

Combining the Best in Triplex Recognition: Synthesis and Nucleic Acid Binding of a BQQ–Neomycin Conjugate

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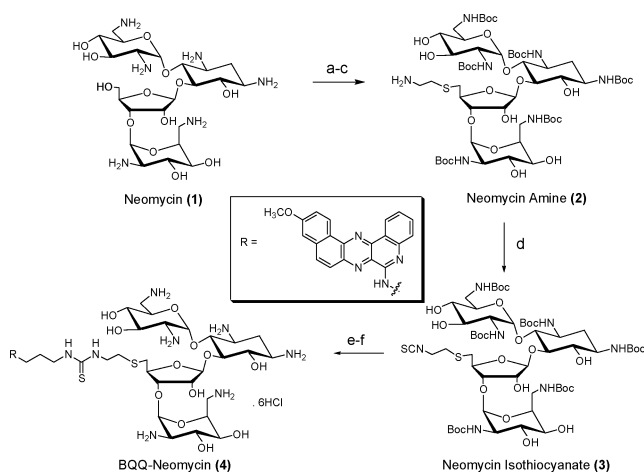
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Recognition of duplex DNA by oligonucleotides (major groove binders—DNA triple helices) is a promising approach to a chemical solution for DNA recognition.¹ Triple helix formation has been the focus of considerable interest because of possible applications in developing new molecular biology tools as well as therapeutic agents, and because of the possible relevance of H-DNA structures in biological systems.^{2–5} Several intercalators as well as various DNA minor groove ligands have been shown to bind to DNA triple helices.^{4,6,7} Intercalators usually stabilize to a greater extent triple helices containing T•A•T triplets, whereas minor groove binders usually destabilize triplexes.⁸ In our quest for new ligands for triple helix stabilization, we have investigated aminoglycoside antibiotics (neomycin, Scheme 1).^{9–11}

The stabilization of the poly(dA)•2poly(dT), poly(rA)•2poly(rU),⁹ a mixed base DNA 22mer,¹¹ and DNA/RNA hybrid duplexes/triple helices¹⁰ by neomycin has recently been reported by us. Among all of the aminoglycosides, neomycin was shown to be the most effective triplex groove binder that remarkably stabilized DNA and RNA triple helices with little effect on the double helices. This work has complemented the success in the development of triplex specific intercalators in the past decade, among which the pentacyclic BQQ-based structures have been presented as one of the best in triplex binding selectivity and potency.^{7,12} We have recently reported that a pyrene–neomycin conjugate can stabilize poly(dA)•2poly(dT) triplex much more effectively and specifically than neomycin or pyrene at low concentrations.¹³ Because pyrene by itself is not a potent intercalator (but a transient one), we hypothesized that by replacing pyrene with a more specific triplex binding intercalator such as BQQ,⁷ new classes of molecules with high triplex affinity can be identified. A BQQ–neomycin conjugate has therefore been synthesized by forming a thiourea linkage between neomycin and benzo[*f*]quino[3,4-*b*]quinoxaline derivative (BQQ) (Scheme 1). We report that the BQQ–neomycin conjugate is more potent in stabilizing DNA triple helices than neomycin, BQQ, or a combination of both.

The following assumptions were made in the design of the conjugate: The amino groups on rings I, II, and IV of neomycin are necessary in stabilizing and in recognizing the triplex groove (aminoglycosides without any of these amines do not stabilize triplexes as efficiently).¹¹ The 5'-OH on ring III was thus chosen to provide the linkage to the intercalating unit. The intercalator (Scheme 1, BQQ-amine¹²) was linked to neomycin isothiocyanate **3** (prepared in four steps from neomycin)¹⁴ in the presence of 4-(dimethylamino)pyridine (DMAP) as a catalyst. The synthesis rests on the selective conversion of ring III 5'-OH of neomycin into a good leaving group (TIBS-2,4,6-triisopropylbenzenesulfonyl) as previously reported by Tor.¹⁵ The displacement of TIBS by aminoethanethiol, conversion into isothiocyanate, followed by coupling with the primary amine in BQQ,¹² and deprotection with HCl gives the target conjugate **4** in good yields (Scheme 1).

Scheme 1^a



^a Reagents and conditions: (a) (Boc)₂O, DMF, H₂O, Et₃N, 60 °C, 5 h, 60%; (b) 2,4,6-triisopropylbenzenesulfonyl chloride, pyridine, room temperature, 40 h, 50%; (c) HSCH₂CH₂NH₂•HCl, NaOEt/EtOH, room temperature, 18 h, 50%; (d) 1,1'-thiocarbonyldi-2(1*H*)-pyridone, CH₂Cl₂, room temperature, overnight, 86%; (e) BQQ, DMF, DMAP, room temperature, 7 h, 83%; (f) 4 M HCl/dioxane, HSCH₂CH₂SH, room temperature, 5 min, 58%.

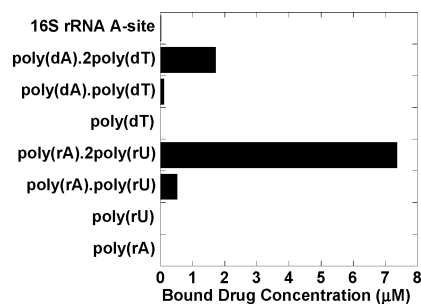


Figure 1. Competition dialysis of various nucleic acids with 1 μM BQQ–neomycin. 180 μL of different nucleic acids (75 μM per monomeric unit of each polymer) was dialyzed with 400 mL of 1 μM BQQ–neomycin (150 mM KCl, 0.5 mM EDTA, 10 mM sodium cacodylate, pH 6.8) solution for 72 h. At the end of dialysis, 150 μL of nucleic acid samples was carefully removed and placed in microfuge tubes, and the samples were taken to a final concentration of 1% (w/v) sodium dodecyl sulfate (SDS). Each mixture was allowed to equilibrate for 2 h. Each nucleic acid solution was then further diluted to an overall volume of 4 mL by BPES buffer. The concentration of BQQ–neomycin after dialysis was determined by fluorescence (FluoroMax-3).¹⁶

To ascertain the selectivity of this novel conjugate, we used the competition dialysis method developed by Chaires.¹⁶ Figure 1 shows competition dialysis results obtained for various nucleic acids with BQQ–neomycin. No binding is observed with single-stranded DNA or RNA. Duplex DNA only weakly binds to BQQ–neomycin as compared to triplex. BQQ–neomycin binding to triplex DNA is greater than to duplex DNA or single strands. RNA triplex similarly

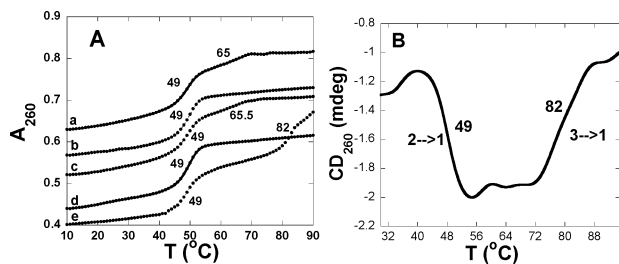


Figure 2. (A) UV melting profile of a 2:1 strand mixture **I** ($dA_{22} \cdot 2dT_{22}$) at 260 nm with 150 mM KCl, 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8: (a) 4 μ M BQQ, (b) 4 μ M neomycin, (c) 4 μ M neomycin + 4 μ M BQQ, (d) no ligand, (e) 4 μ M BQQ–neomycin. (B) CD melting profile (right) of $dA_{22} \cdot 2dT_{22}$ at 260 nm in the presence of 150 mM KCl, 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8. $dA_{22} = 1 \mu$ M/strand; $dT_{22} = 2 \mu$ M/strand; [BQQ–neomycin] = 4 μ M.

has a higher affinity for the drug than does RNA duplex, even higher than the 16S ribosomal RNA A-site, the natural target for aminoglycosides.

Thermal denaturation of complex **I** ($dA_{22} \cdot 2dT_{22}$) was then studied {using a 2:1 pyrimidine (dT_{22}) to purine (dA_{22}) strand ratio} by UV and CD spectroscopy. Figure 2a shows the UV melting profiles of this 2:1 strand mixture **I** in the presence of 4 μ M neomycin, BQQ, and BQQ–neomycin. In the absence of ligand, the melting profile of the mixture is monophasic, which represents the duplex dissociation at $T_{m2 \rightarrow 1} = 49$ °C. No triplex transition is observed. In the presence of 4 μ M BQQ–neomycin, a new transition emerges at ~ 80 °C. From UV and CD melts (Figure 2b), this new transition was identified as the dissociation of triplex $dA_{22} \cdot 2dT_{22}$ directly to single strands. In the presence of 4 μ M BQQ–neomycin, the duplex melts at 49 °C, and the triplex melts at 80 °C, as was previously observed with a few other triplex specific intercalators (coralyne).¹⁷ A negative CD (260 nm) transition at 47 °C represents duplex dissociation, while a positive transition at 80 °C represents triplex dissociation. A similar melting profile is obtained when dA_{22} and dT_{22} are present in a 1:1 ratio, suggesting that disproportionation of some of the duplex to the triplex occurs in the presence of the drug.

Additionally, the transition at 80 °C shows pronounced hysteresis (indicative of slow triplex annealing), whereas the duplex melting and annealing transitions overlap (Supporting Information). This suggests that as little as 4 μ M of the ligand leads to a ΔT_m of >60 °C (assuming a triplex T_m of <10 °C in the absence of ligand). BQQ alone shows triplex melt at 65 °C, whereas neomycin is unable to induce the triplex under these low concentrations. At higher BQQ–neomycin concentrations, duplex and triplex transitions merge to a $T_{m3 \rightarrow 1}$ transition.

The UV melting of a mixed base triplex **II** (Figure 3) was then monitored at 260, 280, and 284 nm. Melting curves of triplex **II** in the presence of different ligands (BQQ, BQQ–neomycin, and neomycin) at 8 μ M concentration are shown in Figure 3. The melting profiles are clearly biphasic, as is typically observed with this triplex.¹¹ Without any ligand, the triplex melts at 30 °C, and the duplex melts at 56 °C. In the presence of 8 μ M BQQ (Figure 3b,d), a broad triplex melt is observed from 32 °C upward.

In the presence of 8 μ M BQQ–neomycin, the melting profile exhibits a broad transition before the duplex, but a much sharper transition after the duplex melt ($T_{m3 \rightarrow 1} = 71$ °C), with the duplex transition at 56 °C (similar to what is observed with $dA_{22} \cdot 2dT_{22}$ triplex). BQQ–neomycin stabilizes T·A·T as well as C·G·C⁺ mixed base triplex DNAs with drug induced triplex stabilization occurring

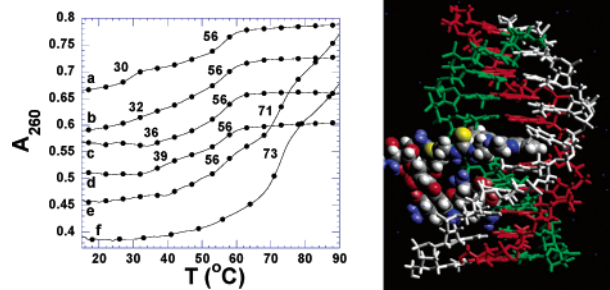


Figure 3. (Left) UV melting profiles of 22mer triplex **II** (shown below) at 260 nm in the presence of 150 mM KCl, 10 mM sodium cacodylate, 0.1 mM EDTA, pH 6.8: (a) no ligand, (b) 8 μ M BQQ, (c) 8 μ M neomycin, (d) 8 μ M neomycin + 8 μ M BQQ, (e) 8 μ M BQQ–neomycin, (f) 10 μ M BQQ–neomycin. $dR_{22} = dY_{22} = dT_{22} = 1 \mu$ M/strand. (Right) A computer model of BQQ–neomycin docked in a T·A·T DNA triplex. dR_{22} : 5'-d(AAAGGAGGAGAAGAAGAAAAA)3'. dY_{22} : 3'-d(TTTCCTCCTCTCTCTCTTTT)5'. dX_{22} : 5'-d(TTTCCTCCTCTCTCTCTTTT)3'.

even after the duplex melts. At higher concentrations of the drug (10 μ M), only one transition (73 °C, $T_{m3 \rightarrow 1}$) is observed (Figure 3f).

An ITC titration of the drug binding to $dA_{22} \cdot 2dT_{22}$ triplex (300 mM NaCl,¹⁸ Supporting Information) yields an isotherm that can be fit to a two binding site model, with one of the K_{ds} 's showing nanomolar drug binding (27 ± 2 nM). A model depicting the possible binding of the conjugate to a T·A·T triplex is shown in Figure 3. As BQQ stacks between the triplex bases, neomycin stays bound in the larger triplex W–H groove. The design of such dual recognition ligands then opens a new paradigm for recognition of triplex nucleic acids (DNA, RNA, or hybrid) and should aid in the development of even more selective and potent conjugates.

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Supporting Information Available: UV melts, scans, and synthesis/characterization of the conjugate (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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