should allow the identification of high affinity, high specificity inhibitors of the important anticancer target telomerase.

ACKNOWLEDGMENTS

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