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Nucleic acid-binding ligands identify new mechanisms to inhibit telomerase

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Abstract—We screened a small library of known nucleic acid-binding ligands in order to identify novel inhibitors of recombinant human telomerase. Inhibitory compounds were classified into two groups: Group I inhibitors had a notably greater effect when added prior to telomerase assemblage and Group II inhibitors displayed comparable inhibition when added before or after telomerase assemblage. Hoechst 33258, a Group I inhibitor, was found to interact tightly ($K_D = 0.36\,\mu M$) with human telomerase RNA (hTR) leading us to propose that hTR is the molecular target for this and other Group I inhibitors. Our results suggest that hTR can be exploited as a small-molecule drug target and provide several new structural motifs for the further development of novel telomerase inhibitors.

Human chromosomes terminate in DNA/protein complexes called telomeres, which serve to protect the chromosome ends.[1] Human telomeric DNA is composed of 5–15 kb of the repeated sequence 5′ TTAGGG/AATCCC followed by a 100–150 nucleotide 3′ overhang. Because of the end-replication problem, the normal DNA replication machinery does not efficiently reproduce telomeric DNA. Instead, the G-rich strand of telomeric DNA is synthesized by the specialized ribonucleoprotein complex telomerase, which utilizes a portion of its integral RNA subunit as the template.[2] Telomeric DNA is lost at a rate of 50–100 bp per cell cycle in cells with limited telomerase activity, and the erosion of telomeric DNA leads to either senescence or apoptosis. Highly proliferative cells require high levels of telomerase activity to offset this loss of telomeric DNA. It follows that a majority of all cancer types have increased telomerase activity compared to normal tissues.[3] Importantly, inhibiting telomerase activity in cancer cells causes telomere shortening and cessation of cell growth.[4] Thus, telomerase is a novel anticancer drug target and telomerase inhibitors can potentially affect the majority of all cancers.

In devising a new approach toward telomerase inhibition, we considered the available targets and the past approaches taken to mediate telomerase activity. Telomerase is a multisubunit ribonucleoprotein complex, but the minimal requirements to establish telomerase activity in vitro include only the catalytic protein subunit, hTERT (human telomere reverse transcriptase) and the RNA subunit, hTR (human telomerase RNA), which contains the template for reverse transcription.[5,6] Several approaches to inhibit telomerase have been investigated and can be broken down into four major categories: G-quadruplex stabilizers,[7] antisense oligonucleotides,[8] reverse transcriptase inhibitors,[9] and non-reverse transcriptase inhibitors.[10,11] The most effective of these to date are phosphoramidate, locked nucleic acid (LNA), and 2′-methoxyethyl oligonucleotides that are complementary to the template portion of hTR.[8] Given the current drawbacks of oligonucleotide-based therapeutics, small-molecule telomerase inhibitors continue to be an area of great interest. In order to explore novel mechanisms for the inhibition of telomerase, we have developed a parallel screen to identify molecules that inhibit telomerase by affecting proper assemblage of the holoenzyme complex.[12] Here, we describe the use of this

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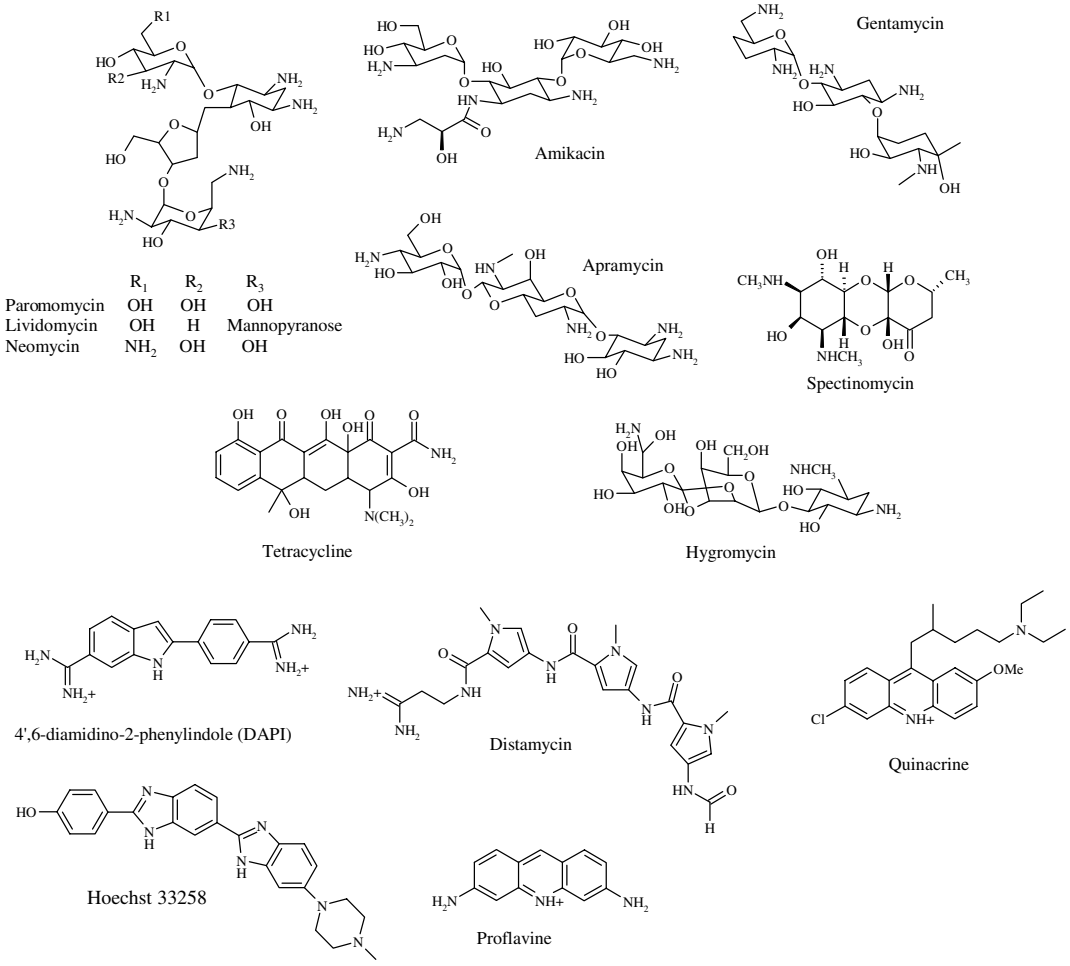

Figure 1. Compounds tested as telomerase inhibitors.

screen to sieve through a small library of nucleic acid-binding ligands. Our main objective in this study was to identify small molecules that inhibit telomerase by interacting with hTR, leading to a dysfunctional telomerase complex.

Our initial effort to determine if hTR can be a small-molecule drug target was based on the premise that nucleic acid-binding ligands can interact with hTR either before or after telomerase assemblage, resulting in a nonfunctional telomerase complex. To test this proposal, we screened a small library of known nucleic acid-binding ligands as inhibitors of recombinant telomerase. Telomerase activity was reconstituted by adding in vitro transcribed hTR to hTERT that was translated in vitro using rabbit reticulocyte lysates.[12] Potential inhibitors, including several aminoglycosides, DNA intercalators including the known telomerase inhibitor proflavine as a control, and DNA minor groove binders (Fig. 1) were added either before assemblage of the telomerase complex (Fig. 2) or after assemblage (Fig. 3). Compounds that were inhibitory were divided into two groups: compounds in Group I inhibited telomerase to a greater extent (>2-fold difference) when added prior to assemblage whereas those in Group II inhibited to a similar extent (<2-fold difference) irrespective of the order of

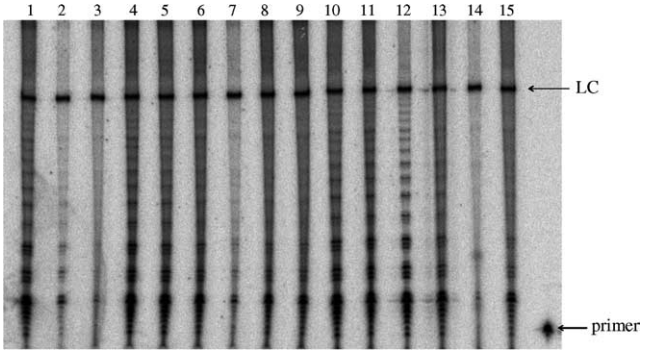

Figure 2. Inhibition of telomerase by nucleic-acid ligands added before telomerase assemblage. 50 μM of a designated inhibitor was added to recombinant hTR and hTERT followed by a 90 min incubation in rabbit reticulocyte lysate to allow assembly. After assemblage, telomerase activity was assayed by following the extension of primer 5′-TTAGGGTTAGGGTTAGGG with 0.5 mM dATP, 0.5 mM dTTP, 2.9 μM dGTP, and 0.33 μM [α-^{32}P]-dGTP (3000 Ci/mmol, 10 μCi/μL). Lane 1, no inhibitor; 2, quinacrine; 3, Hoechst 33258; 4, spectinomycin; 5, amikacin; 6, lividomycin; 7, diamidino-2-phenylindole; 8, gentamycin; 9, neomycin; 10, hygromycin; 11, tetracycline; 12, distamycin A; 13, apramycin; 14, proflavine; 15, paromomycin. LC is a loading control and ^{32}P-labeled primer was loaded in the last lane to mark the starting point for primer extension.

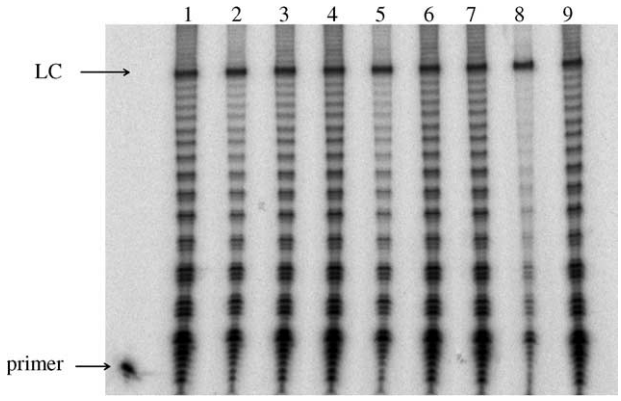

Figure 3. Inhibition of assembled telomerase by nucleic acid-binding ligands. 50 μM of designated inhibitor was added to recombinant telomerase preassembled in rabbit reticulocyte lysate. Telomerase was assayed as described in Figure 2. Lane 1, no inhibitor; 2, quinacrine; 3, Hoechst 33258; 4, lividomycin; 5, diamidino-2-phenylindole; 6, gentamycin; 7, neomycin; 8, proflavine; 9, paromomycin. LC is a loading control and ^{32}P-labeled primer was loaded in a lane to mark the initiation of primer extension.

addition. To determine if these compounds were specific inhibitors of human telomerase, we examined their ability to affect telomerase from two other organisms. An inhibition study with recombinant telomerase from the ciliated protozoan *Tetrahymena thermophila*[13] conducted by adding inhibitors either before or after assemblage revealed a similar distribution of inhibition as observed for human telomerase (Table 1). One notable exception was that none of the aminoglycosides tested were inhibitors of the *T. thermophila* enzyme suggesting a specific interaction between the inhibitory aminoglycosides and hTR. The small library was also tested against endogenous telomerase purified from the ciliated protozoan *Euplotes aediculatus*.[14] With purified *E. aediculatus* telomerase we found that the DNA minor groove-binding drugs distamycin and Hoechst 33258 were excellent inhibitors as were the intercalators quinacrine and proflavine. Other drugs tested had no affect on *E. aediculatus* telomerase activity.

After identifying several new telomerase inhibitors, we turned our attention to elucidating their modes of inhibition. Because Hoechst 33258 was previously identified as an RNA-binding ligand that exhibits an easily monitored change in fluorescence upon binding to nucleic acids, we used fluorescence spectroscopy to directly test the hypothesis that it interacts specifically with hTR.[15,16] We examined the change in fluorescence of Hoechst 33258 in the presence of several nucleic acid components involved in the telomerase reaction cycle including full-length hTR, a G-quadruplex formed from the human telomeric DNA sequence 5′-(GGGATT)$_3$GGG, and the RNA–DNA duplex representing the *E. aediculatus* telomerase-primer duplex 5′-dGGTTTTGGGGTTTTG/r3′-CCUUUUCCCCUUUUC, which was chosen because it forms the most stable duplex of the three telomerase complexes in these studies. Of these, only hTR produced a large and saturable increase in the fluorescence intensity of Hoechst 33258, indicating specificity for hTR (Fig. 4). A plot of fluorescence versus concentration of hTR gave an approximate dissociation constant (K_D) of 0.36 μM assuming a single binding site or multiple noncooperative binding sites. We conclude from this study that hTR is the target for inhibition by this ligand. Because the

Table 1. Percent inhibition of telomerase by selected nucleic acid-binding ligands[a]

	Inhibitor group[b]	*Human pre*[c]	*Human post*[c]	*Tetrahymena pre*[d]	*Tetrahymena post*[d]	*Euplotes*[e]
Intercalators						
Proflavine	II	95%	90%	90%	90%	52%
Quinacrine	II	82%	50%	93%	80%	75%
DNA minor groove binders						
Hoechst 33258	I	81%	34%	50%	15%	86%
Diamidino-2-phenylindole	II	90%	72%	70%	70%	0%
Distamycin A	n/a	10%	n.d.[f]	0%	0%	75%
Aminoglycosides						
Neomycin	I	70%	23%	0%	0%	0%
Gentamycin	I	64%	19%	0%	0%	0%
Paromomycin	I	60%	29%	0%	0%	0%
Lividomycin	I	50%	12%	0%	0%	10%
Apramycin	n/a	30%	n.d.	0%	0%	0%
Amikacin	n/a	25%	n.d.	0%	0%	0%
Spectinomycin	n/a	0%	n.d.	0%	0%	0%
Hygromycin	n/a	0%	n.d.	0%	0%	0%
Other classes						
Tetracycline	n/a	10%	n.d.	0%	0%	0%

[a] Telomerase from several sources was assayed in the presence of 50 μM of the indicated nucleic acid-binding ligand. 100% inhibition indicates no activity observed, and 0% inhibition indicates activity levels equal to no inhibition.
[b] Inhibitors were designated as Group I or Group II as defined in the text and n/a indicates that the designation is not applicable.
[c] Determined using recombinant hTR and hTERT in a direct assay.[12]
[d] Determined using recombinant *T. thermophila* TERT and TR in a direct assay.[13]
[e] Determined using purified endogenous telomerase from *E. aediculatus* in a direct assay.[14]
[f] Not determined.

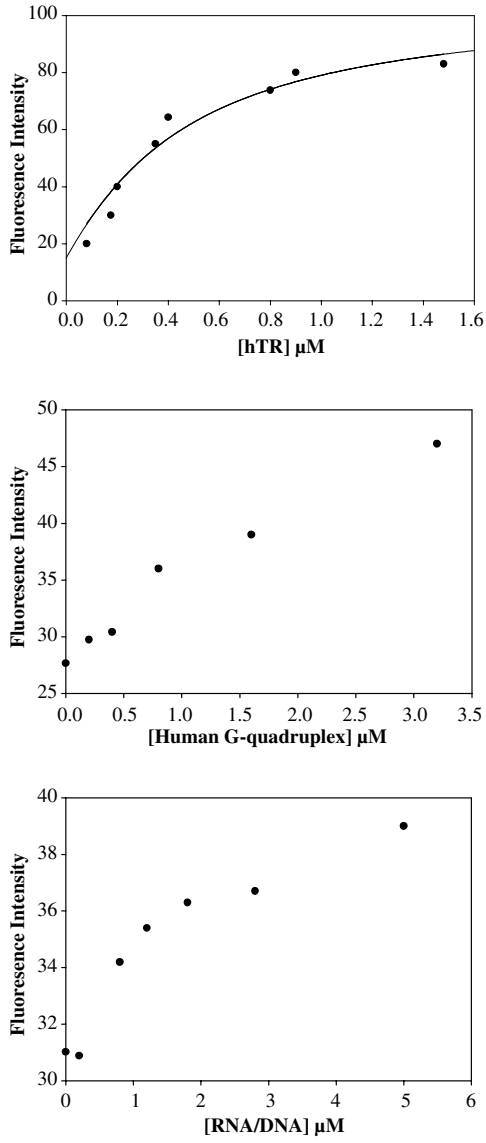

Figure 4. Hoechst 33258 binds to hTR but not to other nucleic acid components present in the telomerase reaction. The fluorescence intensity of Hoechst 33258 (0.2 μM) was monitored as a function of the concentration of either hTR, $(GGGTTA)_3GGG$, or 5′-dGGTTTTGGGGTTTTG/r3′-CCUUUUCCCCUUUUC as indicated. Hoechst was excited at 350 nm and monitored at 460 nm. The hTR curve was fit to the quadratic equation for a binding isotherm: $F = F_0 + \Delta F((([\text{Hoechst}]_0 + [L]_0 + K_D) - (([\text{hTR}]_0 + [\text{Hoechst}]_0 + K_D)^2 - 4[\text{hTR}]_0[\text{Hoechst}]_0)^{1/2})/2$, where F_0 is the fluorescence intensity in the absence of hTR, F is the fluorescence intensity in the presence of hTR, ΔF is the difference between the fluorescence of 0.2 μM Hoechst in the presence of saturating [hTR] and the fluorescence in the absence of hTR. $[\text{hTR}]_0$ and $[\text{Hoechst}]_0$ are initial concentrations.

other Group I inhibitors are also known to bind RNA in general and we directly observed a specific interaction between Hoechst 33258 and hTR, we suggest that all Group I inhibitors act by interacting with hTR leading to formation of a dysfunctional telomerase complex after assemblage. Furthermore, the reticulocyte lysate used to reconstitute telomerase contains tRNA, ribosomal RNA, and mRNA, thus the interactions between the Group I inhibitors with hTR are likely to be specific.

The mechanisms of inhibition of the Group II inhibitors await further investigation. However, several points can be highlighted. The intercalators quinacrine and proflavine appear to be telomerase inhibitors that lack species specificity. Because the telomeric repeats produced by telomerase from each of these species can fold into G-quadruplexes, one possible mode of action for these molecules is quadruplex stabilization. Proflavine has been reported as an inhibitor of human telomerase ($IC_{50} = 3.9\ \mu M$), and a variety of proflavine derivatives have been shown to stabilize G-quadruplex structures.[17] In our direct assays, the banding patterns of the telomerase products generated in the presence of quinacrine and proflavine were not consistent with typical quadruplex stabilizing compounds, which tend to cause enrichment of products associated with four repeats of the telomeric sequence.[18] However, we cannot rule out this mode of inhibition at this time. Interestingly, the minor groove binder distamycin was a strong inhibitor of *E. aediculatus* telomerase, but did not inhibit either recombinant human or *T. thermophila* telomerase. This was a surprise since a distamycin derivative was previously shown to inhibit human telomerase.[19] By contrast, the minor groove binder DAPI, which does not inhibit viral reverse transcriptases,[20] did inhibit both recombinant human and *T. thermophila* telomerase, but did not inhibit *E. aediculatus* telomerase. This suggests that the diamidino-2-phenylindole skeleton would be a useful starting point for the synthesis of selective telomerase inhibitors.

In conclusion, we have utilized a two-part parallel screen to identify inhibitors from a small library of known nucleic acid-binding ligands. Two general types of inhibitors were identified. Several of the compounds, the Group I inhibitors, appear to inhibit telomerase by specifically interacting with unbound hTR. Spectroscopic studies of the interaction between Hoechst 33258 and hTR, increased inhibition of human telomerase compared to inhibition of telomerase from other species, and the increased inhibition of Group I inhibitors when added before assemblage of the telomerase complex support this conclusion. Group II inhibitors were similarly effective when added before or after telomerase assemblage. The most likely explanation for the differences in Group I and Group II inhibitors is that the Group I binding sites are present in hTR and are only available in unbound hTR. By contrast, Group II binding sites, which may be protein, RNA, quadruplex DNA, or RNA–DNA duplex, are available in the holoenzyme. These results provide an impetus to further examine hTR as a target for small-molecule drugs. Future studies will investigate the precise mechanisms of inhibition by the nucleic acid-binding ligands identified here as telomerase inhibitors.

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