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Accelerated Publications

Accelerated Assembly of G-Quadruplex Structures by a Small Molecule†

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ABSTRACT: In the presence of alkali cations, notably potassium and sodium, DNA oligomers that possess two G-rich repeats associate into either a tetrameric parallel G-quadruplex or a variety of dimeric antiparallel G-quadruplexes. The formation of such structures is normally a very slow process. Some proteins, such as the β-subunit of the *Oxytricha* telomere-binding protein, promote the formation of G-quadruplex structures in a chaperone-like manner. In this report, we present data concerning the role of a perylene derivative, PIPER, in the assembly of G-quadruplex structures as the first example of a small ligand behaving as a driver in the assembly of polynucleotide secondary structures. Gel-shift experiments demonstrate that PIPER can dramatically accelerate the association of a DNA oligomer containing two tandem repeats of the human telomeric sequence (TTAGGG) into di- and tetrameric G-quadruplexes. In so doing, PIPER alters the oligomer dimerization kinetics from second to first order. The presence of 10 μM PIPER accelerates the assembly of varied dimeric G-quadruplexes an estimated 100-fold from 2 μM oligomer. These results imply that some biological effects elicited by G-quadruplex-interactive agents, such as the induction of anaphase bridges, may stem from the propensity such compounds have for assembling G-quadruplexes.

DNA quadruplexes, also known as G4 structures, are composed of stacked tetrads, each of which consists of the planar association of four guanines in a cyclic Hoogsteen hydrogen-bonding arrangement (*1*, *2*). Monovalent cations, notably potassium and sodium, greatly stabilize G-quadruplex structures, presumably by coordinating with eight carbonyl oxygens projected between tetrads from guanine residues in the tetrad (*3*, *4*). A variety of G-quadruplex motifs exist in vitro and can be classified in terms of both molecularity and strand orientation. DNA possessing a single guanine tract may associate to form a tetramolecular G-quadruplex with strands oriented parallel with respect to each other (*5*, *6*). In addition to this structure, a sequence that contains two guanine-rich repeats can form a guanine−guanine hairpin, which may in turn dimerize to form a number of G-quadruplex isomers (*7*−*9*). Sequences with either four G-rich repeats or long guanine tracts can fold into antiparallel intramolecular quadruplexes (*10*, *11*).

Although the in vivo existence of a G-quadruplex has not been proven unequivocally, regions of the human genome with the potential to form a G-quadruplex abound. Examples include the telomere (*10*, *12*), the immunoglobulin switch region (*13*), the *c-myc* promoter (*14*), and the fragile X-syndrome triplet repeats (*15*). The prevalence of G-rich regions throughout many genomes implies that G-quadru-

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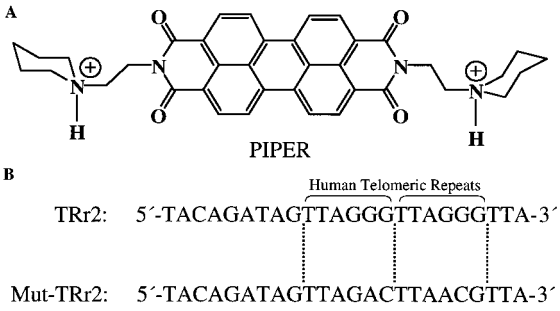
Scheme 1



plexes may indeed have a role in several biological events (*5*, *12*, *16*), particularly when regulatory sequences possess the capacity to form such structures (*14*). Furthermore, several proteins that interact specifically with G-quadruplex DNA have been identified, including both RAP1 from *Saccharomyces cerevisiae* (*17*) and the β-subunit of telomere-binding protein (TBP-β) from *Oxytricha* (*18*). These two factors also greatly accelerate the assembly of G-quadruplexes from competent nucleic acids and are thus considered to be molecular chaperones (*19*−*21*).

Some small molecules, such as porphyrins and anthraquinones, have been shown to interact specifically with G-quadruplex DNA (*22*−*26*). It is thought that such compounds stabilize preformed G-quadruplex structures by stacking external to the G-tetrads (*22*, *27*). Our laboratory recently investigated one such compound: *N*,*N*′-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER, Scheme 1A). PIPER was found to stack on the 3′-terminal G-tetrad surface of a tetrameric parallel G-quadruplex. Continued experimentation revealed that PIPER can inhibit telomerase by interacting with a G-quadruplex intermediate of an oligomer substrate (*28*). Here we present the first evidence that a small ligand can act as a driver in polynucleotide assembly, specifically by promoting the association of a DNA oligomer possessing two tandem repeats of the human telomeric sequence (TTAGGG) into G-quadruplex structures.

MATERIALS AND METHODS

Polynucleotides and PIPER Preparation. DNA oligomers, 5′-TACAGATAG(TTAGGG)$_2$TTA-3′ (TRr2) and 5′-TACAGATAGTTAGACTTAACGTTA-3′ (Mut-TRr2), were prepared on a DNA synthesizer (PerSeptive Biosystems Expedite 8909). Oligomers were purified by denaturing 20% polyacrylamide gel electrophoresis (PAGE), diluted to 80 μM, and dispensed into small aliquots. About 200 ng of DNA was 5′-end-labeled with ^{32}P using T4 polynucleotide kinase (New England Labs) and subsequently purified by denaturing 20% PAGE. PIPER was synthesized and purified as previously described (*28*). A 1 mM stock solution of PIPER in distilled water was stored at −20 °C and diluted to working concentrations immediately before use.

Assay of DNA Secondary Structure by Native PAGE Gel Shift. ^{32}P-end-labeled oligomer at a concentration of 8 μM, or otherwise as indicated, was heated to 95 °C for 10 min in 1 × KCl/TE buffer (10 mM Tris·HCl, 1 mM EDTA, 100 mM KCl, pH 8.0). After the DNA had cooled to room temperature, 2 μL of stock PIPER was dispensed to each sample to produce the specified concentrations at a total volume of 20 μL. Reaction mixtures were incubated for 1−4 h at room temperature. After incubation, 2 μL of gel loading solution (50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol) was added to each mixture. Ten microliter aliquots of each sample were subsequently analyzed by native 16% PAGE (the gel was prerun for 30 min). Electrophoresis proceeded for 14 h at 4 °C in TBE/KCl running buffer (45 mM Tris−borate, 45 mM boric acid, 1 mM EDTA, 20 mM KCl, pH 8.3). Gels were dried and then visualized and quantified on a PhosphorImager (Molecular Dynamics Model 445 SI).

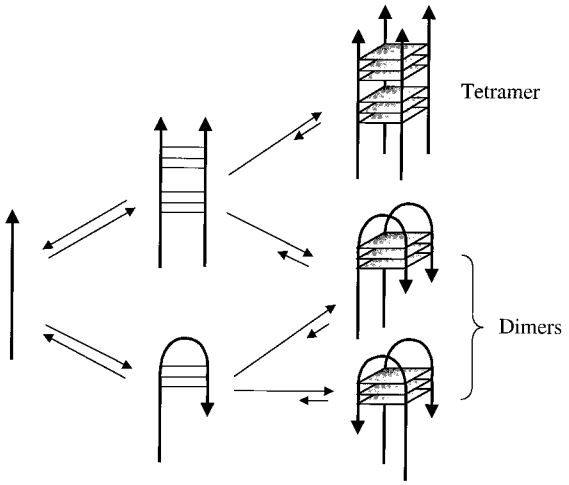
Dimethyl Sulfate (DMS) Methylation Protection. 5′-Labeled TRr2 was heat denatured and then annealed in the TE/KCl buffer containing 10 μM PIPER as described above. After samples had annealed, DMS was added to each with a final concentration of 0.1% (v/v). Methylation proceeded for 10 min at room temperature. The reactions were halted with the addition of 2 μL of 7.2 M β-mercaptoethanol. Immediately thereafter, 2 μL of gel loading solution was added, and samples were then separated by native 16% PAGE. Bands corresponding to monomer, dimer, and tetramer (see below) were visualized by autoradiography and excised. DNA from each band was eluted by overnight agitation at room temperature in TE buffer. Eluted products were precipitated twice in ethanol and then treated with piperidine to induce strand breakage. As a positive control, heat-denatured TRr2 was methylated but not separated prior to strand breakage. After ethanol precipitation, fragments from each sample were resolved by denaturing 20% PAGE and visualized with the PhosphorImager.

RESULTS AND DISCUSSION

Native PAGE assays were performed to investigate the role of the small ligand, PIPER, in the assembly of G-quadruplex structures from the DNA oligomer TRr2 (Scheme 1B), which contains two tandem repeats of the human telomeric sequence. Sequences possessing two guanine tracts can associate into antiparallel and parallel G-quadruplexes that are dimers or tetramers of the oligomer, respectively (*8*, *18*). A parallel tetrameric TRr2 G-quadruplex might form either by association of four linear strands or by dimerization of a parallel guanine−guanine duplex. Several dimeric TRr2 G-quadruplex isomers can form via dimerization of guanine−guanine hairpins or folding of a guanine−guanine duplex (Scheme 2). Four distinct dimeric G-quadruplex isomers of an oligonucleotide containing two guanine tracts were reportedly resolved by native PAGE (*7*).

Potassium Ions and PIPER Each Promote the Formation of Different TRr2 G-Quadruplexes. Native gel-shift analysis of TRr2 incubated in the presence of potassium ions revealed that this oligomer associates into antiparallel dimeric G-quadruplexes in proportion to ion concentration, while additional slow migrating species, presumably tetrameric or high-order structures, form to a lesser extent (D and T, Figure 1A). When PIPER is titrated against fixed concentrations of potassium ion and TRr2 in a native PAGE structural assay, populations of dimeric (D′), tetrameric (T′), and higher order TRr2 species are greatly intensified (Figure 1B). Several interesting changes in TRr2 molecularity and secondary structure are observed at low concentrations (10−20 μM) of PIPER, notably the formation of five unique TRr2

Scheme 2



structures (one tetramer and four dimers), each with an electrophoretic mobility different from those observed in the presence of potassium ions alone. As the PIPER concentration is increased, the prevalence of dimeric and tetrameric TRr2 structures decreases and increases, respectively, and higher order species emerge. Finally, the TRr2 tetramer appears to migrate progressively slower with increasing concentrations of PIPER, suggesting that PIPER may remain bound to the tetramer after assembly (Figure 1B, lanes 6 and 7). A negative control experiment using a mutated TRr2 (sequence shown in Scheme 1B), which lacked any three consecutive guanines, was performed in order to rule out the possibility that shifted bands are simply nonspecific PIPER−oligomer complexes. As expected, a native PAGE shift was not observed when PIPER was titrated against Mut-TRr2 (Figure 1C).

DMS Footprinting Verifies That Native PAGE Band Shifts Correspond to Formation of G-Quadruplexes of TRr2. Given that TRr2 does not possess dyad symmetry, and thus can neither form hairpins nor associate by Watson−Crick base pairing, we reasoned that native PAGE gel shifts represent structural changes mediated by nonstandard base pairing. Specifically, it was surmised that changes in strand molecularity are also indicative of G-quadruplex formation. To test this hypothesis, DMS methylation protection experiments were performed. Since N7 of guanine is critical for the stability of a Hoogsteen base pair, formation of G-quadruplex structures by TRr2 should effectively protect guanine residues from methylation by DMS while guanines of nonquadruplex structures remain susceptible. It was found that none of the guanine residues in either the denatured TRr2 (positive control) or the TRr2 monomer are protected (lanes 1, 2, and 4 in Figure 2). For tetrameric and dimeric TRr2 structures, on the other hand, guanines that are within the two telomeric repeats ($G_{13-15,19-21}$) are protected, while one guanine residue outside the repeats (G_9) remains sensitive to methylation (lanes 3, 5, and 6 in Figure 2). These data verify that shifted bands labeled as dimeric (D and D′) or tetrameric (T′) TRr2 are in fact G-quadruplex structures, a finding that is in agreement with reported analyses of gel shifts observed by evaluating other similar oligomers (*5, 7, 8, 18*).

The Second-Order Dimerization Reaction of TRr2 Becomes a First-Order Reaction in the Presence of PIPER. The β-subunit of the telomere-binding protein (TBP-β) from *Oxytricha* can convert *dimeric* G-quadruplex formation from a second- to a first-order reaction (*21*). To determine if PIPER might have a similar kinetic effect on G-quadruplex formation, TRr2 was titrated against fixed concentrations of potassium ion and PIPER in a native PAGE structural assay. A comparison of the results of incubation in the absence (panel A) and presence (panel B) of PIPER is shown in Figure 3. The four dimeric TRr2 complexes (D′ in Figure 3B) observed in the presence of 10 μM PIPER migrated differently than the two that formed in potassium alone (D in Figure 3A). Two of the four dimers produced in the presence of PIPER migrated faster than either produced in its absence and are thus likely to be distinct isomers of the dimeric G-quadruplex. The remaining two PIPER-mediated dimers, which migrate slower, might also be distinct isomers of the dimeric G-quadruplex, though the possibility that they are instead specific complexes of PIPER and the dimeric G-quadruplex cannot be ruled out. A log−log plot of dimeric TRr2 G-quadruplex concentration versus total oligomer concentration in the absence of PIPER yielded a slope of 1.78 ± 0.04 (Figure 3C), indicating that TRr2 dimerization is normally a second-order reaction. In the presence of 10 μM PIPER, however, the same analysis yields a slope of 1.12 ± 0.03 (Figure 3C). This PIPER-induced alteration suggests that the TRr2 dimerization has switched from a second- to a first-order process, a phenomenon also observed when telomeric oligomers were incubated in the presence of TBP-β (*21*).

Remarkably, the tetramer did not readily assemble without the assistance of PIPER (Figure 3A). The kinetics for the tetramer formation (not shown) were neither first nor second order. Qualitatively, the amount of parallel tetrameric TRr2 G-quadruplex formed increases initially at low oligomer concentrations and then begins to decrease as oligomer concentration rises. One explanation for this result is that labeled strands are being replaced with unlabeled strands in the tetrameric G-quadruplex as the concentration of unlabeled oligomer increases. This explanation assumes that the total population of the tetrameric G-quadruplex is limited by the amount of PIPER available for stabilization. By the same rationale, however, the dimeric G-quadruplexes should also vanish in proportion to oligomer concentration. That dimeric G-quadruplexes are detected increasingly in proportion to oligomer concentration might, in turn, be explained by assuming that a small fraction of dissociated PIPER can catalytically promote oligomer dimerization. Regardless of the precise mechanism, the presence of PIPER dramatically accelerates the assembly of both tetrameric and dimeric G-quadruplexes from TRr2 in general.

TRr2 Dimerization Is Accelerated Approximately 100-fold in the Presence of 10 μM PIPER. More detailed kinetic information concerning the effect of PIPER on TRr2 molecularity and secondary structure was obtained from native PAGE time-course experiments. With concentrations of potassium ions, TRr2, and PIPER fixed at 100 mM, 8 μM, and 5 or 10 μM, respectively, it was found that tetrameric G-quadruplex forms transiently within the first 2 h and is eventually superseded by the formation of dimeric G-quadruplexes (Figure 4A). *Under these conditions*, the first-order rate constant for the formation of dimeric TRr2 G-quadruplexes in the presence of 10 μM PIPER was

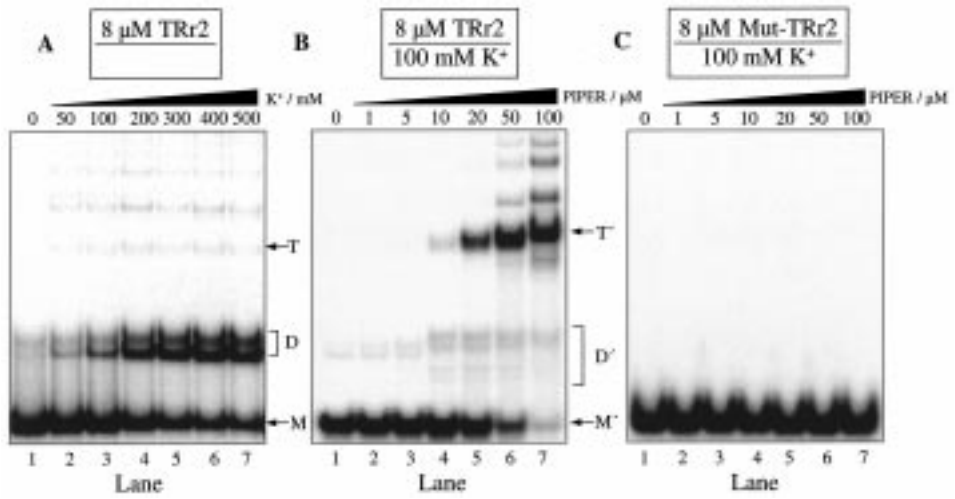


FIGURE 1: Effects of potassium ions and PIPER on the assembly of TRr2 structures illustrated by native PAGE. (A) KCl was titrated against 8 μM TRr2 in TE buffer. Major bands are identified as monomer (M), dimer, (D), and tetramer (T), in accordance with previous studies (*8*, *18*). (B) PIPER was titrated against 8 μM TRr2 in 100 mM K^+/TE buffer. Major bands are identified as monomer (M′), dimer (D′), and tetramer (T′). (C) As for (B), except Mut-TRr2 was used in place of TRr2.

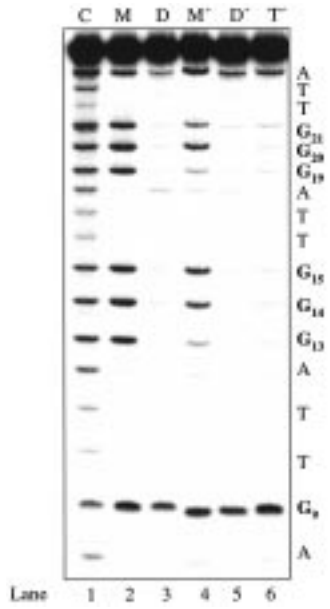


FIGURE 2: DMS methylation protection patterns of the potassium- and PIPER-mediated annealing products of TRr2. Annealed TRr2 was treated with DMS prior to native PAGE separation. Subsequently, DNA from major bands was excised, eluted, and cleaved in piperidine. Fragments were resolved on a sequencing gel. Species that annealed in 100 mM KCl/TE buffer were loaded onto lanes 2 and 3, while those forming in the additional presence of 10 μM PIPER were loaded onto lanes 4−6. Lanes are further labeled as follows: positive control (C), monomer (M and M′), dimer (D and D′), and tetramer (T′). Residue G_9 of TRr2, located outside the two tandem telomeric repeats, served as an internal control.

estimated to be 0.018 h^{-1} (Figure 4B). Likewise, the second-order rate constant for formation of the same set of structures in the absence of PIPER was estimated to be 1.0×10^2 M^{-1} h^{-1}. By comparing initial formation rates of dimeric TRr2 G-quadruplexes from 2 μM TRr2 in the presence or absence of PIPER, we estimate that 10 μM PIPER accelerates tetrameric G-quadruplex assembly 100-fold. To compare the activity of PIPER to that of TBP-β in the assembly of dimeric G-quadruplex species, the rate of dimeric G-quadruplex formation was calculated at an extrapolated TRr2 concentration of 20 nM, though no dimers were observed even at 50 nM TRr2. At this oligomer concentration, in theory, 10 μM PIPER accelerates the dimeric G-quadruplex formation 10^4-fold, or 10^3-fold/μM PIPER. Acceleration of G-quadruplex assembly by TBP-β was estimated to be 5×10^6-fold/μM TBP-β at the same oligomer concentration (*21*). It is important to note that the discrepancy between these two values (10^3-fold) may at least in part be due to substantial differences in protocol, primarily cation buffer, and incubation temperature.

Overall, it appears that in the presence of PIPER the parallel tetrameric TRr2 G-quadruplex is a kinetically favored product, while varied dimeric TRr2 G-quadruplexes are thermodynamically favored. Since the former structure emerges only *to a very small extent* in the absence of PIPER, we propose that the PIPER-mediated assembly of G-quadruplexes from single-stranded TRr2 proceeds via a novel reaction mechanism.

PIPER Plays a Driver-like Role in the Formation of Dimeric TRr2 G-Quadruplexes. Molecular chaperones are generally thought of as macromolecules that assist the noncovalent assembly of a protein into its native structure (*29*). Similarly, macromolecules that assist the formation of G4 DNA, including TBP-β (*21*) and certain G-rich oligonucleotides (*30*), are considered drivers in polynucleotide assembly. As the first example of a small organic ligand driver, PIPER enhances the assembly of dimeric and tetrameric G-quadruplexes from TRr2. Interestingly, PIPER shares some features with TBP-β, notably the ability to promote the formation of both tetrameric and dimeric G-quadruplexes from DNA oligomers possessing two telomeric repeats while converting the reaction kinetics of oligomer dimerization from second to first order. The most likely pathways for the assembly of various TRr2 structures discussed above invoke *both* isomerization and association reactions. Without external factors affecting oligonucleotide assembly, *isomerization reactions* probably proceed at *much faster rates* than *association reactions*. Thus, it is expected that in the absence of PIPER the rate-limiting step for formation of either dimeric or tetrameric TRr2 G-quadruplexes involves a bimolecular collision of monomeric or

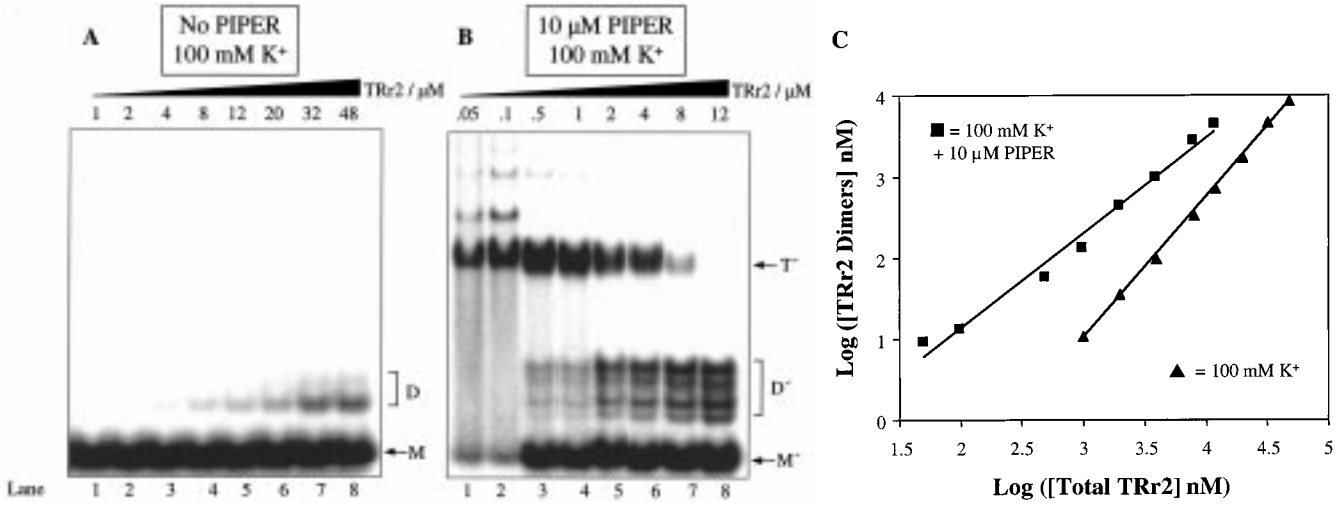


FIGURE 3: Potassium- and PIPER-assisted assembly of G-quadruplexes at different TRr2 concentrations. Specified concentrations of TRr2 were incubated overnight at room temperature in 100 mM KCl/TE buffer either alone (A) or with 10 μM PIPER (B). Dimer formation for both experiments is presented in (C) as log−log plots of dimer concentration versus total TRr2 concentration, both expressed as nanomolar quantities.

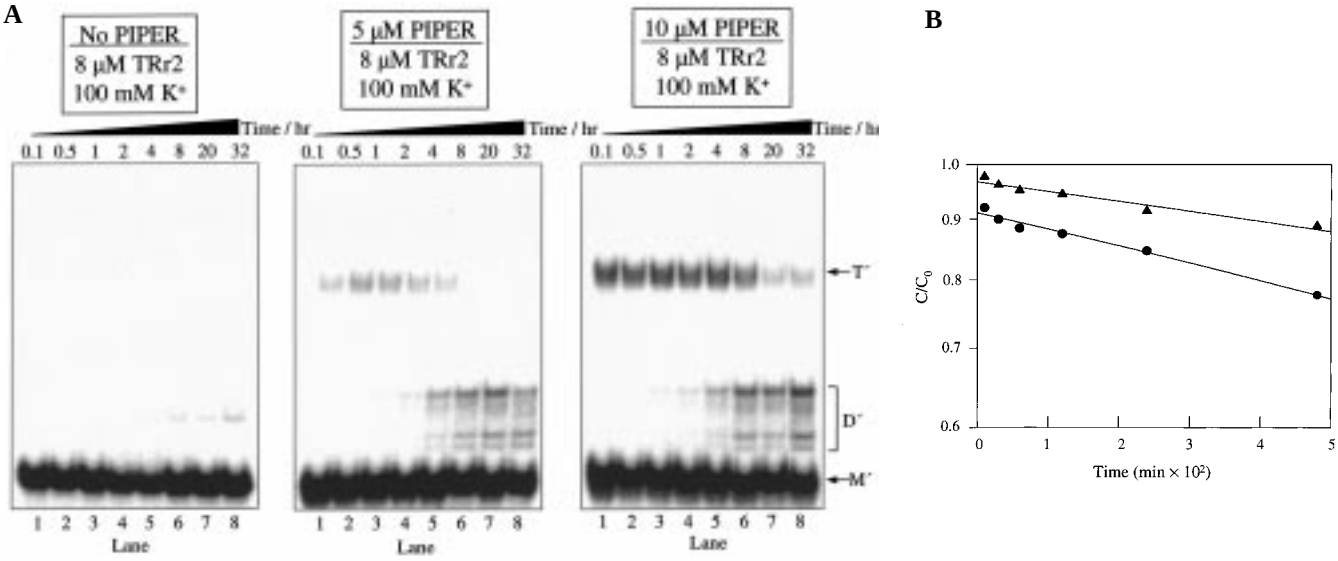


FIGURE 4: Kinetics of the PIPER-assisted assembly of G-quadruplexes from TRr2 determined by native PAGE. (A) 8 μM TRr2 was incubated at room temperature for specified times in 100 mM KCl/TE buffer with either 0, 5, or 10 μM PIPER (left, center, and right panels, respectively). (B) Rate constants of dimeric G-quadruplex formation in the absence and presence of PIPER were derived by plotting the ratio of nondimeric (monomer plus tetramer) TRr2 concentration to total TRr2 concentration (C/C_0) versus time. The *Y*-axis is in logarithm scale. The first-order rate constants for the dimerization reaction assisted by 5 μM (▲) and 10 μM (●) PIPER were 0.012 and 0.018 h^{-1}, respectively.

dimeric intermediates, respectively. Although the precise mechanism by which PIPER acts has yet to be established, we propose that its behavior as a polynucleotide driver is due to a propensity to *accelerate the association of G-rich oligonucleotides*. Consequently, isomerization reactions become the rate-limiting events in G-quadruplex assembly, and overall formation rates thereof are greatly enhanced.

Although the in vivo existence of the G-quadruplex remains a matter of debate, such structures are potential targets for the rational design of novel antitumor agents (*31*). Considerable progress has been made in the design and testing of G-quadruplex-interactive compounds, some of which demonstrably slow the growth of tumor cells (*32*) and induce the formation of anaphase bridges (*33*). Findings presented here imply that G-quadruplex-interactive compounds may elicit biological effects by promoting the formation of G-quadruplex structures in a manner analogous to that of chaperone proteins, such as TBP-β. In addition to the disruption of telomerase activity by rendering single-stranded telomeric sequences inaccessible, the induction and stabilization of G-quadruplexes within G-rich regions of the genome may compromise the integrity of the telomeres or alter gene expression. Experiments are in progress to test these postulates.

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