Inhibition of Human Telomerase by a G-Quadruplex-Interactive Compound

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The ends of chromosomes have specialized sequences, termed telomeres, comprising tandem repeats of simple DNA sequences. Human telomeres consist of the sequence 5′-TTAGGG.[1,2] Telomeres have several functions apart from protecting the ends of chromosomes, the most important of which appear to be associated with senescence, replication, and the cell cycle clock.[3] Progressive rounds of cell division result in a shortening of the telomeres by some 50−200 nucleotides per round. Almost all tumor cells have shortened telomeres, which are maintained at a constant length[4,5] and are associated with chromosome instability and cell immortalization. The enzyme telomerase adds the telomeric repeat sequences onto telomere ends, ensuring the net maintenance of telomere length in tumor cells commensurate with successive rounds of cell division. Telomerase is a DNA polymerase with an endogenous RNA template,[6] on which the nascent telomeric repeats are synthesized. A significant recent finding has been that approximately 85−90% of all human cancers are positive for telomerase, both in cultured tumor cells and primary tumor tissue, whereas most somatic cells appear to lack detectable levels of telomerase.[7] This finding has been extended to a wide range of human tumors (see, for example, refs 8 and 9) and is likely to be of use in diagnosis. Human telomerase has been proposed as a novel and potentially highly selective target for antitumor drug design.[6,10,11] This hypothesis is supported by experiments with antisense constructs against telomerase RNA in HeLa cells, which show that telomere shortening is produced, together with the death of these otherwise immortal cells.[6] Sequence-specific peptide nucleic acids directed against telomerase RNA have also been found to exert an inhibitory effect on the enzyme.[12]

Our laboratories have initiated a structure-based approach to discovering non-nucleoside compounds that will selectively inhibit human telomerase by targeting the nucleic acid structures, such as G-quadruplexes,[13,14] that may be associated with human telomeres or telomerase. In this report we first demonstrate inhibition of human telomerase by the 2,6-diamidoanthraquinone (compound **1**). We also show by ^{1}H-NMR the stabilization of a G-quadruplex by compound **1** and, finally, provide evidence that this compound inhibits the telomerase enzyme by a mechanism consistent with

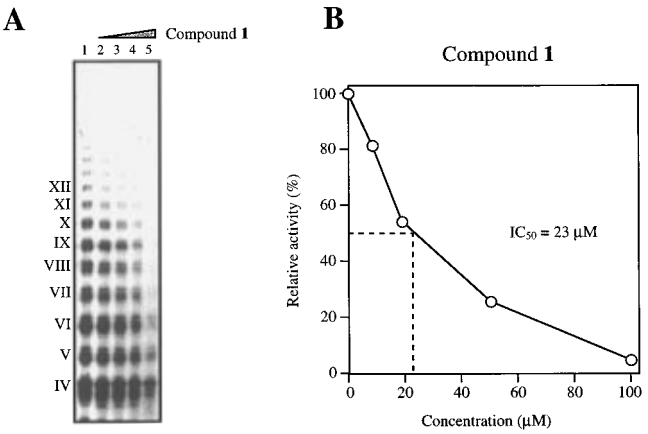
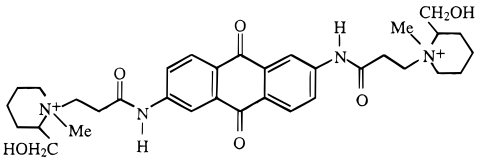


Figure 1. (A) Effect of increasing concentrations of compound **1** on inhibition of telomerase catalyzed extension of an 18-mer primer d[TTAGGG]$_3$ (1 μM). Elongated primer was labeled with 1.5 μM of [α-^{32}P]dGTP (800 Ci mmol^{-1}, 10 mCi mL^{-1}) with 1 mM dATP and dTTP using a standard telomerase assay. Lanes 1−5 are 0, 10, 20, 50, and 100 μM solutions of compound **1**. Roman numerals IV−XII to the left of the gel identify extension products. (B) Graphical representation of data from A.

interaction with G-quadruplex structures. Although G-quadruplexes have been suggested as possible molecular targets for telomerase inhibitors,[15] this is the first published report of a molecule that acts in this way and is also the first report of a non-nucleoside, small molecule inhibitor.



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We have previously developed a series of 2,6-diamidoanthraquinones as DNA-interactive agents.[16,17] They have been shown to act as selective DNA triplex interactive compounds,[18,19] with reduced affinity for duplex DNA and only moderate conventional cytotoxicity in a range of tumor cell lines. Qualitative molecular modeling studies at the Institute of Cancer Research (unpublished results) had also predicted that they might bind by a "threading" intercalation mode to G-quadruplex structures, analogous to their threading behavior with duplexes.[20] This prediction has been reinforced by subsequent detailed modeling studies, which suggest that several stable "intercalation sites" are energetically feasible (J. Trent and S. Neidle, unpublished results).

The relative inhibition of human telomerase by compound **1** was determined in a standard primer extension assay that does not use a PCR-based amplification of the telomerase primer extension products (see Figure 1). The IC$_{50}$ was determined to be 23 μM, and at 100 μM of compound **1** there is an almost complete inhibition of telomerase activity. Several other compounds in this series show similar behavior, and a structure−activity relationship is currently being evaluated.

To examine the binding and mode of interaction with G-quadruplex structures, UV and NMR titrations were carried out. In initial UV titrations, the intramolecular

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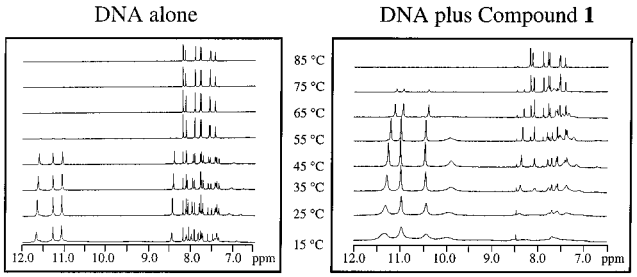

Figure 2. Thermal denaturation of the parallel four-stranded G-quadruplex structure formed by the d[T_2AG_3T] (7-mer) (125 mM KCl, 25 mM KH_2PO_4, 1 mM EDTA, pH 6.9) monitored by NMR. On the left is shown the spectrum for DNA alone and on the right is that for DNA in the presence of compound **1**. The molar ratio of compound **1** to quadruplex is 4:1. The imino proton signals have been assigned previously[14] as G6, G5, and G4 from high to low field. The presence of drug leads to line broadening and an upfield shift of the imino proton signals indicative of intercalation. Furthermore, the melting temperature of the DNA G-quadruplex is increased significantly in the presence of drug. Spectra were acquired in 90% H_2O/10% D_2O on a Bruker AMX 500 MHz spectrometer at the specified temperature using a 1−1 echo pulse sequence with a maximum excitation centered at 12.0 ppm. A total of 128 scans was obtained for each spectrum with a relaxation delay of 2 s. Before acquiring the spectrum at each temperature, the sample was allowed to equilibrate at the new temperature for at least 10 min. The data were processed with an exponential window function using 2 Hz of line broadening.

fold-over quadruplex of the 22-mer d[$AG_3(T_2AG_3)_3$][21] and a four-stranded parallel quadruplex of the 7-mer d[T_2AG_3T] were used.[21] The UV titration of the 7-mer and 22-mer into a 40 μM solution of compound **1** produces similar changes in the UV spectrum of compound **1**. While both quadruplexes induced similar spectral changes, with a hypochromic effect maximally produced at 354 nm, only the 7-mer showed a clear isobestic point at 328 nm; therefore, the 7-mer was used exclusively in the subsequent NMR studies. In comparison to duplex DNA, compound **1** shows a modest preference for binding to quadruplex structures. An NMR titration of compound **1** into a solution of the 7-mer G-quadruplex showed a saturatable upfield shift of all three sets of the G-quadruplex imino protons, suggestive of an intercalation binding mode (Figure 2). The imino-to-imino walk in the two-dimensional NOESY spectrum of the G-quadruplex is not interrupted in the presence of saturating concentrations of compound **1** (unpublished results), suggesting that if intercalation does take place, this may be at the 5′-AG step immediately adjacent to the G-quadruplex structure. Alternative modes of interaction include intercalation within the G-tetrads, but with compound **1** in fast exchange between free and bound forms, and conceivable groove binding. The thermal denaturation behavior of the G-quadruplex in the absence and presence of compound **1** indicates a drug-induced increase in the T_m by about 20 °C (Figure 2).

To address the important question of whether the interaction of compound **1** with the G-quadruplex is the primary mechanism for inhibition of telomerase, an experiment was carried out from which the effect of compound **1** on the pattern of elongation of an 18-mer (3-repeat) primer d[$TTAGGG]_3$ by human telomerase was determined (Figure 3). A concentration of compound **1** (10 μM) that would produce less than 50% inhibition was chosen to lessen the chance of secondary effects and also to maintain a strong signal in the telomerase primer extension assay. In this experiment, a time-course incorporation of [α-^{32}P]-labeled dGTP into telomerase-extended 3-repeat primers was determined (Figure 3A). Telomerase-catalyzed extension of the 3-repeat primer results in the formation of a series of products that give rise to distinct groups of bands on the PAGE autoradiogram of the reaction products. The first of these groups of bands (IV) corresponds to the addition of four bases to the 3-repeat primer. Successive rounds of elongation and translocation of the primer by telomerase produce bands (V and higher) that correspond to the subsequent addition of 6, 12, 18, etc., bases. If the G-quadruplex is the target structure for compound **1**, inhibition should *only* take place at telomere repeats of V or more, presuming at least a partial overlap of the extended primer with the template. At a 10 μM concentration of compound **1**, there is an overall decrease of incorporation of [α-^{32}P]dGTP into the extended 18-mer relative to the control (Figure 3B); however, the overall processivity is not markedly affected. Most importantly, there is no significant effect on either the initial rate of incorporation or the amount of total incorporation of [α-^{32}P]dGTP that occurs at the first and second telomerase-catalyzed extensions, which give rise to the 22-mer and 28-mer (Figure 3C). In sharp contrast, telomerase-catalyzed extensions, which give rise to 34-mers and higher, are significantly inhibited (>50%) in total incorporation of [α-^{32}P]dGTP (see Figure 3C). These data are consistent with the premise that the intramolecular G-quadruplex, which can *only* be formed by telomerase extension after addition of one and one-half mer extensions to the original 3-repeat primer, is the primary target for drug action.

A careful examination of the pattern of bands produced at each extension (IV−XII) also reveals a potentially significant result (Figure 3A,C). In general, a multimer set of three main bands is produced at each 1-repeat extension, presumably a result of some 3′-exonuclease activity associated with the telomerase. In all of the telomerase extensions amenable to analysis except those at V and IX, the pattern of these sets of bands is similar; i.e., the major band corresponds to the 5′-TTAG* telomerase-extended species, with less intense bands for the successive exonuclease-produced 5′-TTA and 5′-TT species (a−c in Figure 3C). However, for the V and IX extensions the pattern of the multimer is fully (V) or partially (IX) reversed, so for these products the most intense band is found at either the 5′-TT (V) or 5′-TTA (IX) species. This effect is more pronounced at later time points (40−60 min) and in compound **1**-treated lanes. This periodicity of 4 repeats (i.e., V and then IX) is suggestive of an ordered structure, most probably the G-quadruplex, that is formed after accumulation of each 4 repeats, which temporarily stalls the telomerase, causing the increased 3′-exonuclease activity at these G-quadruplex-associated pause sites. Both the pausing at the V extension (28-mer), which is enhanced in the presence of compound **1**, and the telomerase inhibition, which occurs only after formation of this extension, are in accord with this suggestion. Since it is well established that G-rich strands can form stable G-quadruplex structures, and we show here that compound **1** interacts to further stabilize these structures, this implicates

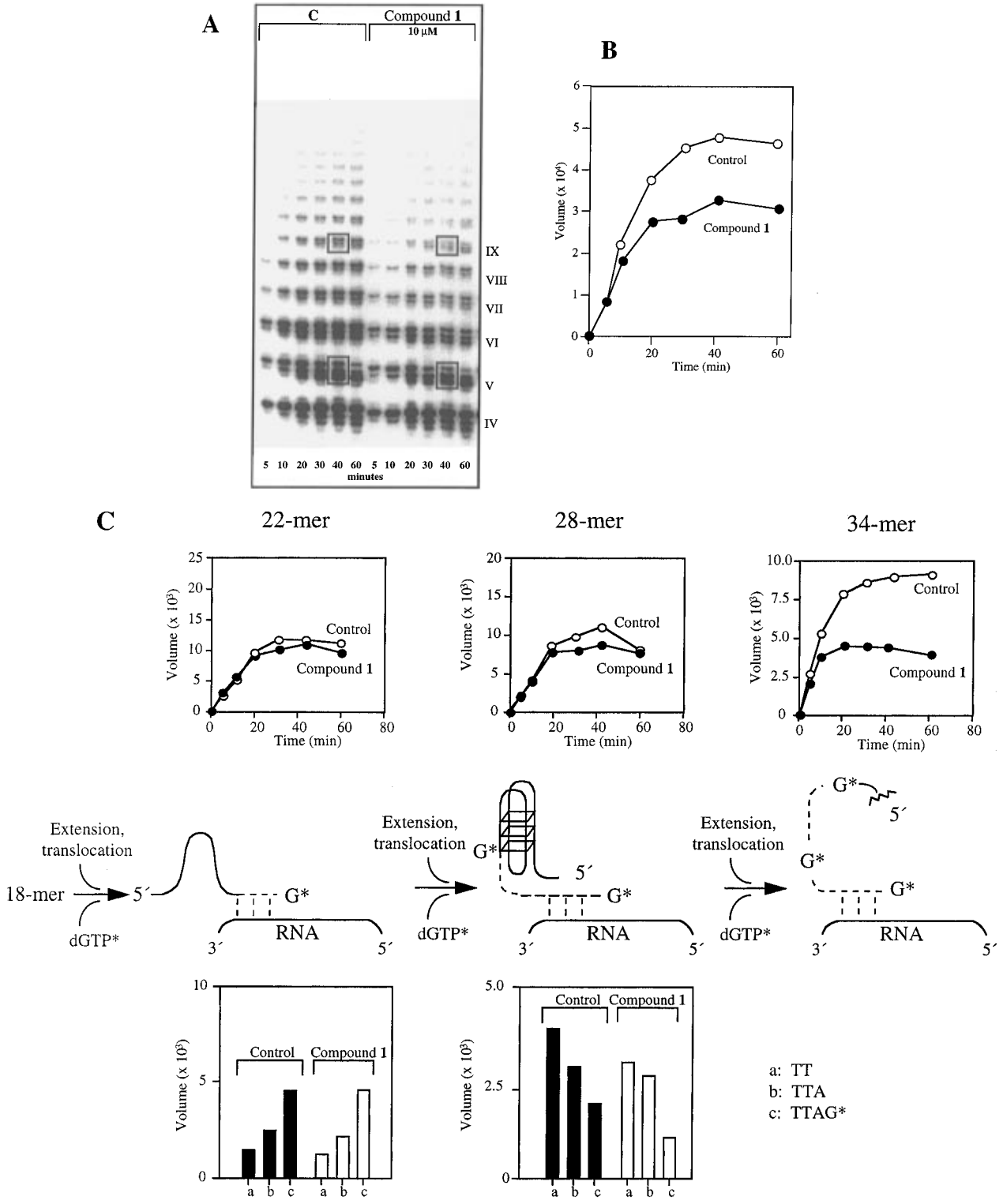

Figure 3. Effect of compound **1** on the time course of telomerase activity using (A) the 18-mer telomeric primer d[TTAGGG]$_3$ (1 μM) without (left-hand lanes) or with compound **1** (right-hand lanes) added at 10 μM. Elongated primer was labeled with 1.5 μM [α-^{32}P]dGTP (800 Ci mmol^{-1}, 10 mCi mL^{-1}) with 1 mM dATP and 1 mM dTTP using a standard telomerase assay. Samples were taken at the time intervals shown at the bottom of the figure, and the extension products (IV−IX) are shown to the right of the gel. The boxes identify the 40-min samples, which show altered multiple-band patterns due to 3′-exonuclease activity. (B) Time course of total amount of [α-^{32}P]dGTP incorporated into the extension products of the d[TTAGGG]$_3$ primer in the presence and absence of compound **1**. (C) Time course incorporation of [α-^{32}P]dGTP into the 22-mer, 28-mer, and 34-mer and comparison of patterns of sets of multimers for the 22-mer and 28-mer in the presence and absence of compound **1**. The diagrams between the two sets of results show the proposed structures of the species formed at each step.

G-quadruplexes as intermediates in the telomerase mechanism for strand extension.

In order to further assess the role of a G-quadruplex as the molecular target for compound **1**, a 5-repeat primer was used in the telomerase extension experiment, in which primer concentration was varied for three different drug concentrations (unpublished results). The results of these experiments are most consistent with a model for competitive inhibition,[22] which pinpoints either the telomerase enzyme or the

5-repeat primer substrate as the molecular target for compound **1**. On the basis of the results of the binding studies of compound **1** with the 22-mer intramolecular fold-over structure, together with the telomerase extension experiments, it seems most likely that the real molecular target for compound **1** is the enzyme-dissociated intramolecular G-quadruplex structure. However, the kinetic analysis does not eliminate a smaller component of the inhibition by compound **1**, due to telomerase-bound G-quadruplex.

The importance of a folded structure such as a G-quadruplex and its stabilization by K^+ in the regulation of telomerase activity has been noted previously.[15a] Compound **1** appears to function as a low molecular weight mimic of K^+ in stabilizing the G-quadruplex folded structure and, consequently, inhibiting telomerase activity. The elongation of the 3-repeat primer by telomerase to attain a 5-mer appears to be a prerequisite for inhibition of telomerase by this compound, suggesting that an intramolecular fold-over structure with a 4-mer 3′-tail (5′-TTAG) is the minimum molecular target for these agents. Although the G-quadruplex targeted by compound **1** appears to be most likely dissociated from the telomerase, the periodicity of 4 repeats for telomerase pausing, which results in increased 3′-exonuclease activity, is suggestive of the existence of such a structure as a telomerase-bound intermediate.

Compounds such as that described here, which interact selectively with G-quadruplex structures and inhibit telomerase, are potentially useful as inhibitors of the proliferation of cells that require telomerase to maintain telomere length for continued growth. In this regard carbocyanine has also been shown to interact with G-quadruplex structures,[23] and we have shown that this is a telomerase inhibitor (unpublished results). Since telomerase appears to be found almost exclusively in tumor cells, this type of agent may be useful as novel antitumor agents. Studies are in progress to determine its effects and those of other G-quadruplex-interactive compounds on telomere length in cell culture.

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Supporting Information Available: Description of Methods, with references (4 pages). Ordering information is given on any current masthead page.

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