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# Small Molecule/Nucleic Acid Affinity Chromatography: Application for the Identification of Telomerase Inhibitors Which Target Its Key RNA/DNA Heteroduplex

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**Abstract**—The purpose of this work is to develop methods for identifying high-affinity nucleic acid binding species from soluble mixtures of compounds. We have developed and applied an affinity chromatography method for identifying small molecules with high affinity for the telomerase RNA/DNA duplex. An affinity resin was derivatized with an RNA/DNA duplex which represents the key structure that forms during telomerase's catalytic cycle. A soluble mixture of compounds was applied to this resin and the compounds which bound to the highest extent were also confirmed to be the best inhibitors of the enzyme. This correlation of affinity for the RNA/DNA duplex with telomerase inhibition both supports the duplex as the target of these compounds, and suggests that the affinity method may be applied for the identification of higher affinity inhibitors from soluble mixtures of compounds. 

The purpose of this work is to develop methods for identifying high-affinity nucleic acid binding species from soluble mixtures of compounds. Such methods will be useful for applying combinatorial chemistry to the targeting of nucleic acids. Combinatorial chemistry appears to be well suited to develop small molecules that target therapeutically important nucleic acids with high affinity. Systematic variation of a small molecule scaffold should introduce a variety of new interactions, some of which will increase the affinity and specificity for the target nucleic acid. This approach is being pursued in a range of laboratories.[1–6]

We have been developing tools for this purpose, with the aim of targeting the RNA/DNA heteroduplex which forms during the catalytic cycle of telomerase, an anticancer target.[7] The purpose of these molecules is to act as inhibitors of telomerase, either by preventing the strand dissociation required of telomerase's catalytic cycle, or by sufficiently distorting the heteroduplex, thereby making it inefficiently extended (Fig. 1). We have tested a range of intercalators, and demonstrated that a fraction of them can bind to this duplex and effect inhibition of telomerase. Our long-term strategy is to use these lead inhibitors as scaffolds upon which to build specific interactions with unique telomerase protein and nucleic acid surfaces. This is only possible because the target structure, the RNA/DNA duplex, is in close contact with these surfaces. These specific contacts will increase both affinity and specificity of the compounds for telomerase over other ribonucleoproteins and nucleic acids within the cell. There is ample precedent for small molecules to cross cell membranes and specifically interact with target nucleic acids (e.g., the work of Dervan et al.[8]). In addition, molecules from this class, intercalators, are known to be bioavailable and are clinically used to treat cancer (e.g., actinomycin D).

We are using two methods to explore how variation in lead intercalators introduces specific interactions with unique telomerase nucleic acid and protein surfaces. These are high-throughput screening of individual combinatorially generated molecules, and affinity separation of tight binding species from soluble mixtures of molecules. This manuscript describes the methodology that we have developed for the affinity separation approach. Although we have developed the method for the purpose of targeting telomerase, it should be applicable to any situation in which identifying high-affinity ligands for nucleic acids from soluble mixtures of compounds is desired.

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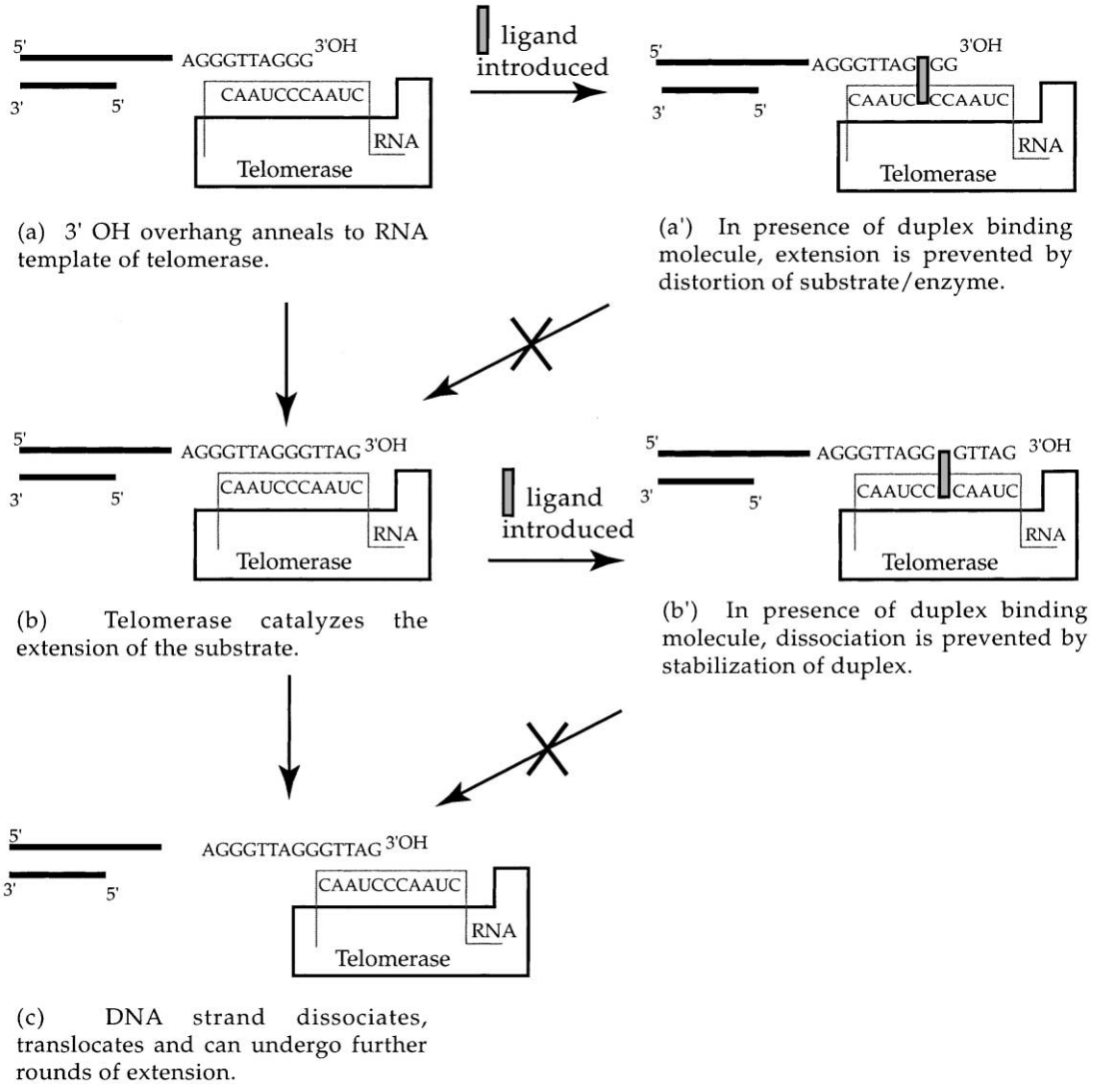


**Figure 1.** Telomerase mechanism showing possible mechanisms of action of heteroduplex binding molecules.

In a recent publication, we tested a range of duplex stabilizing molecules (intercalators) as potential inhibitors of telomerase and found four of them to have lead activity against telomerase.[7] In addition, their kinetics suggest that they do not inhibit by binding to G-quartets, thereby supporting our hypothesis that they inhibit by binding to the RNA/DNA heteroduplex which forms during telomerase's catalytic cycle (Fig. 2). We have used a soluble mixture of this collection of molecules to examine the utility of an affinity method, both for its ability to identify inhibitors of telomerase, as well as its ability to identify the species which bind with highest affinity to the target duplex.

The affinity column was prepared using RNA (sequence $(CCCUAA)_3$) prepared by Dharmacon Research, Inc. (Lafayette, Colorado) and the 5′ biotinylated DNA complement, $(TTAGGG)_3$, prepared by Sigma-Genosys (The Woodlands, Texas). The two strands were dissolved in annealing buffer (10 mM Tris–Acetate, 1 mM EDTA, 0.200 M NaCl, pH 7.5) and annealed at 75 °C for 1 h and then slowly cooled. A DNA/DNA duplex control resin was made identically, except that the strand complementary to $(TTAGGG)_3$ was DNA. These duplexes were immobilized on separate 23 μL aliquots (~6 μL drained bed volume) of Amersham (Piscataway, New Jersey) streptavidin sepharose high performance resin according to the manufacturer's directions. Approximately 1 nmol of each duplex was bound to the resin, as determined by UV quantitation

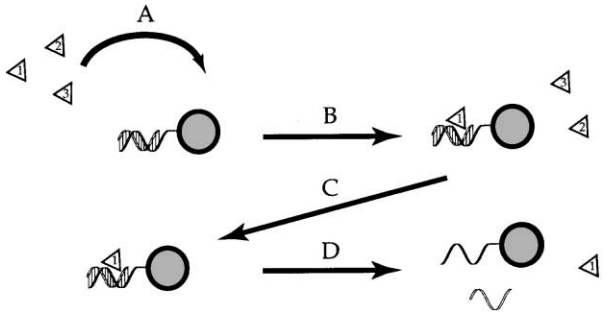


**Figure 2.** Schematic of affinity separation. (A) Mixture of compounds applied to duplex mounted on resin. (B) These compete with each other for limited binding sites. (C) Excess compounds are washed off of resin. (D) Duplex is melted, thereby releasing duplex binding molecules.

of the duplex before and after application of the duplex. The resins were washed with 150 μL of 1× telomerase assay buffer (50 mM Tris, pH 7.2, 1 mM spermidine, 1 mM MgCl, 50 KCl, omitting beta-mercaptoethanol).

An equimolar mixture of the previously assayed intercalators was applied to both columns at a ratio of 0.3 mol of intercalator per base pair of duplex, with a final intercalator concentration of 25 μM for each compound and a final buffer concentration of 1 × telomerase buffer (approximate total volume 170 μL). The column was allowed to equilibrate for 1 h at room temperature and the buffer and unbound compounds were then removed by centrifugation through a spin filter. Simple intercalator–duplex associations are known to be rapid for a range of duplexes and intercalators, with $k_1$ values in the $10^7$ $M^{-1}$ $s^{-1}$ range. This indicates half-times for association on the millisecond time scale at the concentrations used.[9,10] Because all species are in excess of the duplex during the binding stage, compounds compete for the limiting available binding sites, and higher affinity compounds should bind to a greater extent than lower affinity compounds. After removal of the buffer and excess ligands by filtration, the resins were rapidly washed twice with 150 μL of the 1 × telomerase assay buffer. During this wash, the resin was exposed to the buffer for less than 10 s before it was immediately removed using a spin filter. The purpose of this step was to remove any unbound intercalator without allowing a new equilibrium to be attained between bound intercalator and fresh buffer.

Bound compounds were eluted from the resin by heating the resin at 80 °C (approximately 20 °C above the melting temperature for the duplex) for 15 min in the same telomerase assay buffer used during intercalator binding. The heated resin was again drained using a spin filter and microfuge, and the filtrate was collected. The compounds contained in the filtrate were identified and quantitated by HPLC analysis using a C8 column. A 1 mL/min linear gradient starting at 30% acetonitrile/70% water (containing 0.1% trifluoroacetic acid) and ending at 100% acetonitrile over 25 min separated the tested compounds. These were then quantitated using standards of known concentration. This experiment was repeated three times, and the means and standard errors are shown in Table 1. The RNA/DNA affinity column identified ethidium bromide as having the highest affinity, which parallels the telomerase assay results. In addition, the next two high-affinity compounds were the second and fourth most effective telomerase inhibitors. In general, the compounds with the lowest amount of binding to the column were also the compounds of lowest telomerase inhibition potential. The results using the DNA/DNA control column are significantly different (Table 2). In this case, the compound with highest affinity for the column was doxorubicin, which is one of the least effective telomerase inhibitors. In addition, ethidium bromide, which had the highest affinity for the RNA/DNA resin, and was the most effective telomerase inhibitor, has one of the lower affinities for the DNA/DNA resin.

The purpose of the DNA/DNA resin in the above analysis is as a control, to insure that the binding efficiency of individual compounds to the RNA/DNA resin is indeed due to its unique structure, and not instead to the compound's generic duplex binding preference. This appears to be the case, as the ordering of binding amounts to the RNA/DNA resin strongly differs from the DNA/DNA resin. We have repeated these experiments using a different base resin, with the biotinylated RNA/DNA duplex attached to Dynal (Lake Success, New York) magnetic beads (Dynabeads M-280 Streptavidin). The results are essentially identical (data not shown). A second control in which the same mixture of compounds was applied to streptavidin sepharose resin underivatized by nucleic acid showed only limited binding of the compounds (Table 3). These amounts do not alter the ordering of the affinity of compounds in Tables 1 and 2.

We have also attempted to determine intrinsic affinities of the individual species for the resin bound duplexes, using von-Hippel–McGhee analysis.[11] Ethidium, the compound which eluted to the greatest extent from the resin, and which also had the greatest activity against telomerase, also showed the highest affinity to resin ($K_a$ $2\times10^5$ $M^{-1}$), although the other affinities were clustered

**Table 1.** Compounds released from RNA/DNA heteroduplex derivatized resin [sequence $(TTAGGG)_3$]

| Elution rank | Compd | pmol Eluted[a] | Rank in telomerase assay[b] |
|---|---|---|---|
| 1 | Ethidium bromide | 652 (91) | 1 (3.3) |
| 2 | Rivanol | 296 (33) | 2 (8.2) |
| 3 | Acridine yellow | 173 (27) | 4 (21.7) |
| 4 | Doxorubicin | 107 (28) | · |
| 5 | Daunorubicin | 84 (18) | · |
| 6 | Actinomycin D | 38 (15) | · |
| 7 | 7-Amino actinomycin D | 23 (7) | · |
| 8 | Acridine orange | 8 (1) | 3 (12.2) |

[a]Values in parentheses are standard error values.
[b]Dot indicates minimal telomerase inhibition detected (<50% inhibition at 50 μM). Values in parentheses are $IC_{50}$ values in μM as previously determined.

**Table 2.** Compounds released from DNA/DNA duplex derivatized resin [sequence $(TTAGGG)_3$]

| Elution rank | Compd | pmol Eluted[a] | Rank in telomerase assay[b] |
|---|---|---|---|
| 1 | Doxorubicin | 667 (11) | · |
| 2 | Daunorubicin | 362 (5) | · |
| 3 | 7-Amino actinomycin D | 352 (22) | · |
| 4 | Rivanol | 312 (11) | 2 (8.2) |
| 5 | Acridine orange | 228 (9) | 3 (12.2) |
| 6 | Acridine yellow | 173 (9) | 4 (21.7) |
| 7 | Ethidium bromide | 146 (4) | 1 (3.3) |
| 8 | Actinomycin D | 33 (7) | · |

[a]Values in parentheses are standard error values.
[b]Dot indicates minimal telomerase inhibition detected (<50% inhibition at 50 μM). Values in parentheses are $IC_{50}$ values in μM as previously determined.

**Table 3.** Compounds released from underivatized resin

| Control (resin without any duplex attached) | | | |
|---|---|---|---|
| Elution rank | Compd | pmol Eluted[a] | Rank in telomerase assay[b] |
| 1 | Rivanol | 26 (7) | 2 (8.2) |
| 2 | Doxorubicin | 14 (1) | • |
| 3 | Daunorubicin | 9.7 (10) | • |
| 4 | Acridine yellow | 8.0 (2) | 4 (21.7) |
| 5 | Ethidium bromide | nd | 1 (3.3) |
| 6 | 7-Amino actinomycin D | nd | • |
| 7 | Acridine orange | nd | 3 (12.2) |
| 8 | Actinomycin D | nd | • |

[a]Values in parentheses are standard error values. nd indicates none detected in HPLC.
[b]Dot indicates minimal telomerase inhibition detected ($<50\%$ inhibition at 50 μM). Values in parentheses are $IC_{50}$ values in μM as previously determined.

too closely and with too much overlap to allow a statistically significant comparison.

In this work, we have examined the utility of an affinity chromatography method for selecting high-affinity heteroduplex binding inhibitors from soluble mixtures of compounds. The potential advantage of such affinity methods is that hundreds or thousands of compounds may be applied to a single batch of resin, and high-affinity species identified in a single step. Soluble combinatorial synthesis can alleviate much of the labor and cost of 'single-bead/single-compound' libraries.

In our system, we are seeking small molecules with specificity for the rare RNA/DNA duplex that forms during telomerase's catalytic cycle. Such compounds will act as a platform on which to introduce functionalities which can interact with telomerase protein and nucleic acid surfaces and thereby further increase the specificity and affinity of the compounds for telomerase. In order to select such compounds from soluble mixtures, a second generation affinity column which incorporates the protein portion of telomerase, as well as the target RNA/DNA duplex, may be necessary. Our studies suggest that affinity chromatography is an effective method to identify high-affinity nucleic acid targeting species from soluble mixtures of compounds.

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