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Synthesis and Spectroscopic Studies of the Aminoglycoside (Neomycin)−Perylene Conjugate Binding to Human Telomeric DNA

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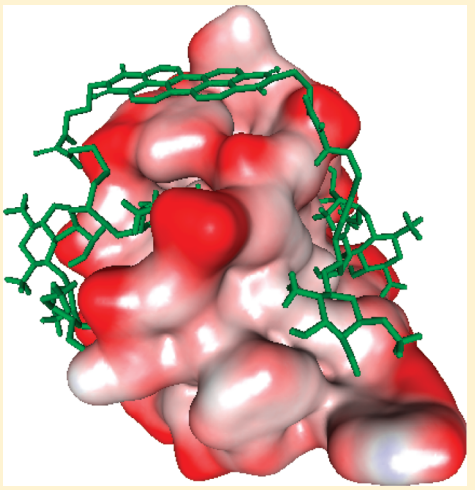
Supporting Information

ABSTRACT: Synthesis of a novel perylene−neomycin conjugate (**3**) and the properties of its binding to human telomeric G-quadruplex DNA, 5′-d[$AG_3(T_2AG_3)_3$] (**4**), are reported. Various spectroscopic techniques were employed to characterize the binding of conjugate **3** to **4**. A competition dialysis assay revealed that **3** preferentially binds to **4**, in the presence of other nucleic acids, including DNA, RNA, DNA−RNA hybrids, and other higher-order structures (single strands, duplexes, triplexes, other G-quadruplexes, and the i-motif). UV thermal denaturation studies showed that thermal stabilization of **4** increases as a function of the increasing concentration of **3**. The fluorescence intercalator displacement (FID) assay displayed a significantly tighter binding of **3** with **4** as compared to its parent constituents [220-fold stronger than neomycin (**1**) and 4.5-fold stronger than perylene diamine (**2**), respectively]. The binding of **3** with **4** resulted in pronounced changes in the molar ellipticity of the DNA absorption region as confirmed by circular dichroism. The UV−vis absorption studies of the binding of **3** to **4** resulted in a red shift in the spectrum of **3** as well as a marked hypochromic change in the perylene absorption region, suggesting that the ligand−quadruplex interaction involves stacking of the perylene moiety. Docking studies suggest that the perylene moiety serves as a bridge that end stacks on **4**, making contacts with two thymine bases in the loop, while the two neomycin moieties branch into the grooves of **4**.



Over the past few decades, a large number of human and other eukaryotic chromosomal end sequences (also known as telomeric sequences) have been identified.[1] These telomeric ends are uniquely characterized with the abundance of repetitive guanine bases. It is now well established that telomeric DNAs, under physiological conditions, can adopt unique structures called G-quadruplexes.[2] G-Quadruplexes are formed by stacking G-quartets. The edges of guanine bases can serve as H-bonding donors (N2 and N1) or acceptors (O6 and N7), and a coplanar arrangement (G-quartet) results among four guanines via Hoogsteen H-bonds (Figure 1) in an inter- or intramolecular arrangement. In humans, the telomeric ends consisting of a tandem hexameric 5′-d(TTAGGG) repeat unit are mostly duplex in nature, yet the extreme 3′-ends are single-stranded overhangs. The 3′-end of the chromosomal DNA, apart from capping the chromosal ends and prohibiting the end to end fusion processes,[3] also plays the role of primer sequence for the reverse transcriptase activity of an enzyme called telomerase.[4] Telomerase, which remains dormant in normal somatic cells, assumes the role of an "evil enzyme" by conducting unwanted repair of telomeric ends in cancer cells, thereby making them immortal. Both the duplex and single-stranded regions of the telomers, in conjunction with telomere end binding proteins such as TFRF1, TRF2, POT1, and other assisting proteins, form the so-called "telosome" complex and have been proposed to play an important role in the interaction of telomerase at the ends of chromosomes.[5] Intervention of telomerase interaction at the telomeric ends has become one of the popular approaches to control cancer proliferation.

Formation of G-quadruplexes has been shown to inhibit the activity of telomerase, which plays a pivotal role in the proliferation of tumor cells[6] and has become a potential therapeutic target in oncology. For more than a decade, small molecules have received wide attention because of their ability to interact and stabilize G-quadruplex DNA structures as well as their potential to inhibit telomerase activity. Ligands that facilitate G-quadruplex formation and subsequently inhibit telomerase activity could be potential anticancer drugs.[7] A large number of small molecule G-quadruplex binders have emerged since the early reports of telomerase inhibition from the laboratories of Hurley and Neidle.[8,9] One such small molecule, telomostatin, has been reported to have minimal inhibitory concentration for telomerase inhibition as low as 5 nM.[10] Various other ligands that contain fused aromatic platforms containing heteroatoms in most cases have been reported to interact with G-quadruplex structures. For instance, quinacridines,[11] BRACO-19,[12] porphyrins,[13] and telomostatin-based macrocycles[14,15]

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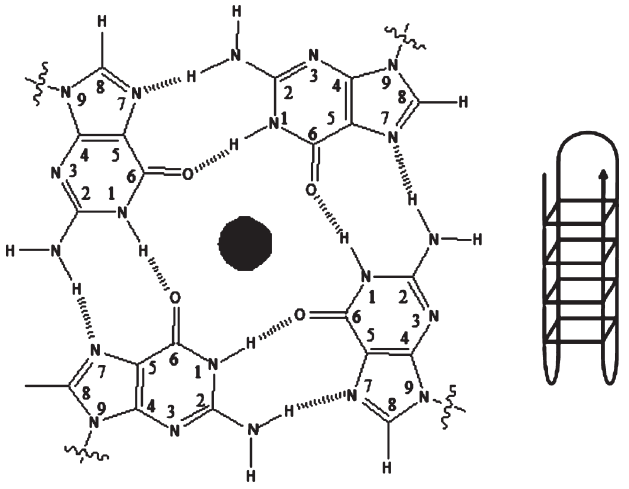

Figure 1. Hydrogen bonding interactions (shown by dashed lines) present in a G-quartet. The guanine bases use the Hoogsteen face for hydrogen bonding. The circle (colored black) in the middle of G-quartet represents a metal cation.

are a few examples of molecules that preferentially bind G-quadruplexes. A recent review by Monchaud et al. describes the current state of G-quadruplex binding ligands.[16]

Most ligands that bind to G-quadruplexes have been proposed to end stack or form interactions in the loops.[17,18] On the other hand, molecules that selectively recognize G-quadruplex grooves are much more limited, and features that allow ligands to be G-quadruplex groove specific are poorly understood.[19] Discovery of G-quadruplex groove specific binders is, thus, an important endeavor for two prime reasons: (i) to develop ligands that advance the knowledge of recognition principles of quadruplex grooves and (ii) to generate conjugates with mulitple moieties that could bind simultaneously through groove recognition and stacking interactions, thus leading to ligands with higher affinities and specificities. Dual recognition allows us to conveniently develop potent ligands by exploiting the noncompetitive binding[20] of individual pharmacophores for the nucleic acid target. We have recently reported that using such a design, specific and high-affinity ligands can be developed. These include pyrene−neomycin,[21] BQQ−neomycin,[22] and anthraquinone−neomycin[20] conjugates for binding DNA triplexes and a methidium−neomcyin conjugate designed to target hybrid nucleic structures with subnanomolar affinities.[23]

Aminoglycosides are aminosugars, long known for their effective antiobiotic action. They have been largely known for interacting with bacterial rRNA (A-site). They have, however, been shown to bind other nucleic acid targets.[24,25] Recent studies have shown that apart from its euabacterial RNA target, neomycin (and other aminoglycosides) also binds to various nucleic acids, including DNA triplexes,[25−29] RNA triplexes,[26,27,29] hybrid nucleic acids,[23,26] and single strands.[30] We have shown that aminoglycosides prefer to bind targets that share characteristics of A-form nucleic acids[31] irrespective of the parent nucleic acid (DNA or RNA). Recently, we have developed various neomycin conjugates by tethering neomycin with other DNA binding moieties or oligonucleotides, because neomycin also aids lipid-mediated oligonucleotide delivery.[32] Such conjugates recognize different nucleic acid structures[21,22,33−40] with binding affinities much higher than those of their parent constituents.[20] Recent studies have shown that neomycin and paromomycin bind in the wide groove of G-quadruplex DNA (formed from the telomeric sequence of *Oxytricha nova*) with moderate affinities ($K_a \sim 10^5$ M^{-1}).[41] Neomycin binds with this G-quadruplex DNA in a 1:1 stoichiometry and forms a single ion pair with the quadruplex, as determined by salt-dependent studies. Our studies revealed that neomycin binds to both parallel and antiparallel G-quadruplexes with differing affinities, and the binding of neomycin to the antiparallel quadruplex occurs in the wide groove. A recent study has shown that aminoglycosides can inhibit telomerase activity.[42] In light of these findings, we herein report the development of a perylene−neomycin conjugate (**3**) (Figure 2) and its ability to specifically recognize human telomeric G-quadruplex DNA (**4**) using spectroscopic techniques.

Human telomeric DNA is highly polymorphic in nature and can adopt various G-quadruplex confomations.[43] For instance, in the presence of sodium ions, 5′-d[AGGG(TTAGGG)$_3$] (**4**), a fragment of human telomeric DNA, adopts an antiparallel structure that contains both lateral and diagonal loops.[44] Different structures of the quadruplex have been reported on the basis of the technique (NMR or X-ray),[44,45] the salt used (Na^+ vs K^+), or the flanking bases.[46−50] Nevertheless, because of the importance of developing inhibtors of human telomeric DNA, ligand binding to this structure continues to be examined[51] and submicromolar inhibition of telomerase by a macrocycle-capped neomycin has been reported.[52] In this report, we show that by covalently linking two neomycin units to a perylene diimide unit, a ligand that specifically binds to **4** is obtained and its binding to **4** is better than that of each of its constituent units.

MATERIALS AND METHODS

General Methods. Unless otherwise specified, chemicals were purchased from Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used without further purification. Neomycin B trisulfate was purchased from MP Biomedicals (Solon, OH). Di-*tert*-butyl dicarbonate (Boc anhydride) was purchased from Advanced ChemTech (Louisville, KY). All solvents were purchased from VWR (West Chester, PA). Silica gel (32−65 μM mesh size) was purchased from Sorbtech (Atlanta, GA). Reactions were conducted under N_2 using dry solvent, unless otherwise noted. ^{1}H NMR spectra were recorded on a JEOL (Tokyo, Japan) ECA 500 MHz FT-NMR spectrometer or Bruker Avance-500 spectrometer. MS (MALDI-TOF) spectra were recorded using a Kratos analytical (Columbia, MD) KOMPACT SEQ mass spectrometer or Bruker Daltonics/Omniflex MALDI-TOF spectrometer. UV spectra were recorded on a Varian (Walnut Creek, CA) Cary 100 Bio UV−vis spectrophotometer equipped with a thermoelectrically controlled 12-cell holder. Circular dichroism spectra were recorded on a JASCO (Easton, MD) J-810 spectropolarimeter equipped with a thermoelectrically controlled cell holder. Fluorescence spectra were recorded on a Fluomax-3 (Jobin Yvon, Edison, NJ) or Photon Technology International (Lawrenceville, NJ) instrument.

Nucleic Acids. The concentrations of nucleotide solutions were determined using the extinction coefficients (per mole of nucleotide) calculated according to the nearest neighbor method. The concentrations of all the polymer solutions were determined spectrophotometrically using the following extinction coefficients (in units of moles of nucleotide per liter per centimeter): ε_{265} = 9000 for poly(dT), ε_{260} = 6000 for poly(dA)·poly(dT), ε_{253} = 7400 for poly(dG)·poly(dC), and ε_{262} = 6600 for poly(dA-dT)·poly(dA-dT). In all cases where mentioned, the term r_{db} refers to the drug:base molar ratio. Nucleic acid solutions were prepared in either phosphate buffer [8 mM

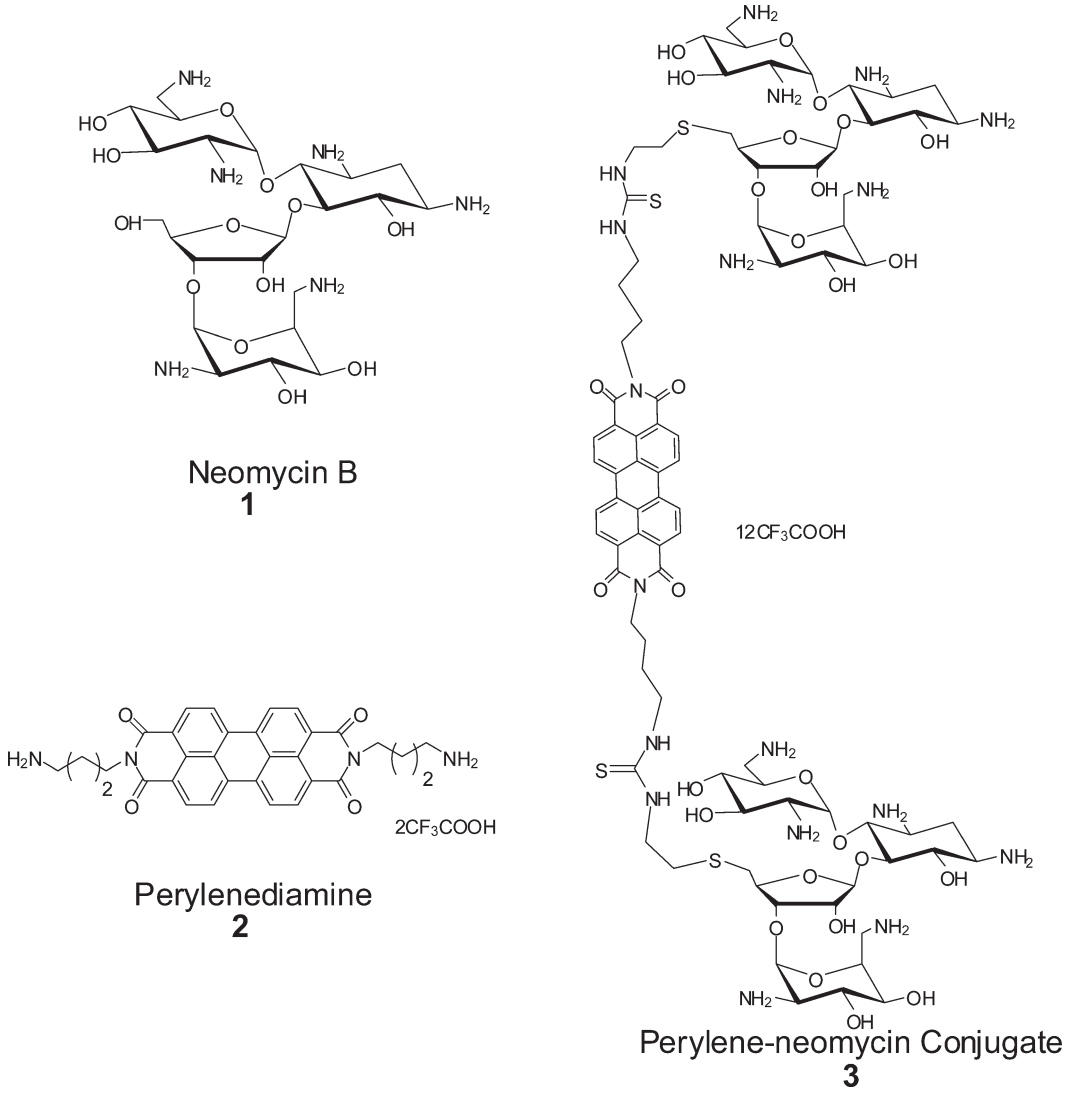

Figure 2. Structures of ligands used in this study.

Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0)] or cacodylate buffer [10 mM sodium cacodylate, 0.5 mM EDTA, and 100 mM NaCl (pH 7.0)]. The quadruplex was formed by heating the human telomeric DNA at 95 °C for 20 min followed by slow cooling to room temperature. The stock solution was then kept at 4 °C for at least 2 days before being used. The formation of the quadruplex was checked by CD spectroscopy.

The numbering adopted for the synthesis of compounds given below is shown in Scheme 1 (**6**). In a 25 mL round-bottom flask, *N*-*tert*-butoxycarbonylbutyl-1,4-diamine (0.92 g, 4.9 mmol) and zinc acetate dihydrate (1.0 g) were added to a suspension of **5** (707 mg, 1.8 mmol) in anhydrous pyridine (15.0 mL), and the mixture was refluxed overnight under an atmosphere of N_2. Thin layer chromatography (TLC) using silica gel showed the completion of the reaction. The reaction mixture was then concentrated. Flash chromatography of the residue [silica gel, 5% (v/v) CH_3OH in CH_2Cl_2] yielded the desired product **6** as a dark red solid (200 mg): $R_f = 0.71$ [silica gel, 10% (v/v) CH_3OH in CH_2Cl_2]; MS (MALDI-TOF) *m*/*z* calcd for $C_{40}H_{40}N_4O_8Na$ ($M + Na^+$) 755.8, found 756.5. This product was taken to the next step without further characterization.

Method A (**2**). **6** (20 mg, 0.028 mmol) was dissolved in TFA (2.0 mL) and the solution stirred for 3 h at room temperature. The reaction mixture was concentrated, and the residue was portioned between deionized (DI) water (30 mL) and ether (30 mL). The aqueous layer was washed twice with ether (30 mL) and concentrated to dryness to yield the desired product **2** in quantitative yield (21.2 mg, 15% for two steps).

Method B (**2**). To a suspension of 3,4,9,10-perylenetetracarboxylic dianhydride (250 mg, 0.64 mmol) in benzene (10 mL) was added putrescine (500 mg, 5.66 mmol). The reaction mixture was refluxed for 3 h, filtered, and washed with benzene. The solid obtained was added to KOH (5 M, 10 mL) and the mixture stirred at room temperature for 8 h. The reaction mixture was filtered, washed with water, and dried. The resulting solid was dissolved in HCl (12 M, 10 mL) and filtered. The filtrate was added to ethanol (200 mL), and the resulting solid was collected by centrifugation and washed with ethanol (50 mL) to yield a dark brown solid (254 mg, 75%): mp >320 °C dec; IR (KBr) 2925, 1682, 1337, 1140 cm^{-1}; ^1H NMR (500 MHz, CF_3COOD) δ 8.78−8.76 (d, 4H, $J = 7.8$ Hz), 8.73−8.72 (d, 4H, $J = 7.8$ Hz), 6.71 (s, br, $N\underline{H}_2$, 4H), 4.36−4.28 (t, br, $NC\underline{H}_2CH_2$, 4H), 3.34−3.26 (t, br, $CH_2C\underline{H}_2NH_3^+$, 4H), 1.96−1.88 (m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$, 8H); MS (MALDI-TOF) *m*/*z* calcd for $C_{32}H_{29}N_4O_4$ ($[M + H]^+$) 533.59, found 533.54.

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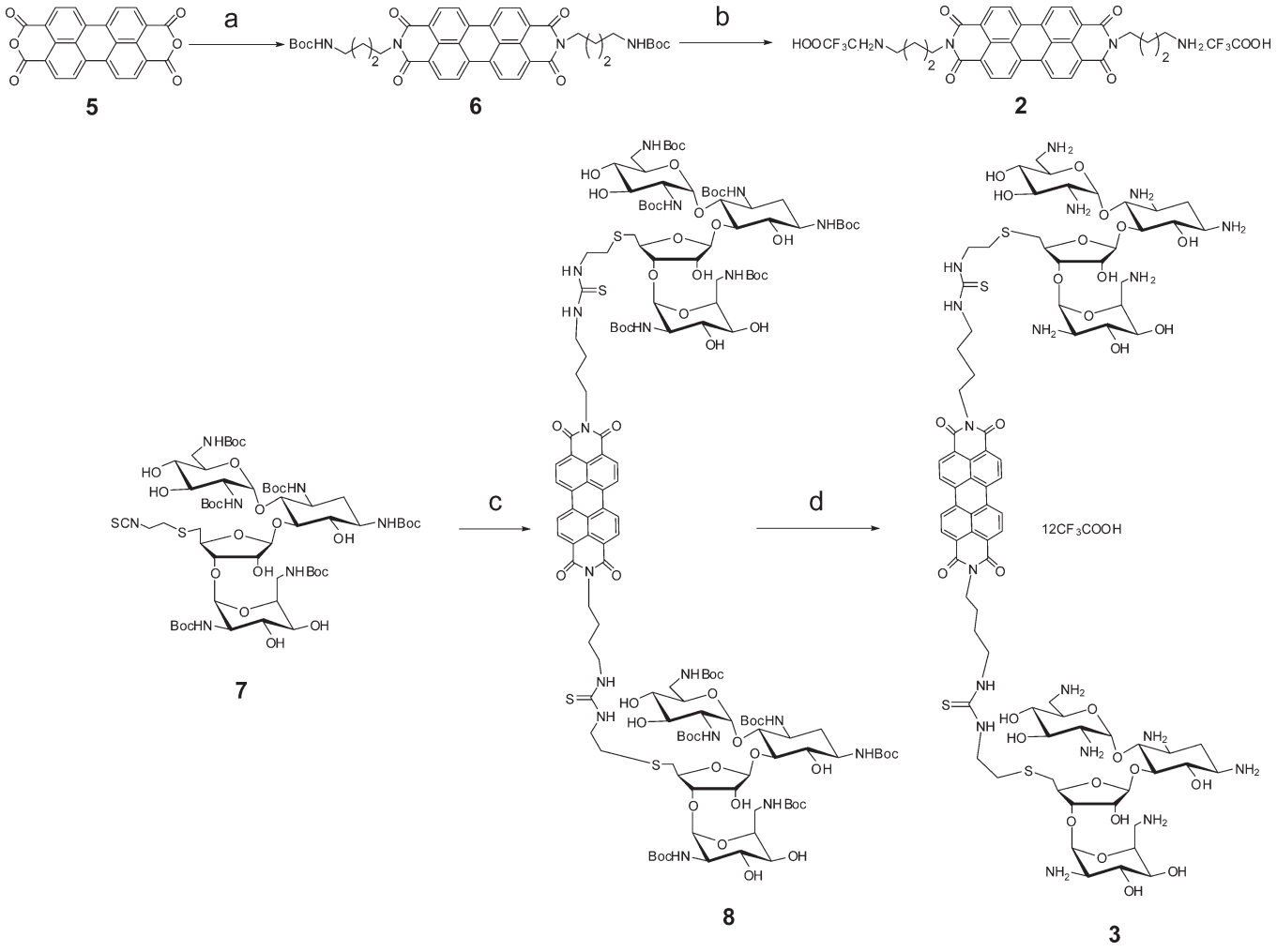
Scheme 1. Synthesis of Perylene−Neomycin Conjugate 3[a]



[a] Reagents and conditions: (a) $NH_2(CH_2)_4NHBoc$, pyridine, reflux, 5 h; (b) TFA, 3 h, room temperature, quantitative; (c) **2**, DMSO/pyridine, 60 °C, overnight; (d) TFA/CH_2Cl_2 (50:50), room temperature, 3 h (32.5% overall yield for steps c and d).

Method A (***3***). In a 25 mL round-bottom flask, **2** (4.2 mg, 5.5 μmol) and 4-dimethylaminopyridine (catalytic amount) were added to a DMSO/pyridine [4 mL, 1:1 (v/v) DMSO:pyridine ratio] solution of **7** (21 mg, 15.9 μmol), and the mixture was stirred overnight under an atmosphere of N_2 at 60 °C. Completion of the reaction was monitored by TLC using silica gel. The reaction mixture was then concentrated. Flash chromatography of the residue [silica gel, 6% (v/v) CH_3OH in CH_2Cl_2] yielded the desired product **8** as a red solid (7.1 mg). The product obtained was taken to the deprotection step without further characterization. Compound **8** (7.1 mg) was dissolved in a TFA/CH_2Cl_2 mixture [2 mL, 1:1 (v/v)] and stirred for 3 h at room temperature. The reaction mixture was concentrated, and the residue was partitioned into DI water (20 mL) and ether (20 mL). The aqueous layer was washed twice with ether (20 mL) and concentrated by lyophilization to yield the desired product **3** (3.9 mg, 32.5% overall yield for two steps).

Method B (***3***). To a solution of **2** (4.0 mg, 6.66 μmol) in a mixture of DMSO and DMF [12 mL, 2:1 (v/v)] was added triethylamine (60 μL), and the mixture was stirred at room temperature for 15 min under an atmosphere of argon. To this mixture was added a solution of **7** (20 mg, 15.2 μmol) in DMSO (2.0 mL) followed by addition of a catalytic amount of 4-dimethylaminopyridine in CH_2Cl_2 (0.5 mL). The mixture was heated at 80 °C overnight. TLC using silica gel indicated formation of the product [R_f = 0.55 in a 9:1 (v/v) CH_2Cl_2/CH_3OH]. Volatiles were removed under a stream of dry nitrogen, and the crude product thus obtained was purified on a silica gel column using a gradient of CH_2Cl_2 and CH_3OH as eluent. The desired product **8** was obtained as a red solid (15 mg). This product was taken to the deprotection step without further characterization. Compound **8** (15 mg 4.7 μmol) was dissolved in a TFA/CH_2Cl_2 mixture [3 mL, 1:1 (v/v)] and stirred at room temperature for 4 h. The reaction mixture was concentrated, and the residue was dissolved in DI water (4 mL) and washed three times with ether (5 mL). The aqueous layer was lyophilized to yield the desired product **3** (8.1 mg, 78% yield for two steps): UV λ = 220, 293, 500, 535 nm; ε (500 nm) = 36283.8 M^{-1} cm^{-1}; ^1H NMR (500 MHz, CF_3COOD) δ 8.37−8.34 (br, 2H), 8.17−8.16 (d, 2H, J = 6.5 Hz), 7.44−7.41 (br, 4H), 6.44−6.22 (br, 2H), 5.72−5.69 (br, 4H), 4.67−4.40 (20H), 4.29−3.55 (24H), 3.38−3.32 (m, 6H), 3.20−3.11 (4H), 3.08−2.88 (6H), 2.66−2.58 (d, br, 2H), 1.72−1.56 (2H), 1.47−1.44 (t, 8H, J = 7.35 Hz), 1.35−1.32 (t, 6H, J = 7.35 Hz), 1.04−1.02 (m, 2H);

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^{1}H NMR (500 MHz, D_2O) δ 7.79−7.76 (br, 2H), 7.60−7.59 (d, 2H, J = 7.3 Hz), 6.71−6.67 (br, 4H), 6.02−5.95 (br, 2H), 5.36−5.31 (br, 2H), 5.23 (s, br, 2H), 4.78−4.60 (peaks masked by the residual HDO peak), 4.40−4.21 (8H), 4.15 (s, br, 2H), 4.03 (t, 2H, J = 9.1 Hz), 3.96−3.90 (t, 2H, J = 9.6 Hz), 3.88−3.80 (4H), 3.75 (s, br, 4H), 3.68−3.59 (2H), 3.56−3.18 (20H), 3.15−3.05 (6H), 2.80−2.60 (6H), 2.46−2.38 (2H), 1.89−1.79 (m, 2H), 1.22−1.03 (6H), 0.85−0.79 (m, 2H); MS (MALDI-TOF) m/z calcd for $C_{84}H_{126}N_{18}O_{28}S_4$ ($[M]^+$) 1961.70, found 1959.83.

Ultraviolet (UV) Spectroscopy. Spectrophotometer stability and wavelength alignment were checked prior to initiation of each melting point experiment. For all experiments, the samples were prepared by dilution of a stock sample that was formed by heating the DNA to 95 °C followed by slow cooling back to room temperature. The melting of DNA with and without the ligand was conducted at a heating rate of 0.2 °C/min. Samples were brought back to 20 °C after each run. All UV melting experiments were monitored at 260 nm. For the T_m determinations, derivatives were used. Data were recorded every 1.0 °C. The UV melting experiments were conducted in phosphate buffer [8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0)].

Competition Dialysis. Competition dialysis experiments were performed in a MINI dialysis flotation device. For each assay, 180 μL of different nucleic acids (75 μM per monomeric unit of each polymer) was placed in the dialysis units and then dialyzed against 400 mL of a 1 μM ligand solution in phosphate buffer for 24 h at ambient temperature (20−22 °C). At the end of the experiment, the nucleic acid samples were carefully removed to microfuge tubes and were taken to a final sodium dodecyl sulfate (SDS) concentration of 1% (w/v). Each mixture was allowed to equilibrate for 2 h. The concentration of the ligand after dialysis was determined by fluorescence spectroscopy. An appropriate correction was made to account for volume changes. The amount of bound drug was determined by difference ($C_b = C_t - C_f$, where C_f is the concentration of bulk drug solution, C_t is the total drug concentration that enters the membrane, and C_b is the concentration of drug bound to the nucleic acids). The data were plotted as a bar graph using Kaleidagraph version 3.5 (Synergy Software). A calibration curve was made before each experiment by plotting the fluorescence intensities at characteristic ligand wavelengths versus corresponding ligand concentrations. C_t and C_f were determined on the basis of a calibration curve.

Circular Dichroism (CD) Spectroscopy. All CD experiments were conducted at 20 °C in cacodylate buffer [10 mM sodium cacodylate, 0.5 mM EDTA, and 100 mM NaCl (pH 7.0)]. We observed that less perylene aggregation was observed in cacodylate buffer than in phosphate buffer. The CD spectra were recorded as a function of wavelength (200−350 nm) and are averages of 25 scans. For CD titrations, aliquots of a stock solution of **3** were added to the preformed quadruplex of **4** (10 μM) to reach the desired concentrations. After each addition, the solution was mixed by magnetic stirring and then allowed to equilibrate for 5 min prior to a scan. Data processing was conducted using Kaleidagraph version 3.5.

Fluorescent Intercalator Displacement (FID) Titrations. All fluorescence titration experiments were performed at 20 °C in cacodylate buffer [10 mM sodium cacodylate, 0.5 mM EDTA, and 100 mM NaCl (pH 7.0)]. The DNA solutions (2.0 mL) were prepared by mixing G-quadruplex **4** (0.25 μM/strand) and thiazole orange (TO, 0.50 μM). Aliquots of a stock solution of **3** were added to the **4**/TO solution to reach the desired concentrations and allowed to equilibrate for 4 min prior to the fluorescence recording (excitation at 501 nm and emission at 510−650 nm). The slit width used was 3.0 mm. The $^{G4}DC_{50}$ values were determined by fitting the experimental data using a dose−response curve (Origin version 5.0). $^{G4}DC_{50}$ represents the amount of ligand required to displace 50% of bound thiazole orange from a G-quadruplex target.

Fluorescence Titrations. The titration assays were performed in a 3 mL quartz cuvette at room temperature (22−24 °C) in phosphate buffer [8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0)] or cacodylate buffer [10 mM sodium cacodylate, 0.5 mM EDTA, and 100 mM NaCl (pH 7.0)]. Aliquots of a stock solution of G-quadruplex **4** were added to a solution of **3** (33 nM in phosphate buffer or 0.25 μM in cacodylate buffer) and allowed to equilibrate for 5 min before the fluorescence was recorded (excitation at 500 nm for phosphate buffer and 496 nm for cacodylate buffer and emission at 530−750 nm). The data were then processed using Kaleidagraph version 3.5.

Molecular Modeling. All dockings were performed as blind dockings (blind docking refers to the use of a grid box that is large enough to encompass any possible ligand−receptor complex) using Autodock Vina version 1.0. All rotatable bonds within the ligand were allowed to rotate freely, and the receptor was considered rigid. The structure of G-quadruplex **4** was obtained from Protein Data Bank entry 143D. All ligand structures were created using Discovery Studio Visualizer version 2.5 and energetically minimized with the Vega ZZ program[53] using a conjugate gradient method with an SP4 force field. The optimized ligand and G-quadruplex structures were then converted into proper file formats for AutoDock Vina using Autodock Tools version 1.5.4.[54] The docking validation (correctly identifying the binding site and scoring the receptor−ligand interactions) was confirmed by using AutoDock Vina to accurately predict the binding of different ligands to their previously established targets.

RESULTS AND DISCUSSION

Ligands that can stabilize G-quadruplexes are currently under intense investigation because of their possible roles in the inhibition of telomerase activity. While a number of ligands that interact with different G-quadruplexes have been discovered in the past decade, ligands with high selectivity for G-quadruplex grooves are limited in number.[55] Most of the G-quadruplex ligands contain planar aromatic moieties and stack to G-quadruplexes with moderate binding affinities. Ligands that can bind the quadruplex in the grooves are scant,[56] and not much is known about their biophysical, biochemical, and structural properties. Herein, we report a G-quadruplex ligand **3** containing both a groove binder and an intercalating unit that selectively recognizes human telomeric DNA **4**. The groove widths and depths present in the quadruplex vary significantly from the groove widths of a DNA or RNA duplex. Therefore, a higher selectivity for quadruplex structures can be envisioned by targeting the quadruplex grooves. When this selectivity is combined with the differences in base stacking surface areas of a G-quadruplex versus a DNA duplex, ligands with much higher selectivity for the G-quadruplex can be designed.

Design and Synthesis of the Neomycin−Perylene Conjugate (3). Perylene diimide was chosen as an intercalating unit

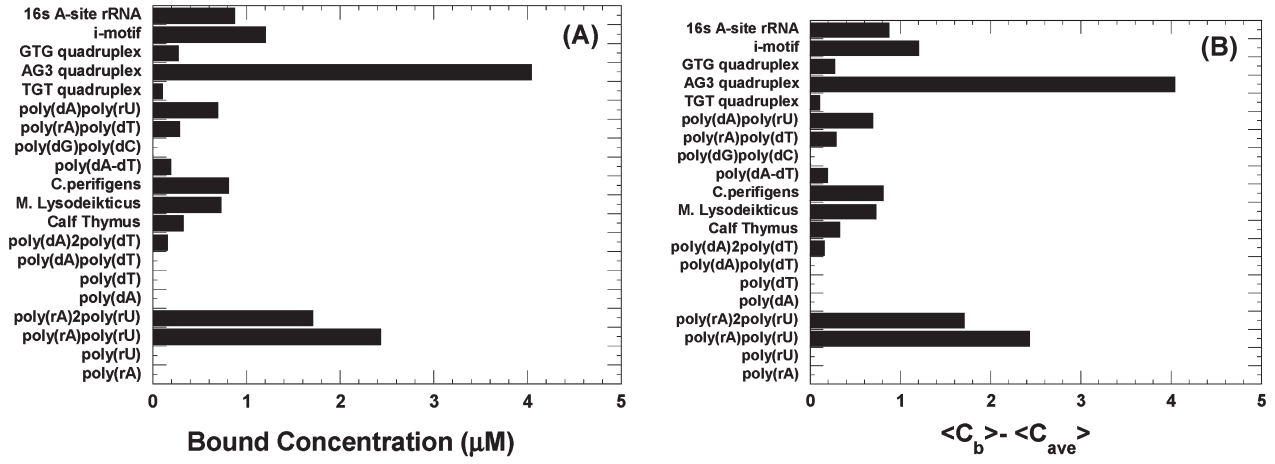

Figure 3. (A) Competition dialysis of various nucleic acids with 1 μM perylene−neomycin conjugate. (B) Difference plot for the perylene−neomycin conjugate. Different nucleic acids (180 μL, 75 μM per monomeric unit of each polymer) were dialyzed with an aqueous solution (400 mL) of **3** (1 μM) in 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0) for 24 h. The accumulated ligand in each dialysis tube was analyzed using fluorescence, and the bound concentrations were determined with the help of a calibration curve.

because its derivatives are known to bind human telomeric sequences such as **4** and inhibit telomerase activity.[8,57−59] A recent study showed that perylene diimide-containing polyamines bind to G-quadruplex DNA with polyamines residing in the grooves.[60] Perylene diimide was also chosen as a stacking moiety because side chains can be conveniently introduced into the scaffold without perturbing perylene's steric and electronic properties to a great extent. In this work, neomycin, an amino sugar, was attached to perylene diimide, making the resulting molecule **3** more water-soluble (hydrophilic) and thus easier to study. Because neomycin possesses moderate binding affinity for G-quadruplex DNA,[41] conjugation of neomycin with perylene diimide should yield a more potent G-quadruplex binding ligand that has a dual recognition mode. We also envisioned that conjugation of neomycin to perylene diimide could alter the known aggregation properties of perylene in aqueous solutions.[61] In addition, **3**, containing two neomycin units, is also expected to incorporate quadruplex groove binding. The synthesis of the desired conjugate was performed in a manner where both neomycin and perylene moieties were modified independently and then linked together using a thiourea linkage (Scheme 1). Compound **2** was synthesized by reacting 3,4,9,10-perylenetetracarboxylicdianhydride **5** with $BocNH(CH_2)_4NH_2$ in pyridine under reflux followed by removal of Boc protecting groups using trifluoroacetic acid (TFA). Neomycin isothiocyanate **7** was prepared using previously established procedures.[38] Conjugate **3** was synthesized by coupling **7** with **2** followed by removal of Boc groups using a TFA/CH_2Cl_2 mixture in moderate yields. Alternatively, compound **2** can be synthesized in one step from **5**, followed by coupling of **2** to **7** to give intermediate **8**, which can be then deprotected to give target ligand **3** in better yields (Scheme S1 of the Supporting Information).

Competition Dialysis Assay. Competition dialysis, an extension of equilibrium dialysis, has been developed by Chaires for the rapid screening of a ligand for its nucleic acid structure selectivity.[62] While a more generalized protocol was developed a decade ago,[63] a recent report has also highlighted its applications in sorting out G-quadruplex specific ligands.[64] To promptly assess the binding preference of conjugate **3**, competition dialysis experiments were conducted. In our experiment, 20 nucleic acids that represent a wide variety of structures were screened against conjugate **3**. The nucleic acids (whose formation was checked using CD and UV spectroscopy) were taken in dialysis tubes and then immersed in a common dialysate solution of **3**. The ligands were allowed to slowly diffuse into the dialysis tubes over a period of 24 h. The subsequent determinations of bound ligand concentrations (after the surfactant treatment) were conducted using fluorescence. The results from competition dialysis are presented in Figure 3. As seen in panels A and B, ligand **3** exhibits the strongest preference (bound concentration of 4 μM) for human telomeric sequence {AG3 quadruplex = 5′-d[$AGGG(TTAGGG)_3$], **4**} among all the nucleic acids. **3** binds to polynucleotide duplex and triplex RNA structures [poly(rA)·polyr(U) and polyr(A)·2polyr(U)] with observed bound concentrations of 2.4 and 1.6 μM, respectively. The binding of **3** to single-stranded DNA and RNA was not observed. Conjugate **3** does bind to native DNA duplexes (*Clostridium perfringens* and *Micrococcus lysodeikticus*), a hybrid duplex [poly(dA)·poly(rU)], and 16S A-site rRNA; however, the binding preference of **3** ($\leq$1 μM) for these nucleic acids is much weaker than the binding of **3** to **4**. Even more significantly, **3** shows an only weak preference for two of the other intermolecular G-quadruplexes (GTG quadruplex 5′-d[$G_{10}T_4G_{10}$] and TGT quadruplex 5′-d[$T_{10}G_4T_{10}$]), even though neomycin has been shown by us to have a weak affinity for **4**.[41] It is also noteworthy that **3** also binds with weak affinity ($\sim$1.2 μM) to the i-motif, a quadruplex structure formed between cytosines.

The competition dialysis assay clearly indicates preferential binding of conjugate **3** to human telomeric G-quadruplex DNA **4**. Moderate affinities of **3** for duplex and triplex RNA sequences can be attributed to the neomycin moiety, which is a strong binder of ribonucleic acids (A-form structures).[31] Nevertheless, conjugate **3** stands out for its predilection for human telomeric intramolecular G-quadruplex DNA **4**. It is noteworthy that **3** binds to intermolecular G-quadruplexes with different structures such as TGT quadruplex (parallel) and GTG quadruplex (antiparallel) approximately 40-fold less than to **4**, making it specific for the recognition of human telomeric quadruplex DNA.

The relative trend of preference for human telomeric DNA by **3** is consistent with the similar competition dialysis findings in

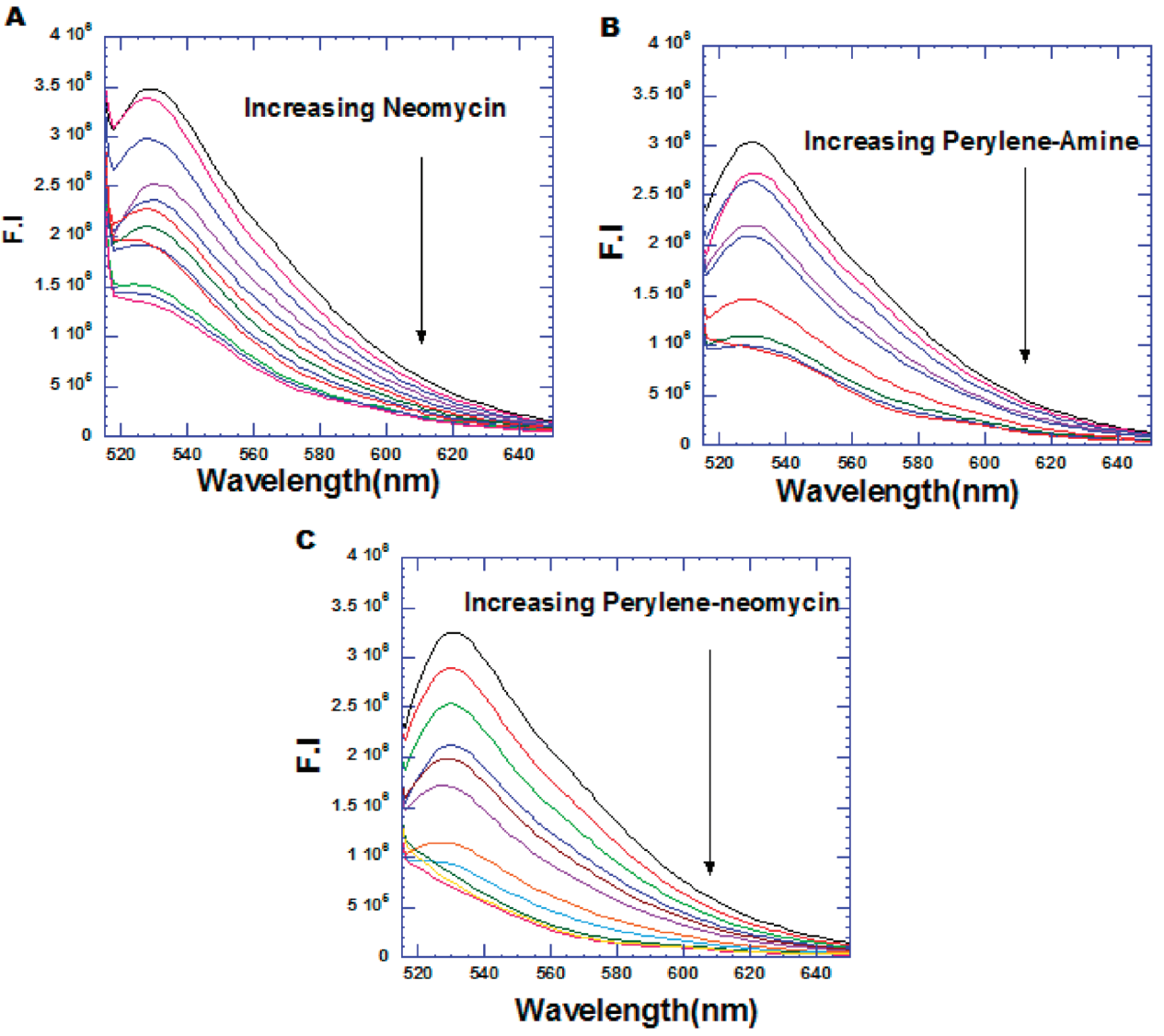

Figure 4. FID titration plots for ligands (A) **1**, (B) **2**, and (C) **3** in 10 mM sodium cacodylate, 0.5 mM EDTA, and 100 mM NaCl (pH 7.0). For each titration, human telomeric DNA (0.25 μM) was mixed with thiazole orange (0.50 μM) in a 1:2 ratio. The bound thiazole orange was then gradually displaced with test ligand until no more fluorescence change was observed. The obtained raw data were fitted with the dose−response curve using Origin version 5.0. All experiments were conducted at 20 °C. Thiazole orange excitation was achieved at 501 nm, and the resulting emission spectra were recorded between 510 and 650 nm.

the Chaires laboratory where another perylene derivative, PIPER, was found to have the strongest preference for the human telomeric DNA.[64]

Fluorescent Intercalator Displacement Assay. Competition dialysis described above unambiguously revealed the binding preference of **3** for human telomeric DNA **4**. However, the contribution of neomycin could not be realized from this technique given the lack of a chromophore in neomycin. We, therefore, utilized a fluorescent intercalator displacement (FID) assay[65] to compare the binding of **3** to **4** with its two parent constituents, neomcyin (**1**) and perylene diamine (**2**). The FID assay allowed us to gauge the contributions of each pharmacophore in conjugate **3**. The assay was originally used for identifying DNA duplex binding ligands[66] but has recently also been extended for screening quadruplex specific ligands.[67] In our experiments, thiazole orange (TO) was first bound to G-quadruplex **4**, and the prebound TO was successively displaced from **4** by addition of the ligand of interest with increasing amounts (Figure 4). The amount of ligand needed to displace 50% of the prebound TO is defined as $^{G4}DC_{50}$. The $^{G4}DC_{50}$ values for **1**−**3** are listed in Table 1.

Table 1. $^{G4}DC_{50}$ Values for 1−3 Derived from the FID Titrations As Shown in Figure 4

ligand	$^{G4}DC_{50}$ (μM)
1	9.48 ± 0.75
2	0.18 ± 0.02
3	0.04 ± 0.01

The $^{G4}DC_{50}$ value of conjugate **3** determined by the FID assay was 4.1-fold lower than that of **2** and 220-fold lower than that of neomycin, suggesting that the strong binding preference of **3** for G-quadruplex **4** predominantly results from the affinity of the perylene diimide moiety. A direct comparison between affinities

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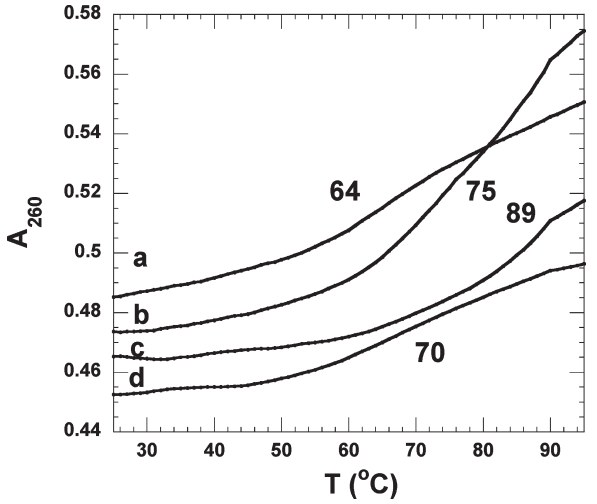

Figure 5. UV thermal denaturation profiles of G-quadruplex **4** at 260 nm in 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0): (a) no ligand, (b) **3** (2 μM), (c) **3** (10 μM), and (d) **3** (1 μM). The samples were heated to 95 °C and cooled to 20 °C at a rate of 0.2 °C/min. All UV melting experiments were monitored under 260 nm, and data points were recorded every 1.0 °C. For the T_m determinations, derivatives were used.

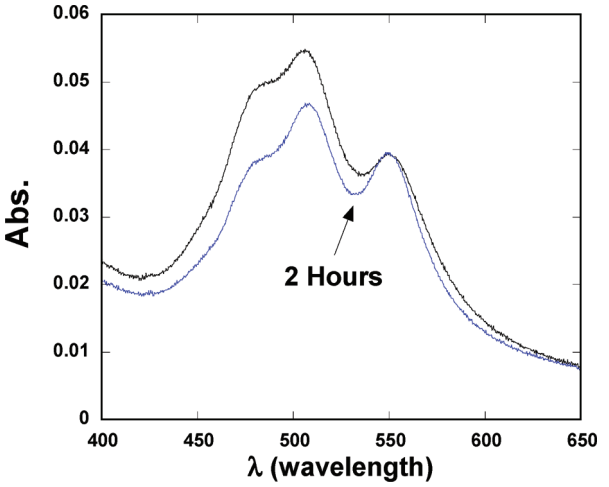

Figure 6. Absorption spectra of solutions of the perylene−neomycin conjugate in 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0): no quadruplex (black line) or in the presence of quadruplex 5′-d[$AG_3(T_2AG_3)_3$]-3′ (blue line). The spectrum was recorded at room temperature and corrected with buffer absorption.

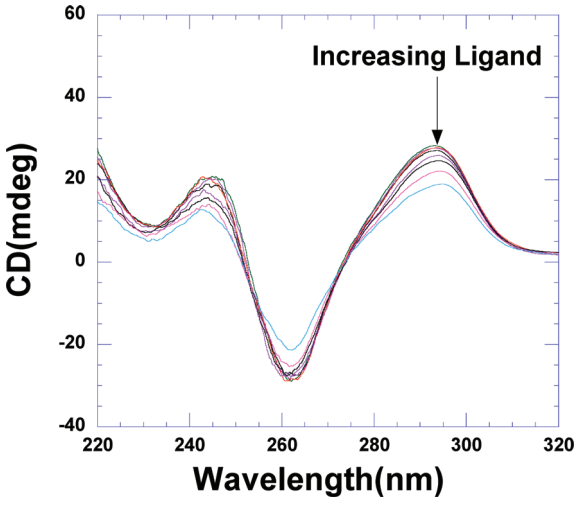

Figure 7. CD titration of G-quadruplex DNA **4** with **3** in 10 mM sodium cacodylate, 0.5 mM EDTA, and 100 mM NaCl (pH 7.0). Ligand was successively added to the DNA solution and the mixture stirred with a magnetic stirrer followed by equilibration for 5 min. Each CD measurement represents an average of 25 scans. The titration was performed at 20 °C.

and $^{G4}DC_{50}$ values cannot be made because the ligands have different stoichiometries of binding to the quadruplex. To exclude the possibility that the observed fluorescence decrease results from quenching by polyamines, we have previously shown that nonspecific binding of polyamines to a G-quadruplex does not result in a fluorescence change.[41]

The results obtained from FID assays were also consistent with the observation that both perylene amine **2** and conjugate **3** have a strong preference for binding to human telomeric DNA (Table 1). This also suggests that low $^{G4}DC_{50}$ values obtained for ligand **3** are also suggestive of a large contribution from the perylene moiety of the ligand to human telomeric DNA binding. The high concentrations required by neomycin to displace the probe are consistent with its low affinity for human telomeric DNA as also determined by ITC experiments.[41]

UV Thermal Denaturation Studies. The effect of **3** on the stabilization of G-quadruplex **4** was also investigated by UV thermal denaturation. The UV thermal denaturation curves of G-quadruplex **4** were recorded in the absence and presence of **3** (Figure 5; also see Figure S2 of the Supporting Information for derivative plots). The temperature at which **4** dissociates into random coils was noted as 64 °C in the absence of ligand. The denaturation temperature of **4** increased as a function of an increasing concentration of **3**. For instance, increments of 6, 12, and 25 °C were observed in the presence of 1, 2, and 10 μM **3**, respectively. The observed thermal stabilization of **4** by **3**, in addition to the binding studies, suggests strong binding of **3** with G-quadruplex **4**.

UV−Vis Absorption Studies. The UV−vis absorption spectra of conjugate **3** in the absence or presence of **4** were recorded. In the presence of **4**, the absorption spectrum of **3** shows a slight red shift (~5 nm) of λ_{max} at 504 nm, while the other major absorption peak at 550 nm remains unchanged (Figure 6). The peak at 504 nm also undergoes a marked hypochromic change (~14%). Such changes in the absorption spectra of perylene derivatives upon binding to G-quadruplex DNA have also been reported by other groups,[59,60,68,69] indicating the interactions between **3** and **4** occur through an intercalative or stacking fashion. The interaction of conjugate **3** with **4** is also time-dependent. The absorbance spectrum of **3** undergoes a gradual change after the addition of **4**, typically requiring 2 h to achieve equilibrium.

The UV absorption studies show a marked hypochromic shift in the perylene absorption at 500 nm, similar to previous studies,[60] but in contrast with other results.[68] Such hypochromic changes in absorbance have been seen in drug-quadruplex stacking interactions. For instance, numerous porphyrin derivatives that bind to G-quadruplexes by stacking interactions have pronounced hypochromicities upon ligand binding.[70−72] The apparent differences in the absorption behavior between different perylene ligands can be attributed to the different ligand aggregation properties, concentrations of ligands used, and

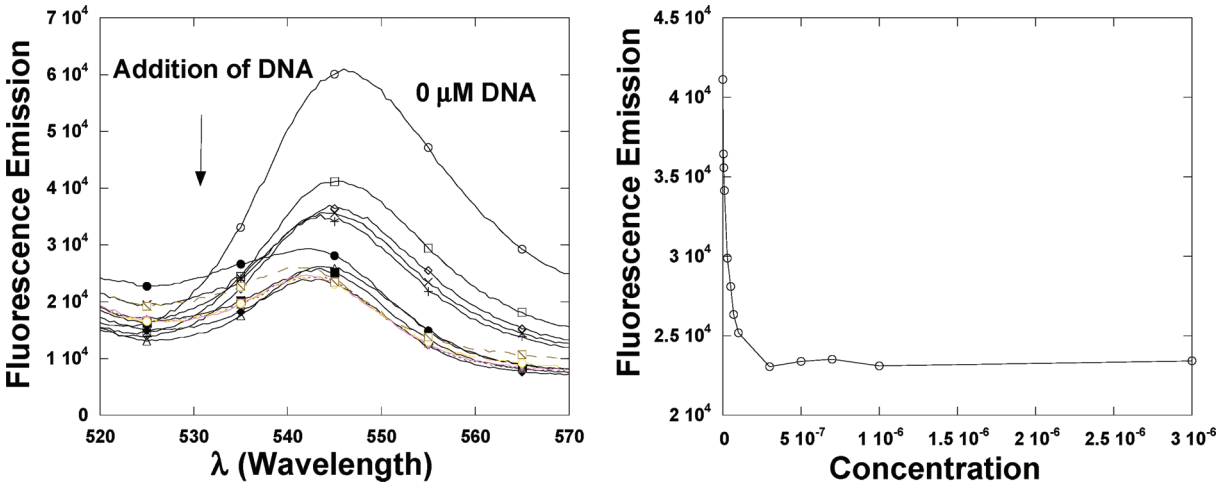

Figure 8. Fluorescence titration of **3** (33 nM) with **4** in 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl (pH 7.0). The concentration of the quadruplex DNA stock solution was 60 μM per strand for titration. The concentration of DNA in the solution varied with each addition of DNA, ranging from 10^{-9} to 10^{-5} M. Ligand **3** was excited at a wavelength of 500 nm, and the resulting emission was recorded from 530 to 750 nm. The raw data were plotted using Kaleidagraph version 4.0. The titration was performed at room temperature (~22 °C).

solution conditions employed. Unlike the perylene ligands previously reported,[68] **3** can also be classified as a more "rigid" perylene derivative because the movement of the perylene is restricted by groove binding of neomycin. The perylene derivatives previously reported have much smaller linkers to reach the qudruplex grooves. In contrast, conjugate **3** has a longer linker that allows binding of two neomycins (the two neomycin units contribute to a total of 12 amino groups). A recent report aimed at studying the effect of straight chain polyamine side chains has proposed a similar groove binding of polyamine ends.[60]

Circular Dichroism (CD) Spectroscopic Studies. To further characterize the binding of **3** with G-quadruplex **4**, CD titration experiments were conducted. The CD spectra of **4** were recorded after aliquots of **3** were successively added to a solution of **4**. In the absence of **3**, the CD spectrum of **4** shows a large positive band at 295 nm and a negative band at 260 nm, suggesting an antiparallel quadruplex structure in the presence of Na^+, which is consistent with previous studies[73] (Figure 7). The absence of any shoulder peaks at ~280 nm is also indicative of single predominant antiparallel quadruplex species in the solution. Addition of **3** to the solution resulted in a decrease in the CD intensity of the band at 295 nm and an increase in the CD intensity at 260 nm, indicating interaction of **3** with **4**. The binding of **3** also resulted in a slight red shift of the band at 295 nm in the spectra of **4**. Taken together, the changes in the CD intensity of the 295 and 260 nm bands as well the slight red shift in the band at 295 nm suggest the complexation of **3** with **4**. Cacodylate buffer was used in the CD titration experiments because the observed aggregation of **3** was less severe in cacodylate buffer than in phosphate buffer. Conjugate **3** was observed to coagulate into dots of red colored solids at high concentrations. The results presented here reflect the CD scans of the ligand−DNA complex free from such coagulation effects. Aggregation is a commonly observed property of perylene-based compounds and has also been proposed to mediate G-quadruplex binding.[69]

With an increase in the concentration of **3**, a continuous decrease in the CD ellipticity was observed at 295 nm. This change in intensity, accompanied by an increase in ellipticity at 260 nm but without any significant red or blue shifts in the spectrum, suggests that the binding of **3** does not perturb the overall conformation of G-quadruplex **4** as the antiparallel structure is maintained throughout the titration. We, however, did not observe strong induced CD signals in the perylene absorption region. This observation could be attributed to the following. (1) It could result from the low concentration ($\leq$5 μM) of **3** used in the CD titration experiments. Precipitation of the ligand-quadruplex complex was observed, and consequently, measurements become more difficult once the concentration of **3** is >5 μM. At such a low concentration, the observed CD absorption of the perylene diimide moiety was very weak and had a high N/S (noise-to-signal) ratio, and reliable readings were therefore not possible in the perylene absorption region. Previous reports of induced CD effects of perylene diimide derivatives used a concentration of 20 μM.[60] Hurley and co-workers also reported a very weak induced CD signal of a perylene derivative upon binding to G-quadruplexes.[8] (2) Binding of **3** with **4** may not significantly alter its achirality. A previous report also suggests that quadruplex intercalation and/or stacking may not necessarily lead to induced CD changes in the ligand absorption region, and likely depends on the experimental conditions employed.[71]

Fluorescence Titration Studies. It has been reported that the fluorescence of perylene derivatives can be quenched upon the addition of nucleic acids, which indicates binding.[68,74] Therefore, the fluorescence of **3** was recorded after addition of aliquots of a stock solution of **4** at 20 °C. The fluorescence of **3** decreased as a function of the increasing concentration of **4**, suggesting an interaction between **3** and **4**. The fluorescence of **3** was almost completely quenched at a very low DNA-to-ligand ratio. The saturation of **4** with **3** was assumed when the fluorescence of **3** did not change with a change in the DNA-to-ligand ratios (Figure 8). Attempts to fit the fluorescence titration data to obtain a binding constant were unsuccessful.

The binding of **3** with **4** resulted in rather rapid quenching of the fluorescence of **3**. Such quenching results from the transfer of energy of the perylene moiety to the stacked bases of DNA.[74] The fluorescence signal was completely quenched at a very low ligand-to-G-quadruplex ratio, indicating the binding of approximately three ligands per quadruplex (Figure S3 of the Supporting Information). A Scatchard plot of the quenching data yields a

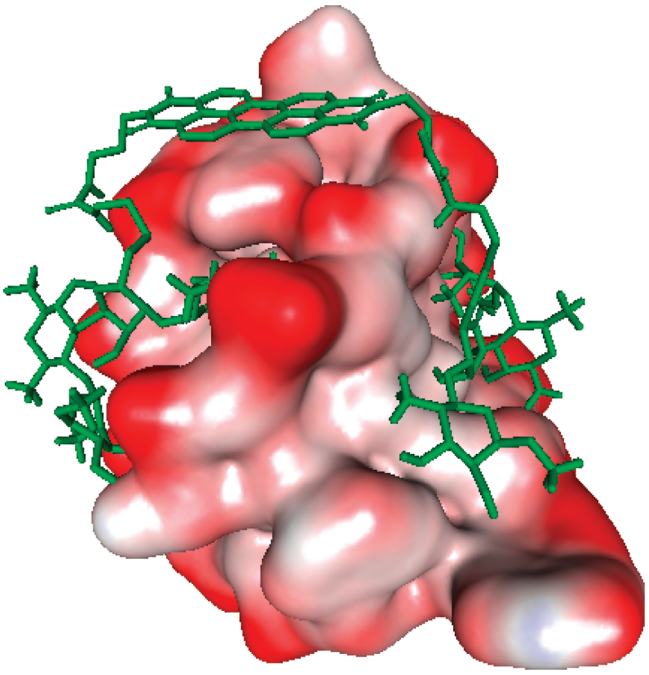

Figure 9. Computer model of **3** docked into human telomeric DNA **4**. Conjugate **3** is colored green.

nonlinear plot. Unfortunately, an association constant could not be determined using this analysis.

Docking Studies. Docking studies were conducted using newly introduced Autodock Vina.[75] The DNA structure was retrieved from the Protein Data Bank (entry 143D). The structure of conjugate **3** was energetically minimized with VegaZZ[53] using the conjugate gradient method. The possible mode of binding was determined by molecular modeling. The perylene moiety of **3** makes stacking interactions with the flipped thymine base (T17) and also makes van der Waals interactions with another thymine base (T5) of G-quadruplex **4** (Figure 9 and Figure S4 of the Supporting Information). The neomycin moieties reside in two grooves. One fits into a larger part of the wide groove, while the other interacts with one of the medium grooves falling between the narrow and wide grooves. This is a conceivable binding mode because neomycin is unlikely to have stacking interactions given its nonplanar structure.

The docking results suggest that neomycin occupies two quadruplex grooves (wide and medium-sized) while the perylene moiety of **3** acts as bridge between these two grooves. The bridging perylene moiety forms a stacking interaction with thymine (T17) and makes contacts with another thymine (T5) via van der Waals interactions. The neomycin binding in the wide groove showed more contacts than the one binding in the medium-sized groove. Interestingly, docking of neomycin alone to human telomeric quadruplex **4** shows that neomycin's most favorable binding occurs in the wide groove.[41] A similar observation has been seen for binding of neomycin to an antiparallel quadruplex resulting from the telomeric sequence of *Oxytricha nova*.[41] Further structural studies are needed to determine the actual mode of binding of **3** with **4**. The weak stacking interactions present between the aromatic platform of the perylene part of the conjugate and the thymine base (T17) may also contribute to the observed absence of a sharp induced CD.

CONCLUSIONS

In this study, we report that a novel perylene−neomycin conjugate **3** has been synthesized by covalently tethering neomycin with perylene diamine **2**. Conjugate **3** exhibited a considerable preference for human telomeric DNA as opposed to various other nucleic acids. The UV thermal melting and absorption binding studies clearly showed the binding of **3** to human telomeric G-quadruplex **4**, and the binding event likely takes place via a dual recognition mode in which the perylene portion of **3** binds via quadruplex stacking. FID experiments showed that **3** supersedes the binding of individual units, indicating the conjugation yields a much improved G-quadruplex binding ligand. CD experiments also revealed the interaction of **3** with **4**; however, the exact mode of binding could not be determined by this method because of the absence of induced CD changes. The fluorescence titrations affirmed the binding of the perylene moiety to the quadruplex. Ligand aggregation at higher concentrations did not allow any direct measurement of association constants using calorimetric techniques. The docking studies indicated stacking of the perylene moiety of **3** with the thymine bases, while the two neomycin units occupy the wide and one of the medium-sized grooves. Further structural studies will be needed to completely characterize the binding, but the severe aggregation and precipitation at higher concentrations will certainly make the structural studies a very challenging endeavor. In summary, our results indicate that dual recognition conjugates (base stacking and groove recognition) can be designed to increase ligand specificity for nucleic acids such as the DNA quadruplex. This approach, as outlined here for the development of G-quadruplex ligands, can be applied to the design of ligands for selective targeting of any conceivable nucleic acid structure where base stacking and groove formation are present.

ASSOCIATED CONTENT

Supporting Information. UV thermal denaturation profiles and UV/NMR/MALDI spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

FID, fluorescence intercalator displacement; UV, ultraviolet; CD, circular dichroism; DI, deionized; TFA, trifluoroacetic acid.

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